Axonal transport of British and Danish amyloid peptides via secretory vesicles

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ABSTRACT

The ABri and ADan amyloid peptides deposited in familial British and Danish neurodegenerative disorders are generated by processing mutant forms of the precursor protein BRI2. Although the pathogenic process that leads to deposition of amyloid in the brains of patients has been studied extensively, the cellular processes and normal function of the precursor protein did not receive much attention. We observed in a variety of transfected cell lines the presence of two independent proteolytic processing events. In addition to the previously described cleavage, which results in the production of carboxyl-terminal ~3 kDa wild-type peptide or ~4 kDa ABri or ADan peptides, we describe a novel amino-terminal cleavage site within BRI2. Both cleavages occur within the cis- or medial-Golgi. Following cleavage, the BRI2-derived carboxyl-terminal peptides are transported via a regulated secretory pathway into secretory vesicles in neuronal cells. Worth noting is that expression of wild-type British or Danish mutants of BRI2 in mouse neuroblastoma N2a cells that do not express endogenous BRI2, induces elongation of neurites, which suggests a role for this protein in differentiation of neuronal cells.

Key words: amyloid • axonal transport • familial British and Danish dementia • BRI2 processing • neurite outgrowth

Familial British (FBD) and Danish (FDD) dementias are early-onset autosomal dominant neurodegenerative diseases. FBD is clinically characterized by progressive cognitive impairment, spasticity, and cerebellar ataxia (1–4). FDD is characterized by progressive ocular and hearing impairments, dementia, and cerebellar ataxia (5). The pathological findings in these patients consist of widespread amyloid angiopathy in the cerebrum, cerebellum, and spinal cord, and the presence of mainly non-neuritic amyloid plaques and neurofibrillary tangles in the hippocampus (5). In FBD patients amyloid fibrillar peptides also deposit in blood vessels of several peripheral tissues, including pancreas and myocardium, which indicates systemic deposition of amyloid in these patients (6, 7). Cerebrovascular amyloid, parenchymal amyloid, and non-amyloid protein deposits are composed of the ABri peptide in FBD and the ADan peptide in FDD (8, 9). The BRI gene family contains at least three members: BRI1, BRI2, and
Mutants of the \textit{BRI}_2 gene [also known as \textit{ITM2B} (11)] are associated with FBD and FDD.

\textit{BRI}_2 is a Type II single-spanning transmembrane precursor protein, which lacks a signal sequence for transport through the membrane of the endoplasmic reticulum (8). Wild-type \textit{BRI}_2 is composed of 266 amino acids with a molecular weight of 30,329 daltons. FBD patients have a point mutation at codon 267 (T for A) in the \textit{BRI}_2 gene (8). The mutation changes the stop codon into an arginine (Stop-267Arg), and as a result the open reading frame of the \textit{BRI}_2 protein is extended for an additional 11 carboxyl-terminal amino acids (277 amino acids; 8). FDD patients have a 10-nucleotides duplication of the DNA sequence encoded between nucleotides 786 to 795 of the wild-type precursor cDNA sequence. This DNA duplication produces the loss of the carboxyl-terminal serine (codon 266) and causes a change in the reading frame that generates an extension of 12 amino acids (9). The ABri and ADan 34 amino acid peptides have the same amino-terminal amino acid sequence but different carboxyl-terminal 12 amino acids. They are generated by endoproteolytic processing within the carboxyl-terminus of mutant \textit{BRI}_2 between arginine 243 and glutamic acid 244 (12). It has been suggested that furin, a secretory pathway endoprotease, may be involved in the endoproteolytic processing of \textit{BRI}_2 and in the generation of the ABri and ADan peptides (12, 13). However, the correlation between endoproteolytic processing, normal biological function, and subcellular localization of wild-type and mutant forms of \textit{BRI}_2 is unclear.

Here we show the existence of two endoproteolytic sites within \textit{BRI}_2 and that both occur in the \textit{cis-} or \textit{medial-} Golgi apparatus. Furthermore, Bri, ABri, and ADan peptides are transported via a regulated secretory pathway into secretory vesicles in neuronal cells. Finally, we demonstrate that stable expression of wild-type \textit{BRI}_2 and the mutant forms of \textit{BRI}_2 cDNA in neuronal cells stimulates neurite outgrowth.

\textbf{MATERIALS AND METHODS}

\textbf{BRI}_2 cDNA expression constructs

\textit{BRI}_2, \textit{BRI}_2-B, and \textit{BRI}_2-D cDNAs (8, 9) were obtained by RT-PCR from total RNA isolated from frozen brain of one individual with FBD (\textit{BRI}_2-B) and one individual with FDD (\textit{BRI}_2-D) by using forward (CGA ATT CGG ATG GTG AAG GTG ACG) and reverse (TTG AAT TCG TAA AGG GTG GGG) oligonucleotides. Wild-type \textit{BRI}_2 cDNA were present in both patients. These cDNAs were subcloned into pCR 2.1 vector (Invitrogen Life Technologies, Carlsbad, CA). After digestion with \textit{EcoRI}, the \textit{BRI}_2 cDNAs were subcloned into the \textit{EcoRI} site of the pCMV-Myc vector (Clontech Laboratories, Palo Alto, CA) encoding an amino-terminal 27 amino acid myc-epitope or into pcDNA3.1 (Invitrogen Life Technologies) vector with carboxyl-terminal \textit{V5-His} epitope. Constructs with amino acid myc-epitope were subcloned into pCR3.1 (Invitrogen Life Technologies), for neomycin (G418) resistance. The resulting plasmids were sequenced in both directions to check the orientation of the insert and that the gene was in frame with the Myc coding sequence.

\textbf{RNA isolation and RT-PCR}

For amplification of \textit{BRI}_2, \textit{furin}, and \textit{GAPDH}, total RNA was isolated from seven cell lines by extraction in TRIZOL Reagent (Invitrogen Life Technologies). cDNA synthesis and DNA
amplification was performed using Superscript™ One-Step RT-PCR System (Invitrogen Life Technologies).

**Transfection of tissue culture cells**

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37°C in 5% CO₂ atmosphere. The media were supplemented with 10% fetal bovine serum (Invitrogen Life Technologies), Penicillin (100 U/ml) and streptomycin sulfate (100 µg/ml; Invitrogen Life Technologies). Cells were transfected by using LipofectAMINE plus reagent (Invitrogen Life Technologies) for either transient or stable expression of the genes. Stably transfected N2a cells were selected with Geneticin (G418, Invitrogen Life Technologies). Overexpression of BRI₂, ABriPP, or ADanPP was confirmed by Western blot analysis of cell lysates.

**Western blot analysis**

Transfected cells were grown to near confluence by 48 h. The cells were harvested in phosphate buffered saline pH 7.3 (PBS), solubilized in RIPA buffer (1% NP-40, 0.5% cholic acid, 0.1% SDS; 150 mM NaCl, 10 mM Tris/HCl, pH 8.0) containing protease inhibitors and were centrifuged at 10,000 x g for 10 min at 4°C. Fifty micrograms of total proteins from cell homogenates were separated by 16.5% Tris/Tricine SDS-PAGE. The proteins were electrophoretically transferred to PVDF membrane (Millipore Corporation, Bedford, MA), and the membrane was blotted with mouse anti-c-myc (2 µg/ml) antibodies (Clontech Laboratories, Palo Alto, CA). Horseradish peroxidase-linked goat anti-mouse was used as secondary antibody (1:5,000) (Amersham Pharmacia Biotech, Piscataway, NJ). Immunoblots were visualized using the ECL system (Amersham Pharmacia Biotech). The Gel Documentation system (Bio-RAD Laboratories, Hercules, CA) and the Quantity One 1-D analysis software were used for comparison of the level of BRI₂ processing.

**Metabolic labeling**

Transiently transfected N2a cells grown on 35 mm dishes were labeled 4 h after transfection. Cells were preincubated with cysteine-free DMEM containing dialyzed 0.5% fetal bovine serum (Invitrogen Life Technologies) and incubated with 0.3 mCi/ml ³⁵S-cysteine (PerkinElmer Life and Analytical Sciences, Boston, MA) in the same medium for various time lengths in the presence or absence of inhibitors: 50 µM furin inhibitor (decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone; Bachem Bioscience Inc. King of Prussia, PA) (50 µM), 5 µg/ml Brefeldin A (Sigma, St. Louis, MO) or 5 µM monensin (Sigma). The media were collected and spun at 4,500 x g for 10 min at 4°C. The cells were harvested in PBS, lysed in 500 µl RIPA buffer containing protease inhibitors, and centrifuged at 10,000 x g for 10 min at 4°C. Cell lysates and media were each divided into two equal volumes and immunoprecipitated with either anti-c-myc or 338 antibodies and with Dynabeads coated with sheep anti-mouse IgG or sheep anti-rabbit IgG (Dynal Biotech, Lake Success, NY). The immunoprecipitated proteins were separated by 16.5% Tris/Tricine SDS-PAGE. The gels were enhanced with ENHANCE (Amersham Pharmacia Biotech) and exposed to X-ray films for 3 to 7 days at –80°C.
Indirect immunofluorescence

Transiently transfected N2a cells grown on coverslips coated with poly-L-Lysine (Clontech Laboratories) were permeabilized and fixed in methanol at –20°C for 3 min. Cells were washed in phosphate buffered saline pH 7.4 (PBS), blocked with 10% bovine serum albumin (Sigma) in PBS for 10 min, and incubated with primary antibody in blocking buffer for 1 h at 37°C. Cells were incubated with secondary antibodies for 1 h at 37°C. The coverslips were mounted on glass slides by using Vectashield mounting medium (Vector Labs Inc., Burlingame, CA). Cells were viewed by using Leica UV microscopy system (Leica DMLB).

Primary antibodies used: ABriPP carboxyl-terminal antibody (338; 1:500 dilution; 8), ADanPP carboxyl-terminal antibody (5282; 1:500 dilution; 9), antibody to the amino-terminal myc-tag (Myc-FITC; 1:100 dilution; Invitrogen Life Technologies), antibody to the carboxyl-terminal V5 tag (V5-FITC; 1:500 dilution; Invitrogen Life Technologies), anti-calnexin (1:200 dilution; Stressgen Biotechnologies, Inc. San Diego, CA), anti-carboxypeptidase E (CPE; 1:250 dilution; BD Bioscience, San Diego, CA). Secondary antibodies used: Texas Red-labeled anti-rabbit IgG (1:200 dilution; Vector Laboratories), FITC-labeled anti-mouse IgG (1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA).

Neurite outgrowth analysis

Minimally two distinct clones of each stably transfected N2a cell line were freshly plated and then cultured for 4 days. Computer-assisted image analysis was used to determine neurite length in five separate fields within the center of each well. Six wells per group were examined, and at least 200 cells were counted for each group. Differences between groups were analyzed by ANOVA test.

RESULTS

Proteolytic processing of BRI2

To examine the production, processing, and secretion of wild-type and variant BRI2, tissue culture cell lines were transiently transfected with amino-terminal myc epitope-tagged BRI2, BRI2-B, or BRI2-D cDNAs. Western blot analysis with anti-myc antibody of cell lysates of N2a cells transfected with BRI2 revealed three bands of ~44, ~41, and ~17 kDa, and ~45, ~41, and ~17 kDa in BRI2-B, or BRI2-D transfected cells (Fig. 1). These bands correspond to full-length BRI2 and two amino-terminal fragments (NFT). The ~41 kDa fragment is the amino-terminal product of a described previously cleavage between amino acid 243 and 244 (12, 13). This cleavage also results in the production of carboxyl-terminal ~3 kDa wild-type peptide or ~4 kDa ABri or ADan peptides. We made cDNA constructs with amino-terminal myc-tag that either contained or did not contain carboxyl-terminal V5-His epitope tag and found that the addition of the carboxyl-terminal tag affected BRI2 processing. Therefore, all the experiments thereafter were conducted by using cDNA constructs without carboxyl-terminal tag.

A lower amount of the ~41 kDa amino-terminal fragment was observed in cells expressing the mutant BRI2 cDNAs compared with cells expressing the wild-type BRI2 (Fig. 1).
To examine the possible contribution of furin to BRI2 processing, we analyzed endogenous furin mRNA expression in various cell lines (Fig. 2). RT-PCR analysis showed furin mRNA expression in all cell lines tested. Whereas LoVo cells express an inactive form of furin (14, 15), expression of BRI2, BRI2-B, and BRI2-D in these cells resulted in the same pattern of BRI2 processing as was seen in other transfected cells (Fig. 2B). A similar processing pattern was observed in a variety of cell lines transfected with BRI2, BRI2-B, and BRI2-D cDNAs: HEK 293, NIH3T3, CHO-K1, HepG2, LoVo, and SK-N-SH (Fig. 2B). Interestingly, no endogenous BRI2 mRNA expression was detected in CHO and N2a cells (Fig. 2A).

A processing product of ~17 kDa (Fig. 1) was also observed in all transfected cells. The ~17 kDa band was not recognized by using antibodies specific for the carboxyl-terminus of ABriPP and ADanPP proteins. This fragment suggests an additional cleavage site within BRI2, amino-terminal to the site resulting in the production of the ~41 kDa fragment. Treatment of cell extracts with PNGaseF did not modify the molecular weight of the ~17 kDa band, suggesting that this new cleavage site occurs amino-terminally to the N-glycosylation site at position 170 (unpublished observation).

**The temporal profile of BRI2 expression, processing, and secretion**

For metabolic labeling experiments, N2a cells transiently transfected with BRI2, BRI2-B, or BRI2-D cDNA were labeled with 35S-cysteine for various lengths of time. Immunoprecipitation with anti-myc antibody, directed against the amino-terminus of the protein, showed that cleavage of the full-length protein occurs within 10 min of labeling to produce a ~41 kDa fragment, and a ~17 kDa product is produced within half an hour of labeling. Both processing products accumulate intracellularly thereafter (Fig. 3). These processing products were not detected in culture media. The ~4 kDa fragment, precipitated with antibodies to the carboxyl-terminus of either ABriPP or ADanPP, is observed in cell lysates within 10 min of labeling and is secreted into the cultured medium in 1 h of chase (Fig. 3). Both wild-type BRI2 and the mutated forms, ABriPP and ADanPP, showed the same temporal profile of processing and secretion (data not shown).

We describe here the existence of two processing reactions of the ~44 kDa BRI2 and the ~45 kDa BRI2-B or BRI2-D. The first, described previously cleavage between amino acid 243 and 244 (12, 13), results in the production of a ~41 kDa amino-terminal fragment and either a ~3 kDa wild-type or ~4 kDa ABri or ADan amino-terminal peptides. The newly described cleavage, producing a ~17 kDa amino-terminal product (Figs. 1 and 3), occurs amino-terminally to the N-glycosylation site at position 170. The intensity of the bands may suggest that the ~17 kDa peptide (Fig. 1) is produced by a proteolytic mechanism that is independent of the production of the ~41 kDa band. However, using antibodies directed against the carboxyl-terminus of the protein, we could not detect bands of ~27 or ~28 kDa, suggesting that the ~17 kDa peptide is produced by further proteolytic cleavage of the ~41 kDa peptide. Two cleavages within BRI2 suggest the existence of a third processing fragment of ~24 kDa. However, we do not have an antibody directed against the center region of BRI2 that would recognize this ~24 kDa fragment.

**Cellular localization of BRI2 processing**

Metabolic labeling experiments in the absence or presence of various inhibitors were performed to determine the intracellular location of BRI2 processing. N2a cells transiently transfected with
BRI2-B cDNA were labeled with $^{35}$S-cysteine for 4 h. The ~45 kDa full-length ABriPP and the ~41 and ~17 kDa amino-terminal fragments were immunoprecipitated by anti-myc antibody from cell lysates of untreated cells (Fig. 4A). The carboxyl-terminal ~4 kDa was precipitated by anti-ABri antibody (338) from cell lysates (Fig. 4B) and media proteins (Fig. 4C).

Decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone, furin inhibitor that directly inhibits all furin family enzymes by binding to their catalytic site (16), does not affect BRI2 processing (Fig. 4).

Brefeldin A blocks protein transport from the endoplasmic reticulum and causes redistribution of Golgi components to the endoplasmic reticulum (17). Metabolic labeling in the presence of Brefeldin A causes accumulation of full-length ABriPP and inhibition of production of the amino-terminal processing products of ~41 and ~17 kDa (Fig. 4A) and of the carboxyl-terminal ~4 kDa ABri (Fig. 4B, C).

Monensin, which inhibits transport from the medial-Golgi to trans-Golgi (18, 19), does not affect ABriPP processing but inhibits secretion of ABri (Fig. 4C).

These results demonstrate that both proteolytic processing reactions of BRI2 occur in the cis- or medial-Golgi apparatus, independently of the presence of the protease furin.

**Intracellular transport of ABri via a regulated secretory pathway into secretory vesicles in neuronal cells**

Experiments for the intracellular localization of BRI2 and Bri were performed by using indirect immunofluorescence. Staining of N2a cells transiently transfected with amino-terminal myc-tagged BRI2 cDNA with anti-myc antibody revealed cytoplasmic staining (Fig. 5A). The cytoplasmic staining partially colocalized with staining with the anti-calnexin-CT antibody used as a marker for the endoplasmic reticulum (Fig. 5A). Staining of N2a cells transiently transfected with carboxyl-terminal V5-tagged BRI2 cDNA with anti-V5 antibody revealed similar cytoplasmic staining, as well as punctate staining along the cell process (Fig. 5B). This staining colocalized with anti-calnexin-CT antibody staining only in the cell soma. To confirm that a carboxyl-terminal fragment containing ABri of ABriPP is present in axons, we double-stained transfected cells with anti-myc antibody and anti-ABri (338) antibody (data not shown). Although high degree of overlap was observed in cell soma, punctate staining in cell processes was observed only with the anti-ABri (338) antibody.

To determine whether the carboxyl-terminal fragment of BRI2 is secreted via regulated secretory pathway, cells were stained with antibody to carboxypeptidase E (CPE), secreted in regulated secretory vesicles (20). Staining of N2a cells expressing BRI-B with anti-ABri antibody (338) colocalized with staining by using anti-CPE antibody (Fig. 5C, D). Similarly, carboxyl-terminal staining of BRI2 with anti-V5 antibody or ADanPP with anti-ADan antibody colocalized with staining for CPE (data not shown).

Taken together, these immunofluorescence microscopic observations demonstrate that full-length and amino-terminal fragments of wild-type and mutant forms of BRI2 are restricted to the cell soma and that only the carboxyl-terminal fragment is secreted via the regulated secretory pathway.
Expression of BRI2 promotes neurite outgrowth in N2a cells

N2a cells that do not express BRI2 mRNA, as demonstrated by RT-PCR (Fig. 2A), were stably transfected with BRI2, BRI2-B, BRI2-D cDNAs, or vector alone. Cells expressing any of the BRI2 cDNAs had longer neurites compared with N2a cells transfected with vector alone (Fig. 6A). Measurements of neurite lengths (described in Materials and Methods) showed a significant induction of elongation of neurites (Fig. 6B).

DISCUSSION

Proteolytic processing of BRI2 plays a crucial role in the pathological progress of FBD and FDD (8, 9). We have studied the processing, subcellular localization, and secretion of processing products of BRI2 by using cultured cell lines. Two cleavage sites were identified resulting in i) an intracellular ~41 kDa amino-terminal peptide and the corresponding ~3-4 kDa carboxyl-terminal fragment secreted into the cultured media and ii) a novel ~17 kDa amino-terminal fragment. A similar pattern of processing of BRI2, ABriPP, and ADanPP was observed in a variety of cell lines. Using intracellular transport-inhibitors and immunofluorescence microscopy we demonstrated that both cleavages occur in cis- or medial-Golgi and, although the amino-terminal fragments accumulate intracellularly, carboxyl-terminal processing products are secreted via a regulated secretory pathway. Finally we demonstrated induction of neurite outgrowth in neuronal cells by BRI2 expression.

In all cell lines tested transfection with either BRI2-B or BRI2-D cDNAs resulted in less production of the ~41 kDa fragment compared with cells transfected with the wild-type BRI2 cDNA. The ~41 kDa fragment is the product of a described previously cleavage between amino acid 243 and 244 (12, 13). This cleavage also results in the production of carboxyl-terminal ~3 kDa wild-type peptide or ~4 kDa ABri or ADan peptides. Both ABriPP and ADanPP differ from wild type BRI2 in the carboxyl-terminal extension that may affect the proteolytic processing of the precursor proteins.

It was previously demonstrated that furin cleaves the APLP1-epitope-tagged BRI2 and associated variants, ABriPP and ADanPP, between amino acids 243 and 244 and that this proteolytic activity was not present in the furin-deficient RPE.40, CHO derived cell line (12, 13). Cotransfection of furin with BRI2 or its mutants led to the appearance of the secreted peptides into the medium (12, 13). However, the sequence amino-terminal to the cleavage site is an atypical furin recognition sequence (21). Kim et al. (22) have shown that other members of the PC family (PACE4, LPC, PC 5/6) process BRI2 and ABriPP, albeit inefficiently. Furthermore, co-expression of human furin in CHO-K1 and N2a cells had a negligible effect on enhancing the production of secreted peptides derived from either BRI2 or ABriPP. Our data indicate that the carboxyl-terminal proteolytic processing occurs in LoVo cells that lack the endogenous furin processing activity (14, 15). Furin cDNA cloned from LoVo cells had two mutations (14, 23) that account for the processing incompetence of LoVo cells. Furthermore, we describe a novel amino-terminal proteolytic activity and demonstrate that the proteolytic processing of BRI2, ABriPP, and ADanPP occurs in the cis- or medial-Golgi and not in the trans-Golgi network where furin activity is predominantly localized (24, 25). In addition, neither proteolytic activity was inhibited by adding a furin inhibitor or by adding monensin, an inhibitor of the transport from the medial-Golgi to the trans-Golgi. Therefore, these data indicate that, although furin can cleave BRI2, a proteolytic enzyme, other than furin, expressed in the cis- or medial-Golgi
apparatus, is involved in cleavage of the carboxyl-terminus of BRI2. This enzyme exhibits a ubiquitous cell-type expression, including in N2a cells that do not express BRI2 mRNA.

It was recently shown that ABri contains a disulfide-bond (26). A soluble form of ABri was identified in serum of FBD patients using immunoprecipitation, mass spectrometry, and Western blot analysis. A peptide with a mass consistent with ABri peptide with oxidized cysteine residues was found (7). The results suggested that some of the soluble molecules might contain a single intrachain disulfide bond. Similarly, ABri peptide with the same molecular mass was identified in conditioned medium of N2a cells transfected with BRI2-B cDNA (12). Disulfide-bonded loops within proproteins and neuropeptides were shown to be essential for sorting of peptides from the trans-Golgi network to regulated secretory pathway (27, 28). Immunofluorescence staining of N2a cells transfected with BRI2-B cDNA revealed co-localization of ABri epitopes with CPE in the axons of N2a cells, suggesting transport of ABriPP carboxyl-terminal fragments via a regulated secretory pathway. Cys5 and Cys22 in the amino-terminus of Bri may be playing a role in Bri sorting into the regulated secretory pathway. These two cysteine residues are conserved in all three BRI isoforms.

Although the neuropathology of the chromosome 13 dementias, FBD and FDD, is similar to that of Alzheimer’s disease (AD), several differences exist. Both exhibit vascular and parenchymal amyloid, pre-amyloid deposition, and neurofibrillary tangles (NFTs) in neurodegenerating neurons (29). In AD, parenchymal Aβ deposits are mainly present in the hippocampus and the cerebral cortex, and Aβ vascular lesions are predominantly seen in leptomeningeal and cortical vessels. Degenerating neurons containing NFTs are frequent in limbic areas and in the neocortex. In FBD, however, severe amyloid angiopathy with perivascular plaque formation occurs throughout the central nervous system. Parenchymal amyloid plaques and pre-amyloid deposits together with NFT pathology predominantly affect limbic structures but rarely the cerebral cortex. The distribution of NFTs in FBD corresponds to Stage IV in the system recommended by Braak and Braak for AD (30). Moreover, systemic deposition of amyloid was observed in FBD patients (7). The presence of soluble amyloid peptides in plasma is also similar between FBD and AD, although the plasma levels of soluble ABri are higher than soluble Aβ. Immunohistochemical analysis of FBD and FDD brain sections demonstrated the presence of proteins of the complement system and their pro-inflammatory activation products that are among the inflammation markers associated with AD lesions (31). The data suggest that, similar to AD, the chronic inflammatory response generated by the amyloid peptides in vivo might play a role in the pathogenesis of FBD and FDD.

The secreted peptides Aβ, Bri and its variants, ABri, and ADan are proteolytic products of precursor proteins, produced intracellularly and constitutively secreted into the extracellular space. However, the processing pathways of these proteins are different. Alternative processing pathways of βAPP have been described. α-Secretase processing of βAPP occurs mainly at the cell surface but also in a late compartment of the constitutive secretory pathway probably in a late trans-Golgi compartment. Following internalization of βAPP via clathrin-coated vesicles, Aβ is produced by a β-secretase. A small fraction of Aβ is generated in the endoplasmic reticulum/intermediate compartment and the trans-Golgi network. γ-Secretase activity occurs during recycling of endosomes to the cell surface, for review see (32). Thus, although the full-length βAPP is transported to the cell surface and various products are secreted, the data presented here demonstrate that two proteolytic processing reactions of wild-type and mutant
forms of BRI2 occur in the cis- or medial-Golgi apparatus and that only carboxyl-terminal fragments are transported via regulated secretory pathway through axons for secretion.

In spite of the similarities between the neurodegenerative disorders, there are differences in the transport and processing of the precursor proteins and the sequence and structure of the secreted peptides that form amyloid fibrils, Abri in FBD, and ADan in FDD are unrelated to those of Aβ in AD. Thus, amyloid formation and the resultant neurodegeneration are not dependent on the primary structure of the peptide or on the cellular transport and production of the peptide. This may suggest that a still unknown factor(s) induce the same pathological pathways leading to similar neurodegenerative disorders.

Despite increasing evidence for a pathogenic role for ABriPP and ADanPP in FBD and FDD, respectively, the physiological function of the protein remains unknown. Significant amino acid sequence identity exists between the carboxyl-terminal hydrophobic domains of the various BRI2 isoforms (10). The three proteins have a conserved sequence (KR) before the predicted cleavage site, suggesting a bioactivity for the secreted peptides. We have shown that stable expression of BRI2 in N2a cells that do not express endogenous BRI2 mRNA, stimulate neurite outgrowth. The induction of neurite outgrowth by expression of BRI2 may suggest a role for this protein in differentiation of neuronal cells. This activity may be independent or may not be altered by the presence of the carboxyl-terminal extension of BRI2 that originates ABri and ADan peptides because similar neurite outgrowth was observed by expression of the mutant forms of BRI2.

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Figure 1. Proteolytic processing of BRI\textsubscript{2}, ABriPP, and ADanPP in N2a cells. Western blot analysis with anti-myc antibodies of lysates of N2a cells transiently transfected with amino-terminal myc-tagged- BRI\textsubscript{2}, BRI\textsubscript{2}-B, or BRI\textsubscript{2}-D. Arrowheads show the full-length precursor proteins and the two amino-terminal processing fragments (NTF) of ~41 and ~17 kDa. The band of ~23 kDa was seen also in blots of cells transfected with vector alone. Molecular masses in kDa are indicated.
Figure 2. BRI₁ mRNA expression and protein processing in various cell lines. A) Absence of endogenous BRI₁ mRNA in CHO and N₂a cells. RT-PCR analysis of endogenous expression of furin, BRI₁, and GAPDH in a variety of cell lines. B) The proteolytic processing of BRI₁, ABriPP, and ADanPP and production of the ∼41 kDa proteolytic product in a variety of cell lines is similar to N₂a cells. Western blot analysis with anti-myc antibodies of lysates of cells transiently transfected with amino-terminal myc-tagged BRI₁, BRI₁-B, or BRI₁-D.
Figure 3. Both the amino-terminal ~17 kDa and the carboxyl-terminal ~4 kDa processing products observed at 0.5 h of labeling. N2a cells transiently transfected with BRI$_2$-B were labeled with $^{35}$S-cysteine for various time periods. Cell lysate and media proteins were immunoprecipitated with either anti-myc antibody, directed against the amino-terminus of ABriPP, or ABriPP carboxyl-terminal antibody (338).
Figure 4. **ABriPP processing occurs in the cis- or medial-Golgi apparatus.** N2a cells transiently transfected with BRFL-B cDNA were labeled with $^{35}$S-cysteine for 4 h in the presence or absence of inhibitors. Cell lysate and media proteins were immunoprecipitated with antibodies to either the amino-terminal myc-tag (A) or to the carboxyl-terminus of ABri (338; B and C). The film was exposed for 15 h (top panels) or 7 days (bottom panels). The full-length ~45 kDa protein, the amino-terminal ~41 and ~17 kDa, and the carboxyl-terminal ~4 kDa processing fragments are shown. Symbols used: untreated cells (–), furin inhibitor (F), Brefeldin A (B), monensin (M), and untreated cells transfected with vector (V). Molecular masses in kDa are indicated.
Figure 5. Subcellular localization of BRI2 and its carboxyl-terminal fragment by immunofluorescent staining of N2a cells transiently transfected with BRI2-B cDNA. A) Amino-terminal staining of BRI2 with anti-myc (FITC) colocalizes with the endoplasmic reticulum marker, anti-calnexin (Texas Red) in cells transiently transfected with BRI2 cDNA. B) Carboxyl-terminal staining of BRI2 with anti-V5 (FITC) colocalizes with the endoplasmic reticulum marker, anti-calnexin (Texas Red) in cells transiently transfected with BRI2 cDNA. C, D) Carboxyl-terminal staining of ABriPP with anti-ABri, 338 (Texas Red in C and FITC in D) co-localizes with carboxypeptidase E (CPE) (FITC in C and Texas Red in D) in regulated secretory vesicles.
Figure 6. Expression of BRI2 promotes neurite outgrowth in N2a cells. A) Induction of neurite outgrowth in N2a cells stably expressing BRI2, ABriPP, or ADanPP compared with cells expressing vector alone. B) Neurites length was measured using NIH-Image J in arbitrary units.