Proteolytic Processing of Familial British Dementia-associated BRI Variants

EVIDENCE FOR ENHANCED INTRACELLULAR ACCUMULATION OF AMYLOIDGENIC PEPTIDES*

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Different mutations in the BRI2 gene cause rare neurodegenerative conditions, termed familial British dementia (FBD) and familial Danish dementia (FDD). The mutant genes encode BRI-L and BRI-D, the precursors of fibrillogenic ABri and ADan peptides, respectively. We previously reported that furin processes both BRI-L and its wild type counterpart, BRI, resulting in the secretion of C-terminal peptides; elevated levels of peptides were generated from BRI-L. In the present study, we show that inducible expression of α1-antitrypsin Portland, a furin inhibitor, inhibits the endoproteolysis of BRI and BRI-L in a dose-dependent manner. Moreover, comparison of the activities of several proprotein convertases reveals that furin is most efficient in endoproteolysis of BRI and BRI-L; PACE4, PC6A, PC6B, and LPC show much lower activities. Interestingly, LPC also exhibits enhanced cleavage of BRI-L compared with BRI. Finally, we demonstrate that BRI-D is also processed by furin and, like BRI-L, the cleavage of BRI-D is more efficient than that of BRI. Interestingly, while the ABri peptide is detected both intracellularly and in the medium, the ADan peptide accumulates predominantly in intracellular compartments. We propose that intracellular accumulation of amyloidogenic ADan or ABri peptides results in the neuronal damage leading to FDD and FBD, respectively.

Familial British dementia (FBD),1 an autosomal dominant neurodegenerative disorder, is characterized by progressive spastic tetraparesis, cerebellar ataxia, and dementia (1). The principal pathological hallmarks of FBD include severe amyloid angiopathy, non-neuritic amyloid plaques affecting cerebellum, hippocampus, and cerebral cortex, hippocampal neurofibrillary tangles, and perivascular white matter changes. The genetic defect underlying FBD was recently identified (2); after excision of the 10-nucleotide (TTTAATTTGT) duplication between codons 265 and 266, just prior to the normal termination codon. This deamer duplication leads to the loss of serine 266 and a change in the reading frame to generate an 11-amino acid longer protein (BRI-D) that has a unique C-terminal sequence. The resulting C-terminal 34-amino acid peptide, termed ADan, is the major component of insoluble amyloid fibrils in brains of FBD patients (7). Thus, ABri and ADan peptides have the same length and identical N-terminal amino acid sequence (first 22 residues), but the C-terminal 12 amino acids are different.

We previously demonstrated that BRI is a type II integral membrane protein and that both wild type BRI and the FBD-associated mutant BRI-L, are processed by furin between amino acids 243 and 244, resulting in the production of C-terminal −3 kDa wild type and −4 kDa ABri peptides, respectively, which are released into the conditioned medium (8). Notably, secretion of ABri peptide derived from the mutant BRI-L precursor is enhanced compared with that of peptide generated from wild type BRI, suggesting that the C-terminal eleven amino acids extension in mutant BRI-L effects furin-mediated proteolysis in a dominant fashion. Although furin is necessary and sufficient for the endoproteolysis of BRI and BRI-L in our cellular model, the fact that the sequence N-terminal to the scissile bond, −KGQKR is an atypical furin recognition sequence (9) provoked us to examine the potential role of other members of the subtilisin/kexin-like proprotein convertase (PC) family in the processing of BRI and BRI-L. Seven members of PC family have been identified including furin, PC1 (or PC3), PC2, PC4, PACE4, PC5 (or PC6; two isoforms of PC6A and PC6B), and PC7 (or LPC/PC8) (9–11). Of these PCs, we chose to assay furin, PACE4, PC6A, PC6B, and LPC, because they are ubiquitously expressed in peripheral tissues and brain and are active in the constitutive secretory pathway (12–14). PC1 and PC2 are expressed only in neuronal and endocrine tissues (15), but their activity is restricted to the regulated secretory pathway. Expression of PC4 is restricted to gonadal tissues (16) and therefore is not relevant for this study.

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‡ The abbreviations used are: FBD, familial British dementia; APP, amyloid precursor protein; α1-FDX, α1-antitrypsin Portland; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; FDD, familial Danish dementia; PC, proprotein convertase; Tricine, N,N,N′-tris(hydroxymethyl)-N′-ethythalamine; GST, glutathione S-transferase.

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In this report, we have analyzed the effects of the selected PCs on BRI processing and examined the potential differences in proteolytic processing of wild type BRI, FBD-associated mutant BRI-L, and FDD-associated mutant BRI-D.

We now report that furin is most efficient in endoproteolysis of BRI and BRI-L; other PCs including PACE4, PC6A, PC6B, and LPC show much lower activities. Interestingly, LPC, like furin, also exhibits enhanced cleavage of BRI-L compared with wild type BRI but PC6A cleaves wild type and mutant molecules with similar efficiency. Furthermore, we demonstrate that the newly described Danish variant, “BRI-D” is also processed by furin and, like BRI-L, the furin cleavage of BRI-D is more efficient than that of wild type BRI. Interestingly, while the ABri peptide is detected both intracellularly and in the medium of transfected cells, the ADan peptide accumulates predominantly in intracellular compartments.

EXPERIMENTAL PROCEDURES

Generation of Expression Constructs—pRK5-BRI and pRK5-BRI-L constructs that encode APLP1-tagged BRI and BRI-L, respectively, were described previously (8). For edysone inducible expression of α1-PDX, FLAG–α1-PDX cDNA (17) (provided by Dr. Gary Thomas) was digested with SmaI and Asp718 and subcloned into pGEM7z vector (Promega, Madison, WI). The insert from this plasmid was liberated with HindIII and XbaI and subcloned into the edysone inducible expression vector pIND (Invitrogen, Carlsbad, California). Expression constructs encoding PACE4, PC6A, PC6B, and LPC were described previously (18).

Antibodies—Glutathione S-transferase (GST) fusion proteins containing the N-terminal cytosolic 52 amino acids of human BRI were expressed in bacteria and purified and used to immunize New Zealand White rabbits to generate cBRI-NT antibody. Antibodies raised against furin, PACE4, PC6, and LPC (MP1) were described previously (18, 19). Anti-FLAG antibody (M2) was purchased from Sigma.

Cell Culture and Transfection—Mouse neuroblastoma N2a cells were maintained in 50% Dulbecco’s modified Eagle’s medium (DMEM) and 50% of OptiMEM (Invitrogen) supplemented with 5% fetal bovine serum (FBS). Furin-defective RPE.40 cells were cultured in F12 medium (Invitrogen, Carlsbad, California). Expression constructs encoding PACE4, PC6A, PC6B, and LPC were described previously (18).

RESULTS

Effects of α1-PDX on BRI Processing—We previously demonstrated that furin is necessary and sufficient for the endoproteolytic processing of the type II membrane proteins, BRI and BRI-L (8). However, the sequence N-terminal to the scissile bond of BRI and BRI-L, −KGIQKR is an atypical furin recognition sequence (9) and hence, it was conceivable that other members of the subtilisin/kexin-like PC family may also play a role in processing BRI and BRI-L. To assess the specificity of furin processing, we first examined the effects of a-antitrypsin (Fig. 1A), a bioengineered furin inhibitor, on the processing of BRI. This inhibitor contains a furin consensus motif in its reactive site loop (Ala355–Ile-Pro-Met358→Arg355–Ile-Pro-Ang360) (13, 21) and potently and relatively specifically inhibits furin (IC50 = 0.6 nM) (17). We generated a stable HEK293 cell line that coexpresses BRI-L harboring an N-terminal Myc tag and a C-terminal CT11 tag and an edysone-inducible, FLAG-tagged α1-PDX polypeptide. In this stable cell line, α1-PDX was readily inducible with an increasing dose of muristerone A (Fig. 1A, upper panel), as the level of this inhibitor was increased, both the C-terminal fragment of BRI-L (Fig. 1A, lower panel) and secreted ABri peptides (Fig. 1B) were markedly reduced. Instead, full-length BRI-L accumulated as a function of α1-PDX expression (Fig. 1A, middle panel). Similar inhibitory effects of α1-PDX were also observed for wild type BRI (data not shown). These results indicate that α1-PDX is capable of inhibiting the protease involved in the processing of BRI and BRI-L.
Effects of Other Proprotein Convertases on BRI Processing—
Although α1-PDX was initially designed as a specific furin inhibitor, several reports have shown that α1-PDX also inhibits the activity of other members of proprotein convertase family (furin, PACE4, PC6A, PC6B, or LPC) or APP (for control) were labeled with [35S]cysteine for 2 h. Cell lysates (upper panels) and conditioned medium (lower panels) were immunoprecipitated with αBRI-NT and CT11 antibodies, respectively and analyzed by 10% Tris/glycine (upper panels) and 16.5% Tris/Tricine (lower panels) SDS-PAGE. The intensity of each band was quantified by Phosphorimager and relative levels of secreted C-terminal peptides normalized to those of full-length molecules were calculated (C). The relative level of ABri compared with the wild type C-terminal peptide for each PC is also shown to demonstrate different efficiencies of PCs against BRI and BRI-L (D). E, expression of the proprotein convertases was confirmed by immunoprecipitation analyses with respective antibodies. Immunoprecipitated materials were run on 7% SDS-PAGE and visualized by Phosphorimager. In panels A, B, and E, molecular mass markers are in kDa.

Effects of Other Proprotein Convertases on BRI Processing—
Although α1-PDX was initially designed as a specific furin inhibitor, several reports have shown that α1-PDX also inhibits the activity of other members of PC family to different extents (17, 21). To more directly examine the potential role of other members of the PC family, particularly those expressed in brain, in the processing of BRI and BRI-L in RPE.40 cells do not express functional furin (22–24), and BRI is not processed in these cells unless furin is coexpressed (8). Cells were labeled with [35S]cysteine for 2 h, and intracellular and secreted BRI-derivatives were analyzed by immunoprecipitation with the αBRI-NT, an antibody raised against the N-terminal 52 amino acids of BRI, and CT11 antibodies, respectively. As we previously reported, BRI and BRI-L are not proteolytically cleaved by furin in RPE.40 cells (Fig. 2, A and B, lanes 1 and 1’), but proteolysis is completely restored by coexpression of furin (Fig. 2, A and B, lanes 2 and 2’). Notably, the N-terminal fragment of BRI was present, albeit at lower levels, when PC6A or LPC was coexpressed (Fig. 2A, lanes 4 and 6). Similarly, the level of the secreted C-terminal peptide was most abundant in medium of cells expressing furin, but lower levels of the peptide were apparent in medium of cells that coexpressed PC6A or LPC (Fig. 2A, lanes 4’ and 6’). Phosphorimaging quantification revealed that PC6A and LPC process BRI at ~20% of the efficiency of furin (Fig. 2C). PACE4 and PC6B failed to show proteolytic activity against BRI (Fig. 2A, lanes 3 and 3’). Interestingly, LPC, like furin, also exhibits higher activity against BRI-L compared with wild type BRI (Fig. 2A and B, compare lanes 6’; quantified in D). However, and for reasons not presently clear, PC6A appears to cleave wild type and mutant molecules with similar efficiency (Fig. 2A and B, compare lanes 6; quantified in D). These data further support our earlier suggestion that the 11-amino acid extension in BRI-L induces local structural alterations that differentially affect proteolytic processing by the PCs. In any event, we demonstrate that the differences in processing efficiency by PCs are not due to failure to express the proteases, as expression of each PC was confirmed by immunoprecipitation analyses using respective antibodies (Fig. 2E). Although it is difficult to quantify the activities of each protease in the transfected cells, we would expect that the expression level of all the...
PCs in these transient transfections should be enough to reach a saturation level to proteolyze the cotransfected substrate.

Proteolytic Processing of the Danish Variant of BRI—We then examined the proteolytic processing of a recently identified Danish variant of BRI (BRI-D) (7). We generated cDNA encoding an APLP1-tagged BRI-D and transiently transfected the construct into CHO-K1 or RPE.40 cells. Detergent cell lysates and conditioned medium were prepared and analyzed by Western blot with αBRI-NT and CT11 antibodies respectively. αBRI-NT antibody detected a ~42-kDa full-length BRI-D protein in lysates of both cell lines and an ~37 kDa BRI-D “NTF” in CHO-K1 cells (Fig. 3A, lanes 1 and 2). However, the BRI-D NTF was not observed in RPE.40 cells (Fig. 3A, lane 3). Parallel analysis of the conditioned medium of transfected CHO-K1 cells revealed the presence of a ~4-kDa ADan peptide (Fig. 3B, lanes 1 and 2). In contrast, we failed to detect any secreted peptides in the medium of RPE.40 cells expressing BRI-D (Fig. 3B, lane 3). However, coexpression of BRI-D with furin in RPE.40 cells led to the production and secretion of the ~4-kDa ADan peptide (Fig. 3B, lane 4), in parallel with the production of a BRI-D NTF in cell lysates (Fig. 3A, lane 4). These results indicated that furin mediates endoproteolytic processing of BRI and its pathogenic variants (BRI-L and BRI-D), resulting in the secretion of respective C-terminal peptides.

We then asked whether BRI-D was also subject to enhanced proteolytic processing by furin, as we had shown earlier for BRI-L (8). N2a cells transiently expressing epitope-tagged BRI, BRI-L, or BRI-D, were labeled with [35S]cysteine for 2 h, and cellular or secreted BRI derivatives were immunoprecipitated with αBRI-NT or CT11 antibodies. The synthetic rate of full-length BRI molecule after 10 min of pulse-labeling is comparable for each BRI variant (Fig. 4A). Surprisingly, and in contrast to cells expressing BRI-L, that secrete significantly higher...
levels of ABri peptide compared with cells expressing wild type BRI (Fig. 4B, compare lanes 1 and 2; quantified in D), the level of secreted ADan peptides in the conditioned medium of cells expressing BRI-D was considerably reduced (Fig. 4B, compare lanes 1 and 3; quantified in D). To explore this apparent discrepancy in levels of secreted ABri and ADan peptides, we examined the levels of peptides in cell lysates; our earlier studies had shown that BRI- and BRI-L-derived C-terminal peptides can be detected in cell lysates, consistent with furin-mediated processing in intracellular compartments (8). We now show that much higher levels of ADan peptide are present in lysates of cells expressing BRI-D compared with the levels of wild type C-terminal peptides in cells expressing BRI (Fig. 4C, compare lanes 1 and 3). Interestingly, phosphorimaging analysis revealed that intracellular levels of both ABri and ADan peptides were elevated by 4 fold compared with wild type peptides (Fig. 4C and E).

To determine whether the intracellular accumulation of ADan peptides is the result of slower transport of the peptide from intracellular stores, we performed pulse-chase analysis. HEK293 cells were transiently transfected with expression constructs encoding BRI, BRI-L, or BRI-D. Parallel dishes of transfected cells were pulse-labeled with [35S]cysteine for 2 h, then chased for 2, 4, 6, or 8 h at 37 °C (Fig. 5). The cell lysates and conditioned medium were prepared at each time point and analyzed by immunoprecipitation with CT11 antibody. In the cell lysates, variable levels of radiolabeled ~40-kDa full-length proteins were detected and these species diminished in levels over the chase period. (Fig. 5A, upper panel). Consistent with the results in Fig. 4, we observed elevated levels of ABri and ADan peptides in the cell lysates after a 2-h pulse compared with the levels of the wild type C-terminal peptide (Fig. 5A, lower panel, compare lanes 1, 6, and 11; quantified in C). Moreover, the levels of secreted ADan peptide in the medium was considerably reduced, while cells expressing BRI-L secrete significantly higher levels of ABri peptide compared with cells expressing wild type BRI (Fig. 5B, compare lanes 1, 6, and 11; quantified in D). Even after 8 h into the chase period, the amount of ADan peptide in the conditioned medium was not increased; most of the ADan peptide remained within intracellular compartments (Fig. 5A, lower panels, lanes 11-15). Collectively, these results support the notion that BRI-D is subject to enhanced processing by furin, but that the bulk of the resultant ADan peptides are retained in intracellular compartments.

**DISCUSSION**

Recent studies revealed that a mutation at the termination codon of the BRI gene encoding a type II membrane protein, BRI, is the underlying genetic defect of FBD, an autosomal...
dominant neurodegenerative disorder (2). The mutant BRI\textsubscript{2} gene encodes BRI-L, the precursor of the ~4-kDa ABri peptide that accumulates in amyloid deposits of brains of FBD patients (2). We previously demonstrated that furin mediates endoproteolysis of both BRI and BRI-L between arginine 243 and glutamic acid 244, resulting in the secretion of ~3 kDa and ~4 kDa C-terminal peptides, respectively (8). The results in the present study provide strong evidence that furin is the major protease responsible for the cleavage of BRI variants. First, proteolytic cleavages of BRI and BRI-L are inhibited in a dose-dependent manner by inductive expression of the furin inhibitor, α1-PDX. α1-PDX is relatively specific furin inhibitor and its IC\textsubscript{50} for furin is much lower than for other members of the PC family (17). Both ~37-kDa N terminal fragments and secreted ~3–4-kDa C-terminal fragments are decreased with increased expression of α1-PDX. Moreover, following coexpression of several PCs and BRI or BRI-L in a furin-defective cell line RPE-40, in which BRI and BRI-L fail to be cleaved, furin is most efficient in restoring endoproteolysis of BRI and BRI-L. Other PCs show much lower activities; PC6A and LPC are ~20% as active as furin, while PACE4 and PC6B fail to proteolyse BRI and BRI-L. It is noteworthy that LPC, like furin, also exhibits enhanced cleavage of BRI-L while PC6A cleaves wild type and mutant molecules with similar efficiency. These results suggest that the C-terminal extension present in the BRI-L alters the local conformation of the precursor in a manner that leads to enhanced proteolysis by furin and other PCs.

Very recently, Vidal et al. (7) described a different genetic defect, a decamer duplication just before the stop codon of the BRI\textsubscript{2} gene in neurodegenerative condition, termed FDD (7). We now report that like wild type BRI and FDB-associated variant BRI-L, the FDD-associated Danish variant (BRI-D) is processed by furin. Interestingly, the BRI-D precursor is also subject to enhanced furin-mediated cleavage. However, and in striking contrast to BRI-L, the ADan peptide largely accumulates in intracellular compartments; the level of secreted ADan peptide in the conditioned medium is much lower compared with those of wild type and ABri peptides indicating that this peptide is poorly secreted. At present, it is unclear why the ABri and ADan peptides behave so differently with regard to secretion. However, it is very possible that the ADan peptide has a higher propensity to aggregate intracellularly, thus precluding its secretion, while the ABri peptide is relatively more soluble following its production, leading to its secretion into the medium. This hypothesis can now be tested by structural and biophysical analyses of synthetic ADan and ABri peptides.

The mechanisms by which ABri or ADan peptides initiate the clinical syndromes and pathophysiological cascades in patients have not been defined. However, it is tempting to suggest that different biochemical properties of two peptides originated from the same precursor molecule, but with different types of mutations, are responsible for similar, but distinct clinical and pathological phenotypes. Abundant evidence has accrued to indicate that different biochemical properties of two peptides originated from the same precursor molecule, but with different types of mutations, are responsible for similar, but distinct clinical and pathological phenotypes. Abundant evidence has accrued to indicate that different biochemical properties of two peptides originated from the same precursor molecule, but with different types of mutations, are responsible for similar, but distinct clinical and pathological phenotypes. Abundant evidence has accrued to indicate that different biochemical properties of two peptides originated from the same precursor molecule, but with different types of mutations, are responsible for similar, but distinct clinical and pathological phenotypes.
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