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Expression of BRI–amyloid β peptide fusion proteins: a novel method for specific high-level expression of amyloid β peptides

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Abstract

In order to develop transgenic animal models that selectively overexpress various Aβ peptides, we have developed a novel expression system that selectively expresses Aβ40 or Aβ42 in the secretory pathway. This system utilizes fusion constructs in which the sequence encoding the 23-amino-acid ABri peptide at the carboxyl terminus of the 266-amino-acid type 2 transmembrane protein BRI is replaced with a sequence encoding either Aβ40 or Aβ42. Constitutive processing of the resultant BRI-Aβ fusion proteins in transfected cells results in high-level expression and secretion of the encoded Aβ peptide. Significantly, expression of Aβ42 from the BRI–Aβ42 construct resulted in no increase in secreted Aβ40, suggesting that the majority of Aβ42 is not trimmed by carboxypeptidase to Aβ40 in the secretory pathway. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

A great deal of genetic, biochemical and pathological evidence indicates that aggregation of amyloid β protein (Aβ) plays a causative role in the development of Alzheimer's disease (AD) (reviewed in [1]. Based on this evidence, a number of AD transgenic mouse models have been developed that deposit Aβ in the central nervous system (reviewed in [2]). These current models primarily rely on overexpression of familial AD linked mutant amyloid β protein precursor (APP) to generate sufficient levels of Aβ to drive age-dependent aggregation and deposition. While these models are extremely valuable tools to study Aβ deposition and APP processing, the high levels of mutant APP overexpression needed to achieve amyloid deposition may confound interpretations of pathological and behavioral studies. Not only could mutant APP overexpression artificially contribute to some of the pathological and behavioral changes that have been described, it is also possible that overexpression of APP and subsequent production of the potentially neuroprotective sAPP derivatives may paradoxically prevent these models from fully recapitulating some features of human AD pathology (e.g., readily appreciable neuronal loss).

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An obvious way to overexpress Aβ in the absence of APP overexpression is to create minigenes encoding the Aβ peptide itself or the Aβ peptide fused to the APP leader peptide. Our experience with these types of constructs is that they do not produce high levels of Aβ and that only trace amounts of Aβ get secreted from cultured cells transfected with these Aβ minigene constructs (T.E. Golde, unpublished observation). Similar results are reported in the literature. In one case, transfection of an Aβ1–43 minigene containing the APP signal peptide resulted in Aβ1–43 expression primarily in the nucleus and cytoplasm with only a small amount of intact Aβ secreted [3]. In another case another Aβ minigene was used to drive expression of Aβ1–42 in a transgenic mice. Although there was evidence for neuronal cell death and evidence for immunohistochemical staining of Aβ epitopes in some neurons, these mice did not develop overt AD pathology [4]. Although the neuronal death phenotype in these mice is intriguing, no follow up reports have been forthcoming, and expression of Aβ has not been documented by any other method than immunohistochemistry.

To develop a system whereby individual Aβ peptides could be efficiently expressed and secreted in the absence of overexpression of the APP, we have created genetic constructs that express fusion proteins between the BRI protein, involved in amyloid deposition in familial British dementia [5], and Aβ (see Fig. 1). The expression of Aβ from these BRI–Aβ fusion proteins was then examined in cell culture systems.

2. Materials and methods

Polymerase chain reaction (PCR) primers were designed to generate a 991 bp fragment of the BRI gene [5], using human brain library cDNA as template. The forward primer, 5′-TTC CCT CGA GTC TCA GCC GCC CGG AGC-3′, and the reverse primer, 5′-ACT GCT CGA GAT GTA AAG GGT GGG GTT ATG-3′, included the recognition sequence for the restriction enzyme *Xho*I in their 5′ sequence. The amplified product was then digested with *Xho*I to create a tagged fragment suitable for cloning into pBS (KS−). The construct sequence was confirmed by direct sequencing on an ABI 377 automated sequencer using Big Dye chemistry and Sequence Navigator software (Perkin Elmer). This construct was

Table 1
ELISA analysis of cells transfected with BRI–Aβ fusion constructs

Cell line/DNA	Transfection[b]	Aβ40 (pM)[c]	Aβ42 (pM)[d]	%Aβ40	%Aβ42
293T/pAG3[a]	Transient	11 ± 3	0	100	0
293T/pAG3 BRI–Aβ40	Transient	145 ± 8	0	100	0
293T/pAG3 BRI–Aβ42	Transient	8 ± 2	161 ± 13	5	95
C17-2/pAG3	Transient	8 ± 1	2 ± 1	80	20
C17-2/pAG3 BRI–Aβ40	Transient	383 ± 26	2 ± 1	99	1
C17-2/pAG3 BRI–Aβ42	Transient	12 ± 3	475 ± 64	2	98
H4/pAg3	Stable	31 ± 5	4 ± 1	89	11
H4/pCDNA3APP695	Stable	694 ± 38	94 ± 3	88	12
H4/pAG3BRI–Aβ40	Stable	18735 ± 177	173 ± 28	99	1
H4/pAG3BRI–Aβ42	Stable	629 ± 9	28059 ± 159	2	98
(2b7) CHO/pAG3APP695wt	Stable	3421 ± 420	392 ± 28	90	10
CHO/pAG3APP695NL,I,his	Stable	12861 ± 1809	2902 ± 395	81	19

[a]The pAG3 vector has been previously described [6].

[b]Transient transfections were performed in triplicate in 6-well plates using Fugene 6 (Roche). Stable lines were transfected with Fugene 6 and selected with either hygromycin (pAG3) or G418 (pCDNA3). Each stable line was derived from a single colony that was selected based on high-level expression.

[c]Aβ40 was assayed using a 3160/BA27 ELISA.

[d]Aβ42 was assayed using a 3160/BC05 ELISA. The small amount of Aβ1–40 detected above background cells transfected with BRI–Aβ42 is likely due to the fact that the Aβ40 'specific' BA-27 antibody will detect Aβ1–42 at low efficiencies (∼1%) [14,15]. A similar cross-reactivity is likely to account for the small amount of Aβ42 detected in the BRI–Aβ40 transfects. In all cases, media samples were conditioned for 16 h.

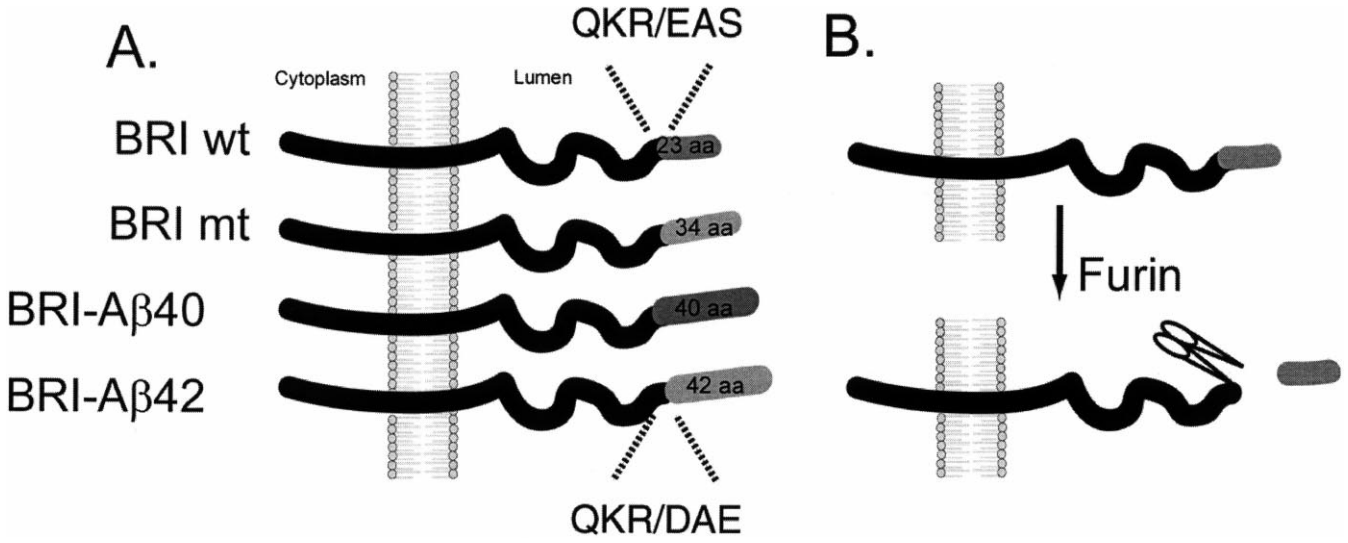

Fig. 1. BRI–Aβ fusion proteins. (A) Schematic of wild-type BRI, mutant BRI, BRI–Aβ40 and BRI–Aβ42 fusion proteins. The amino acids immediately surrounding the furin cleavage site (indicated by a slash) in the BRI and BRI mutant are indicated above BRI wild type. The amino acids forming the cleavage site in the Aβ fusions are indicated below BRI–Aβ42. Note that the substitution at P1′ in the BRI fusions is quite conservative. (B) Schematic of furin cleavage of BRI. BRI is cleaved by furin (and possibly other furin like proteases) to cleave the ABri peptide from the BRI protein. Similar processing in the BRI–Aβ fusions should release authentic Aβ peptides.

used for the template in the subsequent PCR reactions.

Subsequently, fusion constructs encoding the first 243 amino acids of BRI followed by either Aβ40 (BRI–Aβ40) or Aβ42 (BRI–Aβ42) were generated by PCR amplification. The amino terminal portion encoding BRI was amplified using the wild-type BRI cDNA as template and the primers BRIFOR (5′-ATTTTTAAGCTTCTCGAGAGGCTGCAATCG-CAGCGGGAG-3′) and BRIREV (5′-GTCGGA-ATTCTGCATCACGTTTCTGAATACCTTTAAT-AGTTTC-3′). cDNAs encoding Aβ40 and Aβ42 were amplified using APP 695 wild-type cDNA as template and the primer AβFOR (5′-TGGA-GATCTGATGCAGAATTCCGACATGACTC-3′), and Aβ40REV (5′-TAAAAGGATCCCTAGACAA-CACCGCCCACCATGAG-3′) and Aβ42REV (5′-TAAAAGGATCCCTACGCTATGACAACACCG-CCCACCATGAG-3′). The resultant BRI encoding cDNA was digested with *Hin*dIII and *Eco*RI while the Aβ encoding cDNAs were digested with *Eco*RI and *Bam*HI. The fragments were ligated into the expression vector pAG3 using a three-way ligation that resulted in an in frame fusion of the BRI and Aβ sequences. Sequences were confirmed by automated DNA sequencing. These PCRs were performed using the High fidelity PCR kit from Roche. Transfection of cells, Aβ enzyme-linked immunosorbent assays (ELISAs), and immunoprecipitation–mass spectrometry (IP/MS) were carried out as previously described [6]. Western blotting was carried out as described previously using BAN50, BC05 and BA27 antibodies at 1:1000 [7]. An anti-peptide antibody, EN3, raised in rabbit, created to the sequence YKLQRRETIK-GIQ (corresponding to amino acids 229–241 of BRI) was also used at a dilution of 1:1000.

3. Results and discussion

To determine whether the BRI–Aβ fusion proteins produce Aβ, we initially analyzed conditioned media from human embryonic kidney cells (293T) and mouse CNS progenitor cells (C17-2) [8] transiently transfected with BRI–Aβ40 and BRI–Aβ42 using end-specific Aβ ELISAs. These data indicated that Aβ40 was selectively produced from the BRI–Aβ40 construct and Aβ42 was selectively produced from the BRI–Aβ42 construct (see Table 1). To further investigate the expression of Aβ from these constructs we generated stable H4 neuroglioma. As with the transient transfections, each BRI fusion

construct resulted in selective overexpression of the appropriate Aβ peptide (Table 1), with over 98% of the Aβ generated ending with the designed amino acid. We also compared the production of Aβ from the BRI fusion constructs to that generated by 2b7 cells and CHO-APP695NL,I,his cells. The 2b7 clonal CHO line secretes the highest levels of Aβ of any wild-type APP transfected line generated in our laboratory, and the CHOAPP695NL,I,his cells express the highest level of Aβ from any stable cell line we have ever generated, presumably because the NL mutation increases Aβ40 and Aβ42 production whereas the V717I (I) increases the percent Aβ42. Significantly, the specific levels of Aβ40 or Aβ42 were much higher using the BRI fusion methodology. To obtain final conformation of the selectivity of Aβ secretion form this system, IP/MS analysis of the media was performed. As shown in Fig. 2A,B, the mass spectra demonstrate that each construct results in the selective overexpression of the appropriate full length Aβ peptide (Aβ1–40 from BRI–Aβ40 and Aβ1–42 from BRI–Aβ42) with negligible increase in any other peptide peaks. Western blot analysis of the H4 stable lines expressing the BRI–Aβ fusion proteins with antibodies to the amino terminus of Aβ, Aβ40, Aβ42, and BRI indicates that fusion proteins containing the expected epitopes and of the appropriate size (~36 kDa) are produced by expression of the BRI–Aβ constructs (Fig. 2C).

Familial British dementia is an autosomal dominant genetic disorder, characterized by the deposition of the 34-amino-acid ABri peptide, as amyloid in non-neuritic plaques and cerebral vessels. Cloning of the gene encoding the ABri peptide revealed that it is derived from the carboxyl terminus of a type 2 transmembrane protein referred to as BRI [5]. In the mutant BRI protein, a T-A transversion in the stop codon results in an 11-amino-acid extension of the coding sequence. The 266-amino-acid wild-type and 277-amino-acid mutant BRI precursor proteins are

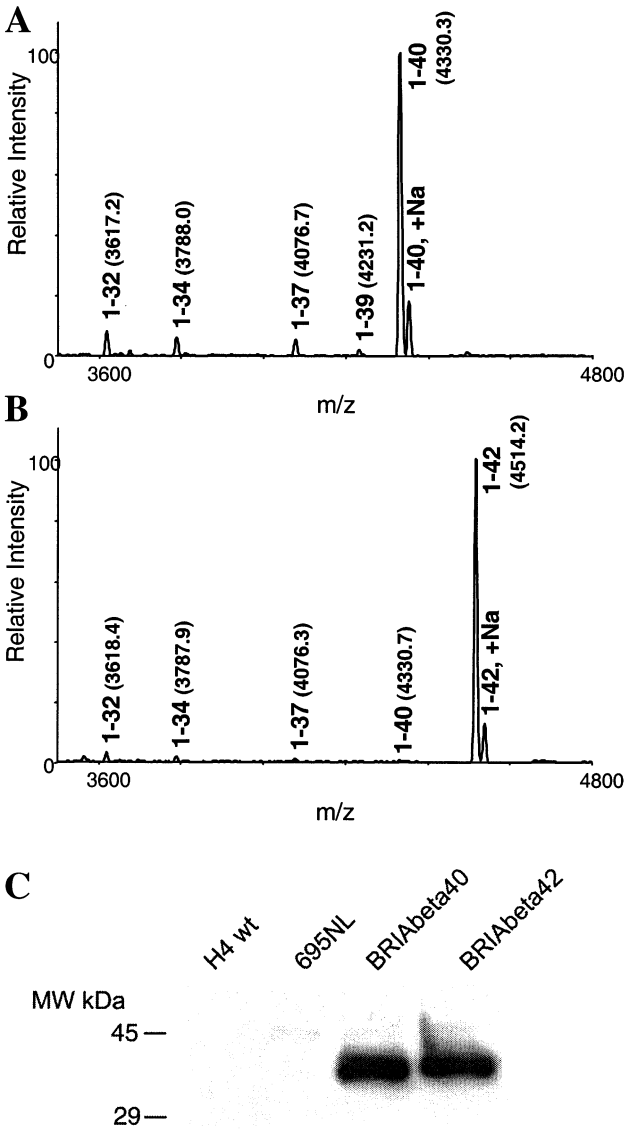

Fig. 2. Analysis of BRI–Aβ fusion proteins. (A) IP/MS analysis of conditioned media from a stable H4 cell line expressing BRI–Aβ40. Aβ species are indicated above the peaks and the mass is enclosed in parentheses. Note that no Aβ42 is detected by IP/MS and that only minor amounts of smaller Aβ peptides (Aβ1–32, Aβ1–34, Aβ1–37 and Aβ1–39) are detected. The shoulder on the 1–40 peak corresponds to the Aβ1–40 peptide complexed with a sodium ion. The levels determined by ELISA analysis of this media are shown in Table 1 (H4/pAG3BRI–Aβ40). (B) IP/MS analysis of conditioned media from a stable H4 cell line expressing BRI–Aβ42 reveals that Aβ42 represents the vast majority of Aβ produced by this construct. Aβ levels determined by ELISA analysis of this media are shown in Table 1 (H4/pAG3BRI–Aβ42). In vector transfected H4 cells, only a small peak corresponding to Aβ1–37 is detected by IP/MS (data not shown). (C) Western blot analysis of BRI–Aβ fusion constructs with BAN50, an antibody that recognizes the amino terminus of Aβ. Additional Western blot analyses with antibody EN3 to the BRI sequence showed identical results to the BAN50 blot (data not shown). Blotting with the antibody BC05 that recognizes Aβ42 and BA27 that recognizes Aβ40 stained only the appropriate BRI fusion proteins (data not shown).

cleaved by furin (and possibly other furin-like proteases) to release both wild-type (23 aa) and mutant Abri (34 aa), respectively (Fig. 1) [9]. As the specificity of furin-like proteases is primarily in the P1–P2 residues [10,11], we speculated that expression of BRI fusion proteins in which the ABri sequence was replaced by Aβ sequence would result in cleavage of the heterologous peptide and subsequent secretion of Aβ. As predicted, the results described herein indicate that BRI–Aβ fusion protein expression results in the selective secretion of the encoded Aβ peptide. Thus, we have proven the utility of the BRI fusion system for expressing Aβ in the secretory system of mammalian cells. Given the aforementioned specificity of the furin cleavage lies in the residues amino terminal to the cleavage site, it is likely that a variety of small peptide sequences can be fused to BRI and subsequently released by furin endoproteolysis. Moreover, this system may prove useful for expression of Aβ in non-mammalian animal models such as *Drosophila* and *Caenorhabditis elegans*. These models do not efficiently process full-length APP into Aβ, but are known to process many proteins containing dibasic furin cleavage sites [12] making it likely that furin cleavage of BRI–Aβ fusions result in a high level of Aβ production in these model organisms.

This study also suggest that Aβ42 produced in the secretory pathway of several mammalian cell types is not trimmed by carboxypeptidases to generate smaller Aβ peptides. This result further supports the notion that Aβ40 and Aβ42 are generated solely by endoproteolysis of APP. Because we do not know whether the BRI–Aβ fusion proteins are produced in exactly the same secretory pathways that produce Aβ from the APP, we cannot completely exclude the possibility that carboxypeptidase trimming of Aβ42 occurs in vivo. Nevertheless, both furin cleavage of other substrates and APP processing to Aβ have been shown to occur in the late Golgi; therefore, it is likely that Aβ production from APP and BRI–Aβ fusion proteins, at least to some extent, occur in overlapping pathways.

The study of familial forms of AD has firmly establishing a pathogenic role for 'long' Aβ species ending at Aβ42 [13]. Yet, multiple Aβ peptides with varying carboxyl and amino termini are produced and deposited in the AD brain. Unfortunately, little is known about the potential role that various truncated Aβ species play in the development of AD; the ability to selectively express specific Aβ peptides from BRI–Aβ fusion constructs may enable the pathogenicity of individual Aβ peptide to be determined in animal models.

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