Alzheimer disease (AD), the most common senile dementia, is characterized by amyloid plaques, vascular amyloid, neurofibrillary tangles, and progressive neurodegeneration. Amyloid is mainly composed of amyloid-β (Aβ) peptides, which are derived from processing of the β-amyloid precursor protein (APP), better named amyloid-β precursor protein (AβPP), by secretases. The AβPP intracellular domain (AID), which is released together with Aβ, has signaling function, since it modulates apoptosis and transcription. Despite its biological and pathological importance, the mechanisms regulating AβPP processing are poorly understood. As cleavage of other γ-secretase substrates is regulated by membrane-bound proteins, we have postulated the existence of integral membrane proteins that bind AβPP and regulate its processing. Here, we show that BRI2, a type II membrane protein, interacts with AβPP. Interestingly, 17 amino acids corresponding to the NH2-terminal portion of Aβ are necessary for this interaction. Moreover, BRI2 expression regulates AβPP processing resulting in reduced Aβ and AID levels. Altogether, these findings characterize the BRI2-AβPP interaction as a regulatory mechanism of AβPP processing that inhibits Aβ production. Notably, BRI2 mutations cause familial British (FBD) and Danish dementias (FDD) that are clinically and pathologically similar to AD. Finding that BRI2 pathogenic mutations alter the regulatory function of BRI2 on AβPP processing would define dysregulation of AβPP cleavage as a pathogenic mechanism common to AD, FDD, and FBD.

AβPP1 is an ubiquitous type I transmembrane protein (1, 2) that undergoes a series of endoproteolytic events (3–5). AβPP is first cleaved at the plasma membrane or in intracellular organelles by β-secretase (6). While the ectodomain is released extracellularly (sAPPβ) or into the lumen of intracellular compartments, the COOH-terminal fragment of 99 amino acids (C99) remains membrane bound. In a second, intramembranous proteolytic event, C99 is cleaved, with somewhat lax site specificity, by the γ-secretase. Two peptides are released in a 1:1 stoichiometric ratio: the amyloidogenic Aβ peptide, consisting of 2 major species of 40 and 42 amino acids (Aβ40 and Aβ42, respectively), and an intracellular product named AID or AICD, which is very short-lived and has been identified only recently (7–9). In an alternative, nonamyloidogenic proteolytic pathway, AβPP is first processed by α-secretase in the Aβ sequence leading to the production of the sAPPα ectodomain and the membrane-bound COOH-terminal fragment of 83 amino acids (C83). C83 is also cleaved by the γ-secretase into the P3 and AID peptides. While Aβ is implicated in the pathogenesis of Alzheimer disease, AID mediates most of the AβPP signaling functions. A pathogenic role for AβPP processing in AD has been ascertained by the finding that mutations in presenilins (10–13), key components of the γ-secretase, and AβPP (14) cause autosomal dominant familial forms of AD. Thus, because of its biological and pathological importance, understanding how AβPP cleavage is regulated is of primary significance.

Membrane-bound proteins prompt Notch cleavage by secretases and the release of a transcriptionally active intracellular fragment (15). Considering the remarkable similarity between AβPP and Notch signaling, we have hypothesized that AβPP processing is similarly regulated. We report herein that the type II membrane protein BRI2 (16), also known as E25 (17) and Itm2 (18), fulfills this description.

**EXPERIMENTAL PROCEDURES**

Split-ubiquitin Yeast Two-hybrid Screening—The split-ubiquitin system provides an attractive alternative to analyze interactions between integral membrane proteins (19). The split-ubiquitin system and human brain libraries were purchased from Dualexysystems Biotech (Zurich, Switzerland). The screenings were performed according to the manufacturers protocol. Briefly, human AβPP (amino acids 1–695), human AβPP (amino acids 1–664; AβPPNcas), or human APLP2 were cloned into pTMV4, pAMBV4, and pAMBV4 bait vectors, respectively, to obtain APP family bait proteins fused to the COOH-terminal half of ubiquitin (Cub), followed by a reporter fragment (LexA, a DNA-binding protein, fused to VP16, a transcriptional activation). Human brain libraries express proteins fused at the NH2-terminal half of mutated ubiquitin (NubG). For each library we screened ~5 × 10^6 transformants. Clones coding for proteins that can interact with AβPP/APLP2-Cub will promote the NubG-Cub interaction followed by recruitment of ubiquitin-specific protease(s), cleavage of the AβPP/APLP2-Cub bait, release of the LexA-VP16 transcription factor, and the transcriptional activation of the two reporter genes (lacZ and HIS3). Library plasmids were recovered from HIS3- and lacZ-positive yeast transformants, identified by nucleotide sequencing, and cloned into pcDNA3.1 with an NH2-terminal FLAG tag and directly tested its ability to interact with AβPP by immunoprecipitation as described below. Screening for co-
activator of both reporter genes resulted in the identification of known APP/PLP-binding proteins, such as Fe65 (20).

**Plasmids**—Full-length BR2 (amino acids 1–266) and BR21–131 were amplified from the two-hybrid clone and cloned into pcDNA3.1-FLAG (21). Mammalian expression vectors αPP, αβPPNcas were described (22). A myc-tag was inserted after signaling sequence of ApoER2 and cloned into pEF-BOS. BACE was cloned from mouse brain cDNA and COOH-terminally myc-tagged by cloning into pcDNA3mycHisB (Invitrogen).

**Antibodies**—The following antibodies are used: αFLAG (mouse monoclonal M2, Sigma), αAPP mouse monoclonals 2C11 (Chemicon) 6E10 (Signet Laboratories), and p2-1 (BIOSOURCE); αmyc mouse monoclonal 9E10, Santa Cruz Biotechnology; EN3 (23) (rabbit polyclonal antibody); rabbit polyclonal antibody αB2 (IgY, Southern Biotechnology); chicken αB2 (IgY, BMA Biomedicals); an unrelated rabbit polyclonal antibody IgG, Southern Biotechnology); EN3 (23) (rabbit polyclonal antibody); rabbit polyclonal αBR2, raised against amino acids 222–232 of human BR2 (24); a mouse polyclonal was raised against a peptide encompassing the cytoplasmic tail of human BR2. The rabbit polyclonal anti-αPLP1 and anti-αPLP2 COOH-terminal antibodies were purchased from Calbiochem.

**Cell Culture and Transfection**—HEK293, HEK293 stably expressing αPP, αβPP, Hela, and N2a cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin, and 10% fetal bovine serum at 37 °C, 5% CO2 (25). FuGENE 6 (Roche Applied Science) or Metafectene (Biontex) was used for transfection.

**Immunoprecipitation and Western Blot**—Unless otherwise noted, all immunoprecipitation procedures were performed at 4 °C. The transfected cells were lysed in Buffer A (20 mM Hepes/NaOH, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 0.5% (w/v) Triton X-100) containing 10% (v/v) glycerol for 30 min, and the lysates were cleared at 20,000 × g for 10 min. For FLAG immunoprecipitation, the cleared lysates were mixed with 20 μl of FLAG-M2 beads (Sigma) for 2 h and washed three times with Buffer A. The precipitates were boiled in 60 μl of 2 × SDS sample buffer and subjected to Western blot. For other immunoprecipitation, the cleared lysates were incubated with antibodies for 1 h and mixed with 20 μl of protein A/G beads (Pierce), washed in Buffer A, and processed as above. Human brains from normal individual were homogenized in Buffer A containing 10% (v/v) glycerol using a Dounce homogenizer. The proteins were extracted overnight with the protein concentration at 5 mg/ml. Extracted proteins were cleared at 20,000 × g for 1 h. The supernatants were incubated with the indicated antibodies and protein A/G beads blocked with PBS containing 1% (w/v) bovine serum albumin. Precipitants were washed and processed as described above.

**Metabolic Labeling**—HEK293APP cells transfected with pcDNA3 or BR12 were incubated in DMEM without methionine and cysteine (Invitrogen) supplemented with penicillin, streptomycin, and 10% fetal bovine serum. After, cells were labeled 30 min by adding to the media were measured using human Aβ ELISA (KMI Diagnostics), according to the manufacturer’s protocol. The transfected cells were lysed and processed as above. The media of labeled cells were collected and precipitated with αAPP monoclonal antibody 22C11 or αβPP monoclonal antibody 2C11 or αAPP. BR2, as well as a ~17-kDa BR2 NH2-terminal fragment (BR2nt), were precipitated by αAPP or together with αAPP.

**RESULTS AND DISCUSSION**

To test whether membrane-tethered proteins might regulate AβPP processing, we have used the split-ubiquitin system to identify interactions between membrane proteins. Screening of a human brain cDNA library for proteins that interact with AβPP family proteins resulted in the identification of BR2 (17) and BR13 (27), members of a gene family of Type II membrane proteins containing a Brichos domain (28). Although the function of BR1 proteins is unknown, BR12 mutations are found in patients with FDD (16) and FDD (29). Of note, neuro-pathological findings in FDD and FDD include amyloid and/or preamyloid parenchymal plaques, congophilic amyloid angiopathy, neurofibrillary tangles, and neurodegeneration, similar to AD. Hence, because mutations in BR1 cause AD-like familial dementia we have further studied the physiological relevance of this BR2-AβPP interaction.

To assess the BR2-AβPP interaction in mammalian cells, Hela cells were co-transfected with BR12 and AβPP constructs (Fig. 1a). Immunoprecipitation of cell lysates with an αFLAG antibody showed that BR12 interacted with full-length AβPP (Fig. 1, b and c), C99 (Fig. 1, b and d), and AβPPNcas, which present a deletion of most of the intracellular region of AβPP (Fig. 1e) but not C83 (Fig. 1, b and d). AβPP runs as a doublet. The lower AβPP band represents nonglycosylated, immature AβPP, the upper form is instead composed of mature, glycosylated AβPP. Of note, only the mature, glycosylated forms of AβPP and AβPPNcas interacted with BR12 (Fig. 1, b–d). It should also be noted that BR12 overexpression dramatically increases the levels of C99 (Fig. 1, b and d). The significance of this finding will be discussed below.

Deletion of most of the BR12 ecto-domain did not abolish the binding to APP (BR21–131, Fig. 1d). The reverse immunoprecipitation with an αAPP antibody revealed that AβPP immunoprecipitates BR12 (Fig. 1e). Additionally, a proteolytic ~17-kDa BR12 NH2-terminal fragment detected in transfected HeLa cells (BR2nt, which is similar in size to BR21–131) was...
also precipitated with AβPP (Fig. 1e). The specificity of these interactions was supported by the evidence that BRI2 did not bind to ApoER2, another type I integral membrane protein (Fig. 1c). These findings attest that while the intracytoplasmic tail of AβPP and most of the AβPP and BRI2 ectodomain are not important for BRI2/AβPP interaction, a 17-amino acid region in the ectodomain of AβPP, juxtaposed to the transmembrane region and containing the NH₂-terminal Aβ sequence, is essential for this binding. These data strongly suggest that BRI2 and AβPP do not interact in trans (i.e., as receptor/ligand expressed on distinct membranes) but, rather, form a molecular complex in cellular membranes.

AβPP and BRI2 are both expressed in mature neural tissues. We therefore sought to determine whether AβPP also interacts with BRI2 in the adult human brain. First, we tested four anti-Aβ2 antibodies to determine whether they could immunoprecipitate human BRI2. For these tests, HeLa cells were transfected with FLAG-BRI2 and immunoprecipitated with the four BRI2 antibodies and controls. As shown in Fig. 2a, only the EN3 anti-BRI2 antibody was able to precipitate BRI2. Next, we made homogenates of human brains and performed immunoprecipitation with either the aAβPPct antibody or EN3. As shown in Fig. 2b, C99 (and larger COOH-terminal AβPP fragments) was precipitated with both anti-AβPP as well as EN3 antibodies, while C99 was not precipitated with a rabbit polyclonal IgG. Of interest, also in this case C83 did not precipitate antibodies, while C99 was not precipitated with a rabbit polyclonal antibody (lane 3). These findings attest that while the intracytoplasmic tail of AβPP-Gal4 will release AID-Gal4 from PP, coAβPPct, or EN3. Total brain lysate (T.L.) and immunoprecipitants were blotted with the aAβPP monoclonal antibody 22C11 (top panel) or aAβPPct (bottom panel).

Inhibition of AID and Aβ production by BRI2 suggests that BRI2 expression reduces cleavage of AβPP by the γ-secretase. However, it is also possible that BRI2 could modulate the β- and α-cleavage of AβPP. As discussed above, cleavage of AβPP by either β- or α-secretase releases sAβPPβ and sAβPPα in the supernatant, respectively. While increased amounts of either sAβPPα or sAβPPβ indicate increased α- or β-cleavage, reduction of either sAβPPα or sAβPPβ reflect decreased α- or β-cleavage. Thus, to determine whether BRI2 affects either β- or α-secretase, we measured the amounts of sAβPPα and sAβPPβ. In these same experiments, we also measured intracellular levels of C99 and C83. HEK293-AβPP cells were transfected with FLAG-BRI2 or a vector control. Transfected cells were pulse-labeled with [3H]methionine-cysteine for 30 min, then chased for 0, 1, 2, and 4 h at 37 °C (Fig. 3c). The cell lysates were immunoprecipitated with aAβPPct antibody at each time point (Fig. 3c). To measure secreted AβPP (sAβPPα and sAβPPβ), supernatants were collected from cells labeled for 4 h and precipitated with the anti-AβPP antibodies P21 (which precipitates both sAβPPα and sAβPPβ) or 6E10 (which only precipitates sAβPPα) (Fig. 3d). BRI2 transfection resulted in decreased amounts of C99 (Fig. 3c) and sAβPPα (Fig. 3d). Conversely, the levels of C99 (Fig. 3c) and sAβPPβ (Fig. 3d) were augmented. Notably, BRI2 was co-immunoprecipitated with the coAβPPct antibody at all time points. Thus, BRI2 expression reduces cleavage of AβPP by α-secretase while increasing its processing by β-secretase. The concomitant inhibition of γ-secretase and increase in β-cleavage of AβPP explains the dramatic increase in C99 levels.

AβPP is a member of a family of proteins that includes APLP1 and APLP2. APLP1 and APLP2 are also γ-secretase substrates (22) and among the numerous γ-secretase substrates are those that bear more sequence similarity to AβPP. Thus, to test whether BRI2 generally affects γ-secretase or specifically inhibits γ-cleavage of AβPP, we transfected BRI2 with either APLP1 and APLP2. Western blot using anti-APLP1 or anti-APLP2 COOH-terminal antibodies indicates that BRI2 expression does not promote accumulation of COOH-terminal
fragments of APLP1 (data not shown) and APLP2 (Fig. 3i). This result supports the notion that BRI2 specifically blocks the γ-activity on AβPP but not on other γ-substrates.

Altogether, our studies suggest that BRI2 and AβPP form a multimolecular complex in cell membranes. While the stoichiometry of AβPP and BRI2 in such complexes has to be investigated and whether BRI2 and AβPP are found in a structure comprising other proteins is unknown, our data suggest that BRI2 functions as an endogenous regulator of AβPP processing. More specifically, we found that BRI2 expression decreases both α- and γ-cleavage of AβPP while increasing its β-processing. Although the detailed molecular mechanisms responsible for these functions must be directly addressed, the finding that BRI2 interacts with a region of AβPP comprising the α- and γ-cleavage sites insinuates that BRI2 physically masks the two target sequences from the secretases. Increased β-activity might be consequential to the reduced α-processing.

Recently, mutations in BRI2 have been found in FBD (16) and FDD (29) patients. Both wild type and mutant BRI2 are processed by furin (30) with this processing resulting in the
secretion of a COOH-terminal peptide. Furin cleavage of wild type BRI2 releases a 23-aa-long peptide. In FBD patients, a point mutation at the stop codon of BRI2 results in a read-through the 3′-untranslated region and the synthesis of a BRI2 molecule containing 11 extra amino acids at the COOH terminus. Furin cleavage of this mutated BRI2 generates a longer peptide, the ABri peptide, which is deposited as amyloid fibrils. In the Danish kindred, the presence of a 10-nucleotide duplication one codon before the normal stop codon produces a frameshift in the BRI2 sequence generating a larger-than-normal precursor protein, of which the amyloid subunit, also released by furin processing, comprises the last 34 COOH-terminal amino acids. The deposition of ABri and ADan amyloidogenic peptides can begin similar neurodegenerative processes. According to this view, amyloidogenesis, and not the particular amyloidogenic peptide, is key in initiating neurodegeneration. However, the finding that BRI2 regulates AβPP processing is intriguing and prompts us to speculate that altered AβPP processing is also a pathogenic factor in FBD and FDD. Intriguingly, in FDD patients Aβ co-deposits with ADan in vascular amyloid lesions (31). Further studies will address whether this hypothesis is correct.

Acknowledgments—We thank Drs. Kami Kim, Gregory Prelich, Tanya Dragic, Marshall Horwitz, all at the Albert Einstein College of Medicine, for kindly allowing extensive use of their equipment.

REFERENCES
1. Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) Nature 325, 733–736
2. Tanzi, R. E., Gusella, J. F., Watkins, P. C., Brun, G. A., St George-Hyslop, P., Van Keuren, M. L., Patterson, D., Pagan, S., Kurnit, D. M., and Neve, R. L. (1987) Science 235, 880–884
3. Selkoe, D., and Kopan, R. (2003) Annu. Rev. Neurosci. 26, 565–597
4. Sisodia, S. S., and St George-Hyslop, P. H. (2002) Nat. Rev. Neurosci. 3, 281–290
5. Gandy, S. (2005) J. Clin. Invest. 115, 1211–1219
6. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loefler, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Trenor, J., Rogers, G., and Citron, M. (1999) Science 286, 735–741
7. Passer, B., Pellegrini, L., Russo, C., Siegel, R. M., Lenardo, M. J., Schettini, G., Bachmann, M., Tabaton, M., and D’Adamo, L. (2000) J. Alzheimers Dis. 2, 289–301
8. Can, X., and Sudhof, T. C. (2001) Science 293, 115–120
9. Cupers, P., Orlans, I., Craessaerts, K., Annaert, W., and De Strouwer, B. (2001) J. Neurochem. 78, 1168–1178
10. Sherrington, R., Roge, E. I., Liang, Y., Roge, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, R., and et al. (1995) Nature 375, 754–760
11. Levy-Lahad, E., Wasco, W., Pookeraj, P., Roman, D. M., Oshima, J., Pettingell, W. H., Yu, C. E., Jondro, P. D., Schmidt, S. D., Wang, K., and et al. (1995) Science 269, 973–977
12. Levy-Lahad, E., Wijsman, E. M., Nemens, E., Anderson, L., Goddard, K. A., Weber, J. L., Bird, T. D., and Schellenberg, G. D. (1995) Science 269, 970–973
13. Roge, E. I., Sherrington, R., Roge, E. A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tuda, T., and et al. (1995) Nature 375, 775–778
14. Goate, A., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., and et al. (1991) Nature 349, 704–706
15. Kopan, R. (2002) J. Cell Sci. 115, 1095–1097
16. Vidal, R., Frangione, B., Rostagno, A., Mead, S., Revesz, T., Plant, G., and Ghiso, J. (1999) Nature 399, 778–781
17. Deleersnijder, W., Hong, G., Cortvriendt, R., Poirier, C., Tylzanowski, P., Pitois, K., Van Marck, E., and Merregaert, J. (1996) J. Biol. Chem. 271, 19475–19482
18. Pittois, K., Deleersnijder, W., and Merregaert, J. (1998) Gene 217, 141–149
19. Siggelaar, I., Kostoszyna, C., Johnson, N., and Heesen, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5187–5192
20. Zambrano, N., Buxbaum, J. D., Minopoli, G., Fiore, F., De Candia, P., De Renzo, S., Farzana, R., Sabo, S., Cheetham, J., Soul, M., and Russo, T. (1997) J. Biol. Chem. 272, 6399–6405
21. Matauda, S., Matauda, Y., and D’Adamo, L. (2003) J. Biol. Chem. 278, 38601–38606
22. Scheinfeld, M. H., Gherzi, E., Laky, K., Fowles, B. J., and D’Adamo, L. (2002) J. Biol. Chem. 277, 44195–44201
23. Pickford, F., Onstead, L., Camacho-Prihar, C., Hardy, J., and McGowan, E. (2003) Neuroscience 128, 95–98
24. Rostagno, A., Tomidokoro, Y., Lashley, T., Ng, D., Plant, G., Holton, J., Frangione, B., Revesz, T., and Ghiso, J. (2005) Cell Mol. Life Sci in press
25. Scheinfeld, M. H., Gherzi, E., Davies, P., and D’Adamo, L. (2003) J. Biol. Chem. 278, 42098–42093
26. Gianni, D., Zambrano, N., Bimonte, M., Minopoli, G., Mercken, L., Talamo, P., Scaloni, A., and Russo, T. (2003) J. Biol. Chem. 278, 9290–9297
27. Vidal, R., Calero, M., Revesz, T., Plant, G., Ghiso, J., and Frangione, B. (2001) Gene 266, 95–102
28. Sanchez-Pulido, L., Devos, D., and Valencia, A. (2002) Trends Biochem. Sci. 27, 329–332
29. Vidal, R., Revesz, T., Rostagno, A., Kim, E., Holton, J. L., Bek, T., Bojesen-Moller, M., Braendgaard, H., Plant, G., Ghiso, J., and Frangione, B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4920–4925
30. Kim, S. H., Wang, R., Gordon, D. J., Bass, J., Steiner, D. P., Lyon, D. G., Thinkankaran, G., Meredith, S. C., and Sisodia, S. S. (1999) Nat. Neurosci. 2, 984–988
31. Holton, J. L., Lashley, T., Ghiso, J., Braendgaard, H., Vidal, R., Geerin, C. J., Gibb, G., Hanger, D. P., Rostagno, A., Anderson, B. H., Strand, C., Aylng, H., Plant, G., Frangione, B., Bojesen-Moller, M., and Revesz, T. (2002) J. Neuropathol. Exp. Neurol. 61, 254–267