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BRI2 homodimerizes with the involvement of intermolecular disulfide bonds

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Abstract

Familial British and Familial Danish Dementia (FBD and FDD) are two dominantly inherited neurodegenerative diseases that present striking similarities with Alzheimer's disease. The genetic defects underlying those dementias are mutations in the gene that encodes for BRI2 protein. Cleavage of mutated BRI2 by furin releases the peptides ABri or ADan, which accumulate in the brains of patients. BRI2 normal function is yet unknown. To unwind aspects of its cellular role, we investigated the possibility that BRI2 forms dimers, based on structural elements of the protein, the GXXXG motif within its transmembrane domain and the odd number of cysteine residues. We found that BRI2 dimerizes in cells and that dimers are held via non-covalent interactions and via disulfide bridges between the cysteines at position 89. Additionally, we showed that BRI2 dimers are formed in the ER and appear at the cell surface. Finally, BRI2 dimers were found to exist in mouse brain. Revealing the physiological properties of BRI2 is critical in the elucidation of the deviations that lead to neurodegeneration.

Keywords

Familial British Dementia; Familial Danish Dementia; Alzheimer's disease; Neurodegeneration; Homodimerization

1. Introduction

BRI2 is a type II transmembrane protein that is genetically linked with two rare autosomal dominant neurodegenerative disorders, Familial British Dementia (FBD) (Worster-Drought and McMenemey, 1933) and Familial Danish Dementia (FDD) (Stromgren et al., 1970). Patients with FBD or FDD bear two different mutations in the *Itih2b* gene (Pittois et al., 1998), which encodes for BRI2 protein. Specifically, a point mutation at the stop codon in FBD (Vidal et al., 1999) and a decamer duplication in FDD (Vidal et al., 2000), result in disruption of the open reading frame of the gene and the production of longer than normal BRI2 precursor

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proteins, referred to as ABriPP and ADanPP, respectively. BRI2 is cleaved by the prohormone convertase furin (Kim et al., 1999) and a soluble peptide of 23aa is released. ABriPP and ADanPP are cleaved at the same site as wild type BRI2 and the amyloidogenic peptides ABri or ADan of 34aa are released. These peptides accumulate in the brains of patients as parenchymal pre-amyloid or amyloid deposits and as vascular and perivascular deposits. More recently, another cleavage of BRI2 was described to occur in the *cis*-medial Golgi, which results in the production of an aminoterminal membrane-bound fragment, with unknown fate and function (Choi et al., 2004).

Concerning the physiological role of BRI2 there is limited information. It has been detected in nerve terminals and has been suggested to play a role in axonal transport (Akiyama et al., 2004). Other studies indicate that it may play a role in neurite outgrowth (Choi et al., 2004) and also to function as a tumor-suppressor (Latil et al., 2003). Furthermore, a splice variant of BRI2 detected in a murine T cell line was found to have pro-apoptotic properties (Fleischer et al., 2002; Fleischer and Rebollo, 2004).

BRI2 possesses two significant structural determinants which suggest that it could dimerize with other BRI2 molecules. First, it contains within its transmembrane domain the GXXXG motif that has been found to contribute in protein homo- and hetero-association. More specifically, in the α -helix of a transmembrane domain, the two glycines of the GXXXG motif appear at the same side, and, since glycine is a small aminoacid, they create a flat surface that allows close contact between two interacting molecules, facilitating extended van der Waals interactions and hydrogen bonding to occur (for a review see, Senes et al., 2004). Second, BRI2 contains nine cysteines, which suggests that at least one of them can be involved in the formation of an intermolecular disulfide bond with another BRI2 molecule or with another protein. Two extracellular cysteines (C248 and C265) are at the C-terminus, part of the peptides released following furin cleavage, and most likely are capable of forming an intramolecular disulfide bond. In fact, ABri and ADan have been found to exist in an either oxidized or reduced form, that correlate with their neurotoxicity (El-Agnaf et al., 2001; Gibson et al., 2004; Ghiso et al., 2001; Tomidokoro et al., 2005). Two other extracellular cysteines (C164 and C223) are believed to form an intramolecular disulfide bond and harbour a functional domain present in BRI2, the BRICHOS domain (Sanchez-Pulido et al., 2002). The remaining cysteines include one extracellular (C89), three transmembrane (C54, C56 and C58) and one intracellular (C38) (Fig. 1A). Among those, C89 is the only one exposed to the oxidizing extracellular environment and, therefore, could participate in the formation of an intermolecular disulfide bond.

Based on the structural elements described above, we studied the possibility that BRI2 forms homodimers, in an attempt to unravel novel aspects of its biological properties. Protein homodimerization has been found to be central in cellular processes, such as receptor function, signal transduction or cell adhesion (Heldin, 1995; Bulenger et al., 2005; Fannon and Colman, 1996). We showed that BRI2 forms homodimers held with disulfide bonds and that the extracellular cysteine C89 is involved in the intermolecular disulfide bonding. The dimers are present at the cell surface and also exist in mouse brain. The two mutated forms of BRI2 in British (ABriPP) and Danish (ADanPP) dementia retain the ability to form dimers, which appear normally at the cell surface. BRI2 dimerization could be important for its cellular function and, as a consequence, its study will give insights in the identification of the physiological role of the protein.

2. Methods

2.1. Chemicals

N-Ethylmaleimide (NEM) was purchased from Pierce (Rockford, IL, USA), and iodoacetic acid (IAA) was purchased from Sigma–Aldrich (Athens, Greece). Where indicated, they were

used to supplement the lysis buffer in a concentration of 20 mM for NEM, and 100 mM for IAA. Brefeldin A (BFA) (Sigma–Aldrich,) was used in a concentration of 5 µg/ml culture medium. All other common chemicals were purchased from Sigma unless otherwise indicated.

2.2. Plasmids and antibodies

Human BRI2 cDNA was subcloned in myc-pRK5 vector (kind gift of Dr. P.F. Worley, John Hopkins, Baltimore, MD) and in flag-pcDNA 3.1 vector (kind gift of Dr. K. Vekrelis, Foundation for Biomedical Research of the Academy of Athens, Athens, Greece). The deletion mutants of BRI2 (designated BRI2 Δ 107–266, BRI2 Δ 1–46) are previously described (Fotiniopoulou et al., 2005). BRI2 cDNA with an internal deletion (designated BRI2 Δ 75–106), and mutation of nucleotides that encode for glycines 60, 67, and 71 to triplets that encode for leucines, as well as BRI2 cDNA with mutations of nucleotides that encode for cysteines 38 (designated BRI2 C38A) and cysteines 89 (designated BRI2 C89A) to triplets that encode for alanines were generated with PCR using the QuickChange Site-Directed Mutagenesis kit (Stratagene, CA, USA). BRI2 cDNA bearing mutations of nucleotides that code for both cysteine 38 and cysteine 89 to nucleotides that encode for alanines (designated BRI2 C38/89A) were generated with PCR using the QuickChange Multi Site-Directed Mutagenesis kit (Stratagene, CA, USA). Primers are available upon request. The anti-myc monoclonal antibodies 9E10 (Iowa University Hybridoma Bank, Iowa, IA, USA) and 9B11 (Cell Signaling Technology, MA, USA) were used for western blot and immunoprecipitation of myc-tagged BRI2, respectively. The monoclonal antibody M2 against flag epitope used to detect flag-tagged BRI2 was from Sigma–Aldrich (Athens, Greece). The anti-BRI2 antibody T-1515 raised against the N-terminus of human BRI2, that was used to detect endogenous BRI2, was a kind gift of Dr. Pfeifer (BMA Biomedicals, Augst, Switzerland).

2.3. Cell culture and transfections

Human Embryonic Kidney cells (HEK293) were purchased from ATCC and cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with heat inactivated 10% Fetal Bovine Serum (both from PAA laboratories GmbH, Linz, Austria), and 100 µg/ml Penicillin/Streptomycin (Sigma–Aldrich, Athens, Greece). Transfections were performed with Escort IV reagent (Sigma–Aldrich, Athens, Greece), used according to manufacturer's instructions.

2.4. Protein extraction and western blotting

Forty-eight hours after transfection, conditioned media was removed. Cells were lysed at 4 °C, in lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, pH 7.6, 1% Triton X-100 (v/v)] supplemented with complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Samples were incubated for 30 min on ice and protein extracts were clarified by centrifugation at 15,000 × g for 30 min at 4 °C. The supernatants were quantified for protein content using the BCA assay kit (Pierce, Rockford, IL, USA). Mouse brains, used to identify endogenous BRI2, were homogenized in RIPA buffer [10 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, pH 7.6, 1% Triton X-100 (v/v), 1% deoxycholic acid and 0.1% SDS] supplemented with 20 mM NEM, and were subsequently sonicated and centrifuged at 15,000 × g for 30 min at 4 °C. Samples from cell or brain extracts were prepared in SDS Laemmli sample buffer (1% SDS, 0.4 M Tris, 40 mM EDTA, 50% glycerol, bromophenol blue) containing 5% β-mercaptoethanol, in experiments performed under reducing conditions, or without β-mercaptoethanol in experiments performed under non-reducing conditions. Extracts were separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Roth GmbH, Karlsruhe, Germany). Membranes were immunoblotted with the primary antibody diluted in a blocking solution containing 10% New-born Calf Serum supplemented with NaN₃ overnight at 4 °C. After washing with PBS devoid of NaN₃, filters were then incubated with horseradish peroxidase-conjugated goat anti-mouse (Zymed), or goat

anti-chicken secondary antibodies (Interchim, France) in blocking solution (1:5000 dilution) without NaN_3 for 1 h at room temperature. Protein bands were detected by chemiluminescence using the enhanced chemiluminescence (ECL) system (Amersham, Upsala, Sweden) on a Fluorochem 8800 imaging station (AlphaInnotech, CA, USA).

2.5. Co-immunoprecipitations (co-IPs)

For the immunoprecipitation experiments, cellular extracts of transfected cells (1000–1500 μg of total protein) were incubated with appropriate dilutions of the IP antibody overnight at 4 °C. Antibody-bound protein complexes were collected with protein G – agarose beads (Sigma, Athens, Greece) following incubation for 1 h at 4 °C and pellets were washed three times with washing buffer containing 50 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.6, 0.5% Triton X-100. The beads were resuspended in SDS Laemmli sample buffer and the recovered proteins were analyzed by immunoblot.

2.6. Biotinylation of cell surface proteins

Biotinylation of cell surface proteins was performed as described previously (Parisiadou and Efthimiopoulos, 2007). Briefly, 2 days post-transfection, cell cultures were washed three times with ice-cold PBS containing 0.5 mM CaCl_2 and 1 mM MgCl_2 (PBS-CM). Then, cells were biotinylated with 0.5 mg/ml Sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) for 30 min on ice-cold water. Fresh biotin solution was added for another 30 min. Unbound biotin was quenched with 50 mM NH_4Cl for 10 min. Cells were rinsed twice with ice-cold PBS-CM and lysed as described above. Cell extracts were centrifuged at $15,000 \times g$ for 30 min and supernatants were incubated with 50 μl of streptavidin–agarose beads (Pierce, Rockford, IL, USA) for 1 h at 4 °C. Following washing, biotinylated proteins were eluted in Laemmli SDS sample buffer and analyzed by western blot.

3. Results

3.1. BRI2 homodimerizes in a cellular system

To determine if BRI2 interacts with other BRI2 molecules in a cellular system, we co-expressed in HEK293 cells myc- and flag-tagged BRI2. We observed that immunoprecipitation of myc-BRI2 with 9B11 antibody (Fig. 1B, panel 1a, lane 3) resulted in co-precipitation of flag-BRI2 (Fig. 1B, panel 1b, lane 3). To exclude artificial post-lysis aggregation of the overexpressed proteins, we mixed the extracts of cells expressing myc-BRI2 alone with the extracts of cells expressing flag-BRI2 alone. In that sample ('mix'), we observed that immunoprecipitation of myc-BRI2 (Fig. 1B, panel 1a, lane 2) did not result in significant co-precipitation of flag-BRI2 (Fig. 1B, panel 1b, lane 2), showing that the observed dimerization existed prior to lysis. The 9B11 antibody did not precipitate flag-BRI2 from extracts of cells expressing flag-BRI2, but not myc-BRI2 (Fig. 1B, panels 1a and 1b, lane 1). Expression of the proteins was verified by WB analysis of cell extracts (Fig. 1B, panels 2a and 2b). In the reverse independent experiment (Fig. 1C), immunoprecipitation of flag-BRI2 with M2 antibody resulted in co-precipitation of myc-BRI2 (Fig. 1C, panels 1a and 1b, lane 3). The above results show that BRI2 forms homodimers in a cellular system.

3.2. Mutation of the GXXXG motif does not affect BRI2 homodimerization

After confirming that BRI2 homodimerizes, we examined whether the transmembrane GXXXG motif of BRI2 is involved in its homodimerization. We found that mutation of G67 or G71 to leucine did not affect the dimerization of myc-BRI2 with flag-BRI2 (data not shown), indicating that the GXXXG motif is dispensable for the homodimerization. However, we cannot exclude the possibility that this motif further stabilizes preformed dimers.

3.3. BRI2 homodimers are held with disulfide bonds

Given the fact that BRI2 contains an odd number (9) of cysteines, we hypothesized that one of them could be free to participate in an intermolecular disulfide bond between two BRI2 molecules. To test this suggestion, extracts of HEK293 cells that express myc-BRI2 were analyzed under reducing (with β -mercaptoethanol) or non-reducing conditions (without β -mercaptoethanol). Under non-reducing conditions (Fig. 2A, panel a, lane 2) we observed BRI2 monomers (44 kDa, 'M') as well as bands of ~88 kDa ('D') that correspond to BRI2 dimers. We also detected a band at ~22 kDa, which most likely corresponds to a furin-derived proteolytic cleavage product of BRI2, identical or similar to the one described before (Choi et al., 2004). In addition, we observed a band of ~66 kDa that, based on the molecular weight, we assume that represents proteolytic fragments of BRI2 linked with a disulfide bridge. Obvious also are fainter bands at higher molecular weights that could represent BRI2 oligomers. To exclude artificial disulfide bond formation during lysis, we added IAA (Fig. 2A, lane 3) or NEM (Fig. 2A, lane 4) in the lysis buffer to block free-thiols and prevent the formation of disulfide bonds during cell lysis. In these cases, dimers continue to appear under non-reducing conditions, which shows that they are not formed during lysis. As expected, under reducing conditions we observe only BRI2 monomers of ~44 kDa and the 22 kDa band (Fig. 2A, panel b). As an antibody specificity control, we analyzed by WB cell extracts that were not transfected with BRI2 (Fig. 2A, lane 1). The above results indicate that BRI2 forms disulfide-linked dimers.

3.4. Analysis of intermolecular disulfide linkages in BRI2 cysteine mutants

To identify the cysteine residue(s) involved in BRI2 homodimerization, all cysteine residues were replaced with alanines, in order to subsequently introduce the cysteines one by one and identify the responsible one(s). However, cysteineless BRI2 was unstable and could not be expressed in cells, possibly because some of those residues are crucial for proper protein folding and exit from the ER. Among the extracellular cysteines, there is evidence that cysteines 248 and 265, which exist within the sequence of the peptide released after furin cleavage form a disulfide bond. In fact, there are studies indicating that ABri and ADan exist in an either oxidized or reduced form (El-Agnaf et al., 2001; Gibson et al., 2004; Ghiso et al., 2001; Tomidokoro et al., 2005). Extracellular cysteines 164 and 223 are believed to form an intramolecular disulfide bond, and create a loop that defines the BRICHOS domain of BRI2 (Sanchez-Pulido et al., 2002). Cysteine C89, however, exists in the oxidizing extracellular environment and, based on the above, is the only one of the extracellular cysteines that seems to be uncoupled. The transmembrane cysteines (C54, C56 and C58) are unlikely to be involved in the homodimerization due to the fact that the environment of the lipid bilayer does not favour disulfide bond formation. Instead, transmembrane cysteines are frequently found to be palmitoylated (Resh, 2006). Intracellular cysteine 38 is also unique in the cytoplasmic part of the protein, but is unlikely to be involved in a disulfide bond, since the redox state of the cytoplasm makes disulfide bond formation difficult. However, intracellular compartmentalization could provide environments with slightly different redox state. It is also known that the redox state of the cytoplasm is able to change transiently, for example, after a stimulus. These conditions could theoretically allow disulfide bond formation between intracellular cysteines. Based on the above, we decided to examine the involvement of both extracellular C89 that lies in the oxidizing extracellular environment, as well as intracellular C38, which is unique in the reducing intracellular environment, in the formation of intermolecular disulfide bonds. For that purpose, we mutated C89 and C38 to alanine, which is an aliphatic, relatively small aminoacid, generally considered non-reactive (construct designated C89A and C38A, respectively). To establish our analysis, we created the double mutant of both C38 and C89 (C38/89A). We expressed myc-tagged BRI2, BRI2 C89A, BRI2 C38A or BRI2 C38/89A in HEK293 cells and performed WB analysis under reducing and non-reducing conditions. Under non-reducing conditions, we detected homodimers of BRI2 and

BRI2 C38A (Fig. 2B, panel a, lanes 1 and 3), but not of BRI2 C89A and BRI2 C38/89A (Fig. 2B, panel a, lanes 2 and 4), which indicates that only C89 is responsible for BRI2 intermolecular disulfide bonding. Under reducing conditions (Fig. 2B, panel b) only BRI2 monomers and the proteolytic fragment of 22 kDa were detected in every case. Next, we examined whether there are non-covalent interactions between BRI2 molecules that hold the dimers together, even in the absence of disulfide bonds. Myc-tagged BRI2, BRI2 C89A or BRI2 C38/89A were co-expressed with flag-BRI2 in HEK293 cells. Following myc immunoprecipitation (Fig. 2C, panel 1a, lanes 2–4), flag-BRI2 was co-precipitated at almost the same degree in every case (Fig. 2C, panel 1b, lanes 2–4). This indicates that non-covalent interactions exist between BRI2 dimers. Expression of transfected proteins was verified by WB analysis (Fig. 2C, panels 2a and 2b). To identify important regions involved in such non-covalent bonds, we co-expressed flag-BRI2 with myc-tagged BRI2, or the deletion mutants, BRI2 Δ 107 (lacks extracellular aa 107–266), BRI2 Δ 75–106 (lacks extracellular aa 75–106), BRI2 Δ 1–45 (lacks intracellular aa 1–45). We observed that immunoprecipitation of myc-BRI2 proteins led to co-precipitation of flag-BRI2 in every case (data not shown). These results indicate that two or more regions from those deleted in our experiments are involved in the non-covalent interactions of BRI2 dimers. However, it is possible that the transmembrane domain, present in all constructs, is involved in the dimerization.

3.5. BRI2 exists as a disulfide-linked dimer in mouse brain

The next important issue we addressed was whether endogenous BRI2 molecules form homodimers. To detect endogenous BRI2, we first tested for the specificity of the commercially available anti-BRI2 antibody T-1515, since published results using this antibody do not exist. Extracts of HEK293 cells transfected with myc-BRI2 or pRK5 vector were subjected to WB under reducing or non-reducing conditions (Fig. 3A), using the anti-BRI2 antibody T-1515, T-1515 pre-incubated with its antigenic peptide or the 9E10 antibody against the myc-epitope of BRI2. Our data show that T-1515 specifically detects dimers (Fig. 3A, panel a) and monomers (Fig. 3A, panel b) of BRI2. After confirming the antibody specificity, we used it to analyze adult mouse brain samples homogenized in lysis buffer supplemented with NEM. Under non-reducing conditions, we observed BRI2 monomers as well as dimers in extracts from mouse brain (Fig. 3B, panel a, lane 2) and in extracts from HEK293 cells transfected with human myc-tagged BRI2 (Fig. 3B, panel a, lane 3). Under reducing conditions only BRI2 monomers were observed (Fig. 3B, panel b). Under both reducing and non-reducing conditions, in mouse extracts we also observed a prominent T-1515 immunoreactive band at about 31 kDa (Fig. 3B, panels a and b, lane 2). Mouse brain BRI2 migrates at a slightly lower molecular weight than transfected human myc-BRI2 (Fig. 3B, panels a and b, compare lane 2 and 3). The difference in the molecular weight is more evident under reducing conditions, when proteins usually appear as sharper bands. We calculated that endogenous mouse BRI2 migrates as a single band at 38 kDa compared to the 44–41 kDa (immature–mature form) of exogenously expressed human myc-tagged BRI2 in HEK293 cells. When the antibody was pre-incubated with its antigenic peptide no signal was detected (Fig. 3B, lane 1).

3.6. BRI2 dimers are formed in the ER and appear at the cell surface

Most proteins that dimerize, form dimers at tandem or right after their translation in the ER. A few exceptions from that rule exist, for proteins that dimerize in Golgi (for example see Musil and Goodenough, 1993). To discriminate between these possibilities, HEK293 cells expressing myc-BRI2 were treated with (Fig. 4A, lane 2) or without Brefeldin A (BFA) (Fig. 4A, lane 1). This drug rearranges Golgi membranes into the ER and does not allow BRI2 to enter the Golgi and be cleaved by furin. As a result, in the presence of BFA only immature, non-cleaved BRI2 was detected (Fig. 4A, lane 2 compared to lane 1). Under non-reducing conditions BRI2 dimers were detected in the presence of BFA (Fig. 4A, panel a, lane 2), which indicates that dimers are formed as early as in the ER. However, the intermediate band of 66

kDa was not observed, which reinforces our view that this band represents proteolytic fragments of BRI2 linked with disulfide bridges, since BRI2 cleavage takes place in post-ER compartments.

Given that many proteins (e.g. receptors) have a functional role in the cell plasma membrane as dimers, we investigated if BRI2 dimers reach the cell surface. Surface proteins of HEK293 cells expressing myc-BRI2, were labelled with biotin. Biotinylated proteins were precipitated with streptavidin–agarose beads followed by WB analysis. As shown in Fig. 4B, BRI2 monomers, dimers and the intermediate ~66 kDa band, are present at the cell surface under non-reducing conditions (Fig. 4B, panel 1a, lane 1). Under reducing conditions, we detected only BRI2 monomers (Fig. 4B, panel 1b, lane 1). The ER-resident protein calnexin was not detected in streptavidin complexes (Fig. 4B, panel 1c, lane 1), indicating that the biotin ester did not penetrate the plasma membrane. Without biotin treatment no protein is detected at the cell surface (Fig. 4B, panels 1a, 1b and 1c, lane 2). In Fig. 4B, panels 2a, 2b and 2c represent WB analysis from total cell extracts.

3.7. ABriPP and ADanPP form dimers that reach the cell surface

Our next aim was to identify if the ability of BRI2 to dimerize is retained in ABriPP and ADanPP mutated forms of BRI2 linked with FBD and FDD, respectively. For that purpose, we expressed myc-BRI2, ABriPP, or ADanPP in HEK293 cells and analyzed cell extracts by WB. Under non-reducing conditions (Fig. 5A, panel a, lanes 2 and 3) ABriPP and ADanPP were detected as monomers and also as disulfide-linked dimers like BRI2 (Fig. 5A, panel a, lane 1). Lastly, we examined if similar to BRI2, ABriPP and ADanPP dimers are present at the cell surface. ABriPP monomers, dimers and the intermediate ~66 kDa band, are present at the cell surface under non-reducing conditions (Fig. 5B, panel 1a, lane 1). Under reducing conditions, only ABriPP monomers are visible (Fig. 5B, panel 1b, lane 1). Again, calnexin was not detected in streptavidin complexes (Fig. 5B, panel 1c, lane 1) and without biotin treatment no protein is detected at the cell surface (Fig. 5B, panels 1a, 1b and 1c, lane 2). WB analysis of total cell extracts is shown in Fig. 5B, panels 2a, 2b and 2c. Identical results were obtained for ADanPP (data not shown).

4. Discussion

BRI2 is a central protein in the pathogenesis of two neurodegenerative diseases, Familial British and Familial Danish Dementias (FBD and FDD). Patients with those dementias express two mutated forms of BRI2, ABriPP and ADanPP that upon cleavage by furin release the peptides ABri and ADan. These peptides are amyloidogenic and accumulate as pre-amyloid or amyloid plaques in the brains of patients. FBD and FDD share striking neuropathological similarities with Alzheimer's disease (AD), which is characterized by brain deposition of A β peptide that results from processing of Amyloid Precursor Protein (APP). We showed previously (Fotinou et al., 2005; Matsuda et al., 2005) that BRI2 interacts with APP and inhibits its processing and the release of A β peptide. This data brings up BRI2 as a molecule that, via regulation of APP processing, may also be implicated in the development of AD. Furthermore, BRI2–APP interaction could be the basis for the striking neuropathological similarities between AD and FBD/FDD. Therefore, studying the biological properties of BRI2 could give insights in the pathological processes involved in these dementias. In addition, it may indicate common cellular pathways involved in neurodegeneration.

The biological role of BRI2 is poorly understood. To reveal novel aspects of BRI2 biological properties, we studied the possibility that BRI2 forms homodimers, based on its structural elements. First, the GXXXG motif that exists in the transmembrane domain of BRI2 is known to mediate transmembrane helix–helix interactions. In most cases examined so far the GXXXG motif is important for homo-association, and in less for hetero-association of protein molecules

(Senes et al., 2004). In some instances this motif was found to have functional significance. For example, mutations of the GXXXG motif of A β -1 interfere with the assembly and activity of γ -secretase (Lee et al., 2004; Edbauer et al., 2004) and cause a loss-of-function phenotype in *C. elegans* (Goutte et al., 2002). Apart from that, BRI2 contains in its aminoacid sequence an odd number of cysteines (9) (Fig. 1A). Since cysteines are considered to be very active aminoacids, it is possible that at least one of them is free to create a disulfide bond with the corresponding free cysteine of another BRI2 molecule.

In the current paper, we showed that BRI2 is able to homodimerize in a cellular system and that those dimers are linked with disulfide bonds. The formation of an S–S bond requires oxidizing conditions that are available at the extracellular environment. In contrast, the transmembrane domain and the internal of the cell are not favourable environments for the formation of disulfide bonds. Thus, we suggested that the three transmembrane cysteine residues of BRI2 (C54, C56 and C58) and the one intracellular (C38) are not likely to be involved in a disulfide bond formation. Of the five extracellular cysteines C248 and C265, exist within the peptide sequence, which is released after furin cleavage, and have been found to form an intrachain disulfide bond (El-Agnaf et al., 2001; Gibson et al., 2004; Ghiso et al., 2001; Tomidokoro et al., 2005). The same is believed for extracellular cysteines C164 and C223, since through the formation of an intrachain disulfide bond they create the loop of the BRICHOS domain of BRI2 (Sanchez-Pulido et al., 2002). Thus, C89 is the only uncoupled cysteine in the extracellular space, available to form an interchain disulfide bond. Indeed, mutation of C89 to alanine inhibited the formation of disulfide-bonded dimers, when mutation of intracellular C38 had no effect. Although we cannot exclude the possibility that other intermolecular disulfide bonds exist, the formation of a bond between cysteines C89 seems to be the prerequisite for other disulfide bonds to be formed. Disulfide bond formation seems to be important for the dimerization of other proteins including several GPCRs, such as the mGluR 1 (Robbins et al., 1999), mGluR5 (Romano et al., 1996), calcium-sensing receptor (Fan et al., 1998; Ward et al., 1998; Goldsmith et al., 1999; Pace et al., 1999; Ray et al., 1999), the muscarinic receptor (Zeng and Wess, 1999) and the human prostacyclin receptor (Giguere et al., 2004). Additionally, the insulin receptor, its homologues IGF-R and IRR (reviewed in Ottensmeyer et al., 2000) and the human transferrin receptor (Jing and Trowbridge, 1987) exist as disulfide-linked dimers.

Next, we sought to determine if apart from disulfide bonds, non-covalent interactions drive dimer formation, as is the case for other proteins like the muscarinic receptors (Zeng and Wess, 1999) and the Ca-sensing receptor (Zhang et al., 2001). Our data showing that C89A myc-tagged BRI2 mutant co-precipitates with flag-BRI2 suggests that non-covalent bonds can hold together BRI2 dimers in the absence of intermolecular disulfide bonds. As a conclusion, BRI2 dimers are held together via both disulfide bonds and non-covalent interactions.

Most proteins dimerize/oligomerize in the ER, although, some, like gap junction connexin 43 (Musil and Goodenough, 1993), polymerize after exit from the ER. BRI2 dimers were found to exist in the ER, which shows that dimerization occurs intracellularly soon after translation of BRI2 mRNA. Those dimers are then transferred to the cell membrane, since we found that BRI2 dimers together with monomers appear at the cell surface.

An important question was whether endogenous BRI2 exists as dimers. Western blot analysis of mouse brain extracts showed that endogenous mouse BRI2 exists as monomers and dimers. Mouse BRI2 monomers and dimers ran slightly slower than human myc-BRI2 used as a control. The molecular weight previously reported for mouse BRI2 is 37 kDa for the immature form and 34 kDa after cleavage by furin (Pickford et al., 2003), whereas we detected a single band at 38 kDa. Similar differences in molecular weight between human and mouse BRI2 have been observed for other proteins, such as presenilin 1 (Thinakaran et al., 1996). Surprisingly, we

detected a prominent band at 31 kDa, under both reducing and non-reducing conditions, which implies that this form of the protein does not form disulfide-linked dimers. We suggest that this band could represent an alternatively spliced form of BRI2 that exists in mouse brain. In support, another form of BRI2 derived from alternative splicing has been previously described in murine T cell line (Fleischer et al., 2002). Consequently, it is possible that different forms of BRI2 occurring from alternative splicing do exist in vivo. Alternatively, that band could represent the product of a proteolytic cleavage event occurring in mouse brain, or it could be a consequence of differential post-translational modification of BRI2 in mouse tissues compared to HEK293 cells.

The mutated forms of BRI2 in FBD and FDD, ABriPP and ADanPP, were also observed to form disulfide-linked dimers present at the cell surface. The issue of whether those dimers are functional remains to be elucidated, since it is possible that the conformational change of the mutated forms of BRI2 compared to wild type BRI2 alters the properties of the dimers.

Other proteins involved in neurodegenerative diseases have also been found to form homodimers. For example, APP was found to be able to form homodimers that appear at the cell surface (Scheuermann et al., 2001), with subsequent studies showing that APP and its mammalian paralogs APLP1 and APLP2 form homo- and heterocomplexes that play a CAM-like role in cell–cell adhesion (Soba et al., 2005). Also, BACE 1, one of the enzymes that cleave APP to release A β peptide was found to exist as dimers, and dimeric BACE 1 presented a higher affinity and turnover rate of its substrate compared to monomeric BACE 1 (Westmeyer et al., 2004). Presenilin, the catalytic entity of the γ -secretase complex, that cleaves APP, in both the amyloidogenic and the non-amyloidogenic pathway, was found to be dimeric too (Schroeter et al., 2003).

The above studies together with numerous others demonstrate that protein dimerization is a feature that could play an important role in the function of proteins. Ongoing studies in our lab aim to identify a BRI2 mutant construct that is unable to form dimers. This construct will constitute an invaluable tool in revealing the physiological importance of dimerization for BRI2. Given that BRI2 dimers are formed in the ER, it is reasonable to assume that BRI2 is presented to furin as a dimer. Thus, dimerization could be a prerequisite for BRI2 processing by furin and the release of the C-terminal peptides. This is of major significance for BRI2, since dimerization could prove a regulating mechanism for the release of the amyloidogenic peptides in FBD and FDD. In addition, as shown for other proteins, dimerization of BRI2 could be important for its stability or intracellular trafficking and expression at the cell surface. Most important, dimers could have a serious involvement in BRI2 cellular function. The presence of dimers at the cell surface, for instance, could mean that BRI2 acts as a receptor-like molecule or is part of a receptor complex that participates in signal transduction pathways.

Deposition of ABri and ADan is an important neuropathological feature of FBD and FDD that most probably plays a central role in the development of the corresponding pathologies. Additionally, we believe that these mutations could lead to dementia by affecting the physiological role(s) of BRI2. Our study contributes in the understanding of the biological properties of BRI2 that may prove important for exerting its function(s) and paves the way for unravelling the malfunctions that lead to disease.

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Fig. 1.

(A) Schematic representation of the cysteine residues of BRI2. (B) Co-immunoprecipitation of differently tagged BRI2 proteins. HEK293 cells were transfected with myc-pRK5 vector and flag-BRI2 (lane 1) or myc-BRI2 and flag-BRI2 (lane 3). Myc-BRI2 was immunoprecipitated using the anti-myc antibody 9B11 (IP9B11). As a control, extracts of cells expressing myc-BRI2 or flag-BRI2 alone were mixed after lysis (lane 2, 'mix') and were also incubated with 9B11. Immunoprecipitates were analyzed by western blot using the anti-myc antibody 9E10 (1a) or the anti-flag antibody M2 (1b). Cell extracts were analyzed by western blot with anti-myc (2a) or anti-flag (2b) antibody to verify expression of the corresponding proteins. Extr: 20 µg of extracts of HEK293 cells transfected with myc-tagged human BRI2 as positive control. (C) Extracts of HEK293 cells transfected with flag-pcDNA3.1 vector (pcflag) and myc-BRI2 (lane 1) or flag-BRI2 and myc-BRI2 (lane 3) were incubated with the anti-flag antibody M2. As a control, extracts of cells expressing myc-BRI2 or flag-BRI2 alone were mixed after lysis (lane 2, 'mix') and were also incubated with M2. Immunoprecipitates were analyzed by western blot using the anti-flag antibody M2 (1a) or the anti-myc antibody 9E10 (1b). Cell extracts were analyzed by western blot with anti-flag (2a) or anti-myc (2b)

antibody to verify expression of the corresponding proteins. Extr: 20 µg of extracts of HEK293 cells transfected with myc-tagged human BRI2 as positive control.

Fig. 2.

(A) Disulfide bonds are involved in BRI2 homodimerization. myc-BRI2 was expressed in HEK293 cells and cells were lysed in lysis buffer without IAA and NEM (lane 2), or in lysis buffer containing IAA (lane 3) or NEM (lane 4). Western blot of the cell extracts under non-reducing conditions (w/o β -merc, a) showed the formation of BRI2 dimers in every case, whereas under reducing conditions (w/ β -merc, b) there are only BRI2 monomers. As a control of antibody specificity, extracts of cells that did not express BRI2 were analyzed by western blot under reducing and non-reducing conditions (lane 1). M: monomers; D: dimers. (B) Analysis of intermolecular disulfide linkages in BRI2 cysteine mutants. Extracts of HEK293 cells transfected with the cDNAs encoding myc-tagged BRI2 (lane 1), BRI2 C89A (lane 2), BRI2 C38A (lane 3) or BRI2 C38/89A (lane 4) were analyzed by western blot under non-reducing (w/o β -merc, a) or reducing (w/ β -merc, b) conditions. Extr: 20 μ g of extracts of HEK293 cells transfected with myc-tagged human BRI2 as positive control. (C) Involvement of non-covalent interactions in the dimerization. HEK293 cells were transfected with flag-BRI2 and either myc-pRK5 vector (lane 1), BRI2 (lane 2), BRI2 C89A (lane 3) or BRI2 C38/89A (lane 4). Immunoprecipitation of myc-tagged BRI2 proteins from cell extracts with anti-myc antibody 9B11 was followed by western blot analysis with anti-myc (1a) or anti-flag antibody (1b). Total cell extracts were analyzed by WB with anti-myc (2a) or anti-flag (2b) antibody to determine expression of the transfected proteins. Extr: 20 μ g of extracts of HEK293 cells transfected with myc-tagged human BRI2 as positive control.

Fig. 3.

(A) Specificity of the T-1515 antibody. Extracts of cells transfected with BRI2 or myc-pRK5 vector were analyzed by WB under non-reducing (w/o β -merc, a) and reducing (w/ β -merc, b) conditions using the anti-BRI2 antibody T-1515 preabsorbed with its antigenic peptide (lanes 1 and 2), the T-1515 antibody (lanes 3 and 4) or the anti-myc antibody 9E10 (lanes 5 and 6). (B) BRI2 exists as a monomer and a dimer in mouse brain. Mouse brain extracts were subjected to WB under non-reducing (w/o β -merc, a) and reducing (w/ β -merc, b) conditions using the anti-BRI2 antibody T-1515 preabsorbed with its antigenic peptide (lane 1) or the T-1515 antibody (lane 2). As a control, we used HEK293 cell extracts expressing human myc-BRI2 (lane 3), which migrates at 44 kDa (mature form) at the monomeric state and at 88 kDa at the dimeric state, while endogenous mouse BRI2 migrates slightly faster.

Fig. 4.

(A) Formation of disulfide-linked dimers in the ER. HEK293 cells were transfected with myc-BRI2. Twenty-four hours post-transfection cells were left untreated (lane 1) or treated with Brefeldin A (lane 2). Cell extracts were analyzed by western blot under non-reducing (w/o β -merc, a) or reducing conditions (w/ β -merc, b) using 9E10 antibody. (B) Disulfide-linked dimers exist at the cell surface. HEK293 cells were transfected with myc-BRI2. Forty-eight hours post-transfection cell surface proteins were biotinylated (lane 1) or not (lane 2) as control of biotinylation specificity. Cell extracts were incubated with streptavidin beads and precipitated proteins were analyzed by western blot under non-reducing (w/o β -merc, 1a) or reducing (w/ β -merc, 1b and 1c) conditions using the anti-myc antibody 9E10 (1a and 1b) or the anti-calnexin antibody (1c). Total cell extracts were also analyzed by western blot under non-reducing (w/o β -merc, 2a) or reducing (w/ β -merc, 2b and 2c) conditions to verify successful expression of proteins. Extr: 20 μ g of extracts of HEK293 cells transfected with myc-tagged human BRI2 as positive control.

Fig. 5.

(A) The British (ABriPP) and Danish (ADanPP) mutated forms of BRI2 form disulfide linked dimers. HEK293 cells were transfected with wild type BRI2 (lane 1), ABriPP (lane 2) or ADanPP (lane 3). Cell extracts were analyzed by WB with anti-myc antibody 9E10 under non-reducing (w/o β -merc, a) or reducing (w/ β -merc, b) conditions. (B) Disulfide-linked dimers of ABriPP exist at the cell surface. myc-ABriPP was expressed in HEK293 cells. Forty-eight hours post-transfection cell surface proteins were biotinylated (lane 1) or not (lane 2). Biotinylated proteins were precipitated with streptavidin beads and analyzed by western blot with anti-myc antibody 9E10 (1a and 1b) or the anti-calnexin antibody (1c) under non-reducing (w/o β -merc, 1a) or reducing (w/ β -merc, 1b and 1c) conditions. Total cell extracts were also analyzed under non-reducing (w/o β -merc, 2a) or reducing (w/ β -merc, 2b and 2c) conditions. Extr: 20 μ g of extracts of HEK293 cells transfected with myc-tagged human BRI2 as positive control.