Transmembrane proteins BRI2 and amyloid precursor protein (APP) co-localize with amyloid β (Aβ) lesions in sporadic Alzheimer disease and mutations in both precursor proteins are linked to early-onset familial cases of cerebral amyloidosis associated with dementia and/or cerebral hemorrhage. A specific interaction between BRI2 and APP was unveiled by immunoprecipitation experiments using transfected and non-transfected cells. The use of deletion mutants further revealed that stretches 648–719 of APP751 and 46–106 of BRI2, both inclusive of the full transmembrane domains, are sufficient for the interaction. Removal of most of the APP and BRI2 extracellular domains without affecting the interaction implies that both proteins interact when are expressed on the same cell membrane (cis) rather than on adjacent cells (trans). The presence of BRI2 had a modulatory effect on APP processing, specifically increasing the levels of cellular APP as well as β-secretase-generated COOH-terminal fragments while decreasing the levels of α-secretase-generated COOH-terminal fragments as well as the secretion of total APP and Aβ peptides. Determining the precise molecular pathways affected by the specific binding between APP and BRI2 could result in the identification of common therapeutic targets for these sporadic and familial neurodegenerative disorders.

Aβ,1 a 39–42 amino acid peptide of unknown biological function normally present in biological fluids, is also the main constituent of parenchymal and vascular amyloid deposits characteristic of Alzheimer disease (AD) (reviewed in Ref. 1). It is an internal fragment of the larger type-I transmembrane amyloid precursor protein APP (also referred as AβPP), which exists in several isoforms of different length, ranging from 695 to 770 residues (2, 3). From all these APP isoforms, Aβ is normally generated by proteolytic processing through the sequential action of β- and γ-secretases. Within amyloid lesions, a number of unrelated components collectively known as amyloid-associated proteins (amyloid P-component, α1-antichymotrypsin, apoE, apoJ, complement components, vitronectin, extracellular matrix proteins, and APP, among others) co-localize with fibrillar and non-fibrillar Aβ, as shown by immunohistochemical studies (4–10). It is not clear whether these proteins are important for the mechanism of amyloidogenesis or just innocent bystanders. Recently, it was reported that a novel protein BRI2 was abundant in dystrophic neurites, in senile plaques, and around vessels in ischemic lesions in AD and was also detected in Lewy neurites in cases of dementia with Lewy bodies and Parkinson disease (11). Of interest, mutations at or near the stop codon of BRI2 are associated with dementia and cerebellar ataxia in kindreds of British (12) and Danish (13) origin.

BRI2 is a type-II transmembrane protein encoded by the single gene BRI2 (also known as ITM2B and E25B) located on the long arm of chromosome 13 (12, 14–16). BRI2 belongs to an evolutionary conserved multigene family comprising at least three homologues in both mouse and humans all bearing the same genomic organization (14, 16, 17). BRI2 is broadly expressed in peripheral organs as well as in the brain, in which it is ubiquitously present in white and gray matter but shows more abundant distribution in hippocampus and cerebellum compared with cerebral cortex (12). The protein product BRI2 contains 266 amino acids, a single spanning transmembrane region (positions 52 and 75), a BRICHOS domain (amino acids 137–231) (17), a single N-glycosylation site (Asn179), and a consensus motif for furin-like proteolytic processing at peptide bond 243–244 (18). Studies aimed at determining the distribution of BRI2 in human brains demonstrated its presence in pyramidal neurons of the CA3 and CA4 layers of the hippocampus and in Purkinje cells of the cerebellum. Although the biological function of BRI2 still remains elusive, its cellular distribution, its presence in dystrophic neurites and amyloid lesions in various neurodegenerative conditions (11), along with its axonal localization in transfected neurons (19) suggest a role in transport along neuronal processes, making BRI2 a potential candidate for protein-protein interactions with other molecules related to neurodegeneration, including membrane proteins like APP.

Using transfected and non-transfected cells in culture, we...
BRI2 Interacts with APP

RESULTS

BRI2 Interacts Specifically with APP—To investigate whether APP interacts with BRI2, we used HEK293 cells stably expressing APP751 that were transfected with myc-tagged BRI2 or its vector prk5. Immunoprecipitations (IPs) of cell extracts were performed using the 9B11 anti-myc monoclonal antibody, preabsorbed anti-myc, or an anti-transferrin receptor antibody as a negative control. Immunoprecipitates were analyzed by Western blot using antibodies against myc-tagged BRI2 (9E10), the COOH terminus of APP751, R1(57), or transferrin receptor. As shown in Fig. 1A, the 9B11 antibody precipitated from extracts of cells expressing myc-tagged BRI2 and APP751, BRI2 (panel 1, lane 2), as well as APP751 (panel 2, lane 2) but not transferrin receptor (panel 3, lane 2). Neither BRI2 nor APP751 (panels 1 and 2, lane 1) were IP from extracts of cells expressing only APP751 (panel 5, lane 1). IPs of 9B11 antibody preabsorbed with its antigenic peptide did not contain BRI2 (panel 1, lane 3) or APP751 (panel 2, lane 3). In addition, IPs with anti-transferrin receptor did not retrieve BRI2 (panel 1, lane 4) or APP751 (panel 2, lane 4). These data clearly suggest a specific interaction between BRI2 and APP751. Panels 4 and 5 show Western blots of extracts from cells either expressing APP751 or APP751 and BRI2 using antibodies against either BRI2 (panel 4) or APP751 (panel 5).-To verify the BRI2-APP751 interaction, the opposite experiment was set; cell lysates were IP with anti-APP antibody R1(57) or preabsorbed R1(57) and immunoprecipitates analyzed by Western blot using the antibody R1(57) to detect APP or 9E10 (to detect myc-tagged BRI2) (Fig. 1B). From extracts of cells expressing APP751 and myc-tagged BRI2, R1(57) precipitated APP751 (panel 1, lane 1) as well as BRI2 (panel 2, lane 1) whereas IPs with preabsorbed R1(57) did not contain APP751 (panel 1, lane 2) or BRI2 (panel 2, lane 2). These data further support the conclusion that BRI2 interacts specifically with APP751. Panels 3 and 4 of Fig. 1B show Western blots of extracts of cells expressing APP751 and BRI2 using antibodies against APP751 (panel 3) or BRI2 (panel 4).

To determine whether the BRI2-APP interaction is APP isoform-specific, we also examined the BRI2 interaction with APP695. As illustrated in Fig. 1C, IPs of BRI2 (panel 1, lanes 2 and 3) from extracts of cells co-expressing myc-tagged BRI2 (panel 3, lanes 2 and 3) and APP695 (panel 4, lanes 2) or APP751 (panel 4, lane 2) resulted in their co-precipitation (panel 2, lanes 2 and 3), a clear indication that this interaction is not APP isoform-dependent. The anti-myc antibody did not precipitate APP695 (panel 2, lane 1) from extracts of cells expressing only APP695 (panel 4, lane 1).

The APP-BRI2 interaction was also observed in N2a cells between exogenously expressed BRI2 and endogenous APP (Fig. 1D). IPs of transfected BRI2 (panel 1, lane 2) resulted in the co-precipitation of APP (panel 2, lane 2). No APP was precipitated from extracts of cells transfected with vector (panels 1 and 2, lane 1).

To examine whether endogenous BRI2 interacts with endogenous APP, we used a polyclonal antibody (5727) recognizing the BRI2 segment 246–265. In extracts of HEK293T cells that express low levels of endogenous BRI2 and APP we performed IPs using preimmune serum, antibody 5727, or preabsorbed 5727 (Fig. 1E). Extracts and IPs were analyzed by Western blot using the antibody 5727 to detect BRI2 (panel 1) and R1(57) to detect APP (panel 2). BRI2 and APP were found to co-IP with the 5727 antibody (panels 1 and 2, lane 3) but not with preimmune serum (lane 2) or preabsorbed 5727 (lane 4).

Amino Acids 648–719 of APP Sequence Are Sufficient for Its Interaction with BRI2—To determine the APP sequence needed for the APP-BRI2 interaction, an APP cytoplasmic domain deletion construct, as well as an APP hybrid protein, were used in IP experiments with BRI2. Specifically, the two constructs used were (i) a deletion mutant lacking the 720–751 end part of the cytoplasmic region (APPΔ720) and (ii) a hybrid protein (APPΔ751) in which the C-terminal end was replaced by BRI2 (Fig. 2A). IPs of APPΔ720 (lane 1) and APPΔ751 (lane 2) were analyzed using antibodies to APP (panel 1) and BRI2 (panel 2). IPs of APPΔ720 were negative for BRI2 (lane 1, panel 2) whereas IPs of APPΔ751 were positive for BRI2 (lane 2, panel 2). These data clearly support that the cytoplasmic domain of APP is necessary for the interaction with BRI2.
protein ending at aa719, with its extracellular region 1–647 replaced by a derivative of human secreted alkaline phosphatase (SEAP-APPΔ720) (20). HEK293 cells were successfully co-transfected with the above constructs and BRI2 or its vector (prK5). As indicated in Fig. 2, cell extracts were incubated with the antibody 9B11 to IP the myc-tagged BRI2. Western blot analysis with anti-myc 9E10 and anti-APP antibody 6E10 revealed that all constructs interact with BRI2 (panels 1 and 2). Expression of transfected proteins was confirmed on the cell extracts (panels 3 and 4). Our data indicates that the fragment 648–719 of APP (751 numbering) is required for its interaction with BRI2.

The 46–106 BRI2 Sequence Is Sufficient for Its Interaction with APP—To narrow down the BRI2 sequence required for the APP–BRI2 interaction, BRI2 deletion constructs BRI2Δ1–45, BRI2Δ107, BRI2Δ147, BRI2Δ187, and BRI2Δ227 were prepared (see “Materials and Methods”) and used in transfection and IP experiments. HEK293 cells stably expressing APP751 were transfected with the above myc-tagged BRI2 deletion constructs or full-length BRI2 and IP using the 9B11 antibody. As illustrated in Fig. 3, A and B, all constructs interacted with APP, as indicated by the immunoreactivity with 9E10 and R1(57) antibodies. Expression of transfected proteins is shown in panels 3 and 4. We concluded that the 46–106 BRI2 sequence is sufficient for the interaction APP-BRI2 to occur.

The BRI2 Protein Decreases Secretion of APP and of Aβ Peptides—To examine whether BRI2 has a modulatory effect on APP695 and APP751 processing, we evaluated total levels of cellular APP, levels of APP COOH-terminal fragments that derive from α- or β-secretase activity (APP/CTFα and APP/CTFβ, respectively), secretion of sAPPα that derives from the

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**Fig. 1.** BRI2 interacts specifically with APP. A, HEK293 cells stably expressing APP751 were transfected with either myc-tagged BRI2 or the myc-tagged BRI2 bearing vector, prK5. IPs with the anti-myc antibody 9B11 (to precipitate myc-tagged BRI2), 9B11 preabsorbed with its antigenic peptide, or anti-transferrin receptor antibody were analyzed by Western blot (WB) using the anti-APP antibody R1(57) (panel 1), the anti-APP antibody R1(57) (panel 2), or the anti-transferrin receptor antibody (panel 3). Extracts of cells expressing APP or APP and BRI2 were analyzed by WB using the antibodies 9E10 (panel 4) or R1(57) (panel 5). B, HEK293 cells stably expressing APP751 were transfected with myc-tagged BRI2. IPs with R1(57) or R1(57) preabsorbed with its antigenic peptide were analyzed by WB using R1(57) (panel 1) or 9E10 (panel 2). Extracts of cells expressing APP and BRI2 were analyzed by WB using the antibodies R1(57) (panel 3) or 9E10 (panel 4). C, HEK293 cells were transfected with myc-tagged BRI2 or prK5 vector and either APP751 or APP695. IPs of the 9B11 antibody were analyzed by WB using the antibodies 9E10 (panel 1) or R1(57) (panel 2). Extracts were analyzed by WB using the antibodies 9E10 (panel 3) or R1(57) (panel 4). D, N2a cells endogenously expressing APP were transfected with myc-tagged BRI2 or prK5 vector. IPs with 9B11 antibody were analyzed by WB using 9E10 (panel 1) or R1(57) (panel 2) antibodies. E, in extracts of HEK293T cells that endogenously express BRI2 and APP we performed IP using preimmune serum (lane 2), 5727 anti-BRI2 antibody (lane 3), or 5727 preabsorbed with its antigenic peptide (lane 4). Extracts (lane 1) and IPs (lanes 2–4) were analyzed by WB using the antibodies 5727 (panel 1) or R1(57) (panel 2). IgG(H) denotes the heavy chain of IgG.

**Fig. 2.** The sequence 648–719 of APP751 is sufficient for its interaction with BRI2. HEK293 cells were co-transfected with the BRI2 vector prK5 and APP 751 (lane 1), BRI2 and APP 751 (lane 2), BRI2 and APPΔ720 (lane 3), or BRI2 and SEAP-APPΔ720 (lane 4). BRI2 protein was precipitated using the 9B11 antibody (panel 1) and co-precipitation of APP proteins was tested by WB with the 6E10 antibody (panel 2). Expression of transfected proteins was verified by WB using the myc-tagged BRI2 recognizing antibody 9E10 (panel 3) or the APP recognizing antibody 6E10 (panel 4). IgG(H) denotes the heavy chain of IgG.
activity of α-secretase, and secretion of Aβ peptides produced by the activity of β- and γ-secretase.

As indicated in Fig. 4A, co-expression of BRI2 with either APP695 or APP751 in HEK293 cells resulted in increased levels of cellular APP (panels 1) and a significant decrease in the levels of total secreted APP (panels 2) as well as of sAPPα (panels 3). Consistently, the levels of APP/CTFβ were found to be significantly diminished (panels 4); levels of APP/CTFα in vector-transfected cells were much higher relative to those of APP/CTFβ, in agreement to the knowledge that the majority of APP is processed by α-secretase rather than by β-secretase. The decrease in the APP/CTFβ levels was accompanied by an increase in the levels of APP/CTFβ (panels 4). The two bands that are designated as APP/CTFβs could derive after β-secretase cleavage of APP at different sites. The co-expression of BRI2 with either APP695 or APP751 in HEK293 cells resulted also in a significant decrease in the secretion of both Aβ1–40 and Aβ1–42, compared with cells expressing only APP695 or APP751 (p < 0.005) (Fig. 4B).

The shortest BRI2 deletion mutants were as efficient as the full-length BRI2 in exhibiting modulatory effect on APP processing. As illustrated in Fig. 4C, BRI2Δ1–45 and BRI2Δ107 retained the effect of wild type BRI2 on APP751 processing. Relative to HEK293 cells transfected with vector (prK5), transfection with wild type BRI2 and BRI2Δ107 increased the levels of cellular APP, while the BRI2Δ1–45 did not have a profound effect (Fig. 4C, panel 2). Similar to wild type BRI2, both deletion mutants decreased dramatically the levels of secreted sAPPα (Fig. 4C, panel 3) and secreted Aβ1–40 (Fig. 4D).

**DISCUSSION**

The data presented above indicate a protein–protein interaction between transmembrane proteins BRI2 and APP. The interaction was found to be specific and was observed with both APP751 and APP695, suggesting that it is not APP isoform-dependent. Moreover, the interaction was not restricted to proteins overexpressed in HEK293 cells, since it was also observed between endogenously expressed proteins in extracts of HEK293T cells. BRI2 is the first type II transmembrane protein found to interact with APP. However, it was demonstrated that APP interacts with the low density lipoprotein receptor-related protein (22), a type-I transmembrane protein. The use of several APP hybrid and deletion mutants revealed that the APP sequence between amino acids 648–719 is sufficient for their interaction. This sequence includes 33 amino acids of the APP extracellular domain (5 amino acids upstream of the first asparagine of the Aβ sequence plus 28 amino acids of the Aβ sequence), the transmembrane domain, and 15 amino acids of the cytoplasmic sequence. Of note, this segment encompasses all sites for secretase activity. The use of various BRI2 deletion mutants revealed that the BRI2 sequence between amino acids 46–106 is sufficient for its interaction with APP. This sequence includes 31 amino acids of the extracellular domain (5 amino acids upstream of the first asparagine of the Aβ sequence), the transmembrane domain, and 6 amino acids of the intracellular domain of BRI2. It is known that single transmembrane do-

**FIG. 3.** The 47–106 BRI2 sequence is sufficient for its interaction with APP. APP751 stably expressing HEK293 cells were transfected with BRI deletion constructs (see “Materials and Methods”). BRI proteins were precipitated with the 9B11 antibody (A and B, panels 1) and coprecipitation of APP was examined by WB using the R1(57) antibody (A and B, panels 2). Expression of myc-tagged BRI2 (A and B, panels 3) and APP751 (A and B, panels 4) was examined by WB analysis using the 9E10 and R1(57) antibodies, respectively. A single asterisk denotes the heavy chain of IgG and a double asterisk the light chain of IgG.

**FIG. 4.** BRI2 decreases secretion of sAPPα and of Aβ peptides. A, HEK293 cells were transiently transfected with the APP751 cDNA (0.5 μg) or APP695 (0.5 μg) and the BRI2 cDNA (3.5 μg) or pRK5 vector (3.5 μg). Cell extracts and media samples were analyzed by WB using the antibodies R1(57), 8E5, or 6E10 to detect the antigens indicated on the right of the panels. B, the levels of secreted Aβ peptides were measured by enzyme immunoassay and expressed as pg/ml medium/mg of total protein (values represent mean of four experiments performed in duplicates). C, HEK293 cells were transfected with the APP751 cDNA and the cDNAs encoding for BRI2, BRI2Δ1–45, BRI2Δ107, or pRK5 vector. Cell extracts and media samples were analyzed by WB using the antibodies 9E10, R1(57), and 6E10 to detect the antigens indicated on the right of the panels. D, the levels of secreted Aβ1–40 were measured by enzyme immunoassay and expressed as pg/ml medium/mg of total protein (values represent mean of two experiments performed in duplicates).
main proteins expressed on the plasma membrane of adjacent cells can interact in trans via their extracellular domains (23, 24). Alternatively, both proteins can be expressed in the same membrane of a single cell and interact in cis (25). The fact that most of the extracellular domains of APP and BRI2 are not required for their interaction suggests that these proteins interact in cis rather than in trans.

It has been shown that the interaction between LRP and APP increases the internalization of APP and the production of Aβ, while it decreases sAPPα secretion (26, 27). This effect has been attributed to an increased APP trafficking through the endocytic pathway that favors the amyloidogenic processing of APP (28). In our case, we found that expression of BRI2 stabilizes APP, while it dramatically decreases secretion of sAPPα. The latter data suggest that BRI2 inhibits the activity of α-secretase. In accordance, we found a significant decrease in the levels of APP/CTFα that was accompanied by an increase in the levels of APP/CTFβ. The latter result can be explained by an increased or unaltered activity of β-secretase and decreased activity of γ-secretase. Expression of BRI2 was also found to decrease secretion of both Aβ1–40 and Aβ1–42 suggesting that it affects the activity of γ-secretase. Our data indicate that BRI2 protein plays a strong regulatory role in the processing of APP. Thus regulation of the APP/BRI2 interaction in vivo may provide an alternative avenue to explore new therapeutic strategies for AD.

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BRI2 Interacts with Amyloid Precursor Protein (APP) and Regulates Amyloid β (Aβ) Production
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