High-level expression and *in vitro* mutagenesis of a fibrillogenic 109-amino-acid C-terminal fragment of Alzheimer's-disease amyloid precursor protein

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We amplified DNA encoding the 3' 109 codons of Alzheimer's-disease amyloid precursor protein (APP) inclusive of the beta protein (A β) and cytoplasmic domains from cDNA using oligonucleotide primers designed to facilitate cloning into the T7 expression vector pT7Ad23K13. We also modified this construct to generate recombinant molecules incorporating two recently described APP mutants by site-directed mutagenesis. Both native C109 (deletion construct inclusive of the C-terminal 109 residues of APP) and constructs with a single mutation at codon 642 (T $\rightarrow$ G, resulting in a substitution of glycine for valine) or a double mutation at codons 595 (G $\rightarrow$ T, substituting asparagine for lysine) and 596 (A $\rightarrow$ C, substituting leucine for methionine) were expressed in *Escherichia coli* to levels of 5–20% of total bacterial protein after induction. The major constituent of expressed C109 protein had an apparent molecular mass of 16–18 kDa by SDS/PAGE and appeared to be the full-length

construct by size and N-terminal microsequencing. Also present was a 4–5 kDa species that co-purified with C109, constituting only ~ 1% of expressed protein, which was revealed by Western-blot analysis with antibodies specific for A β epitopes and after biotinylation of purified recombinant C109. This fragment shared N-terminal sequence with, and appeared to arise by proteolysis of, full-length C109 in biosynthetic labelling experiments. C109 spontaneously precipitated after dialysis against NaCl or water, and with prolonged (> 20 weeks) standing was found by electron microscopy to contain a minor (< 5%) fibrillar component that was reactive with antibodies to a C-terminal epitope of APP. Recombinant C109 appears to duplicate some of the biochemical and physicochemical properties of C-terminal A β -inclusive fragments of APP that have been found in transfected cells, brain cortex and cerebral microvessels.

INTRODUCTION

A β (also designated β -protein or A4) is the major constituent of amyloid fibrils in a spectrum of cerebral amyloidoses, including Alzheimer's disease (AD) and several forms of angiopathy in brain [1]. A β has an apparent molecular mass of ~ 4 kDa and has been found to be 39–42 amino acid residues long, depending on the source used for purification (i.e. vessel wall or plaque core respectively) [2,3]. Both purified A β and synthetic peptides corresponding to segment 12–28 are intrinsically fibrillogenic and possess a cross- β -pleated conformation by X-ray diffraction analysis [4,5]. Although the C-terminal 11–14 residues of A β appear to contribute to insolubility as amyloid [6], recent reports have also described soluble forms produced by cultured cells and present in body fluids [7,8].

A β is derived from AD amyloid precursor protein (APP), a group of 110–140 kDa molecules that have been shown to undergo complex processing in various cultured cells, as well as in cell lines transfected with APP constructs [9]. Under normal circumstances, a major pathway of cleavage occurs at position 16–17 of the A β sequence, which is inconsistent with generation of a fibril-forming subunit corresponding to that found in amyloid deposits [10]. However, different groups have reported the presence of 10–22 kDa APP isoforms with reactivity to C-terminal epitopes inclusive of the A β sequence in PC-12 cells, stably transfected 293 cells, brain cortex and cerebral microvessels

that could be precursors for this fragment [11–13]. Several of these species include the C-terminal cytoplasmic domain of full-length APPs, a domain which has also been shown to include a fibrillogenic sequence [14]. If such molecular species are in fact immediate precursors for A β , this may relate to reports of immunoreactivity specific for the cytoplasmic domain in vessel walls and senile plaques of patients with AD [15,16].

Deletion constructs inclusive of the C-terminal 100 residues (designated C100) of APP have been used to express proteins that incorporate into microsomal membranes [17], form aggregates in transfected cell line [18,19], are mitogenic for 3T3 fibroblasts and neurotoxic to PC12 [20]/P19 [21] cells, and increase levels of β -amyloid immunoreactivity in the brains of transgenic mice [22]. Recently, this construct was found to aggregate specifically in response to addition of free radicals in an *in vitro* translation system [23]. Also, cells stably transfected with a similar construct (C104) caused cortical atrophy and induced Alz50 immunoreactivity when transplanted into the brains of newborn mice [24].

We report the expression of an A β -inclusive fragment (C109) in a prokaryotic-cell system, the characterization of APP-reactive species in this system, production of variant molecules and the potential of C109 to polymerize spontaneously as fibrillar structures.

Some of these results have already been reported in preliminary form [25].

Abbreviations used: AD, Alzheimer's disease; APP, (Alzheimer's-disease) amyloid precursor protein; A β , beta protein; IPTG, isopropyl β -D-thiogalactopyranoside; C109 (etc.), deletion construct inclusive of the C-terminal 109 residues of APP (etc.); KPI, Kunitz-type proteinase inhibitor; MAbs, monoclonal antibody.

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MATERIALS AND METHODS

Bacterial expression of C109

A 355 bp segment of the APP cDNA sequence encoding the 3' 109 codons was PCR-amplified from a pGEM-2 plasmid containing the full-length APP₇₅₁ sequence kindly provided by Dr. Efrat Levy, New York University Medical Center. Amplimers were $\alpha\beta$ 1759 CCGGATCCATATGAAGACGGAGGAGATCTCTGAA and $\alpha\beta$ 2065 TTGGATCCGGTGACCGTTCTGCATCTGCTCAAAGAA. The former is an upstream amplimer that encodes an *NdeI* site in the 5' 13 bases, followed by bases showing sequence identity with bases 2051–2071 of the reported cDNA sequence of APP₇₅₁ [26]. The latter is a downstream amplimer encoding a *BstEII* site in the 5' 15 bases, and bases complementary to nucleotides 2377–2357 of APP₇₅₁ cDNA.

PCR product was digested sequentially with each restriction endonuclease (Boehringer-Mannheim), for in-frame insertion into a T7 recombinant expression vector pT7Ad23K13 with complementary ends (Figure 1), kindly provided by Dr. John Dunn and Dr. Carl Anderson, Brookhaven National Laboratories [27]. In this recombinant plasmid, the insert is engineered to include an ATG initiation signal encoding methionine for protein expression at the *NdeI* restriction site, and a histidine hexamer tail (CACCACCACCATCACCAT) in-frame with the *BstEII* site to facilitate purification of recombinant protein by metal-chelate exchange chromatography. After ligation of the C109 insert into linearized vector DNA, an initial transformation of competent *Escherichia coli* DH5 α cells (a host that lacks T7 RNA polymerase) was performed, and DNA purified by alkaline lysis. Stable recombinant molecules were screened by PCR for the presence of the 355 bp insert, and integrity of reading frame established by dideoxy sequencing. Supercoiled DNA was then retransformed into competent BL-21 (DE3)/pLys S *E. coli*, a host strain containing the T7 RNA polymerase gene under control of the inducible lac UV₅ promoter

and a pACYC184 plasmid (pLys S), which results in low levels of T7 lysozyme, a natural inhibitor of T7 RNA polymerase [28]. C109 protein expression was induced by addition of 0.4 mM isopropyl β -D-thiogalactopyranoside (IPTG), and induction was assessed by SDS/15% PAGE of cell lysates and Western-blot analysis with monospecific antibodies (see below).

Purification and characterization of C109 and related peptides

BL21 (DE3)/pLys S cells carrying C109 and control plasmids were grown to exponential phase (2 h) until reaching an $A_{600} \sim 0.6$, and then induced with IPTG for an additional 3 h. Bacterial pellets were harvested by centrifugation at 4000 *g* for 20 min, washed twice, resuspended in 6 M guanidinium chloride/0.1 M NaH₂PO₄/0.01 M Tris, pH 8.0 (Buffer A). A Ni-NTA agarose (Quiagen) column was pre-equilibrated with Buffer A and loaded with the pellet supernatant obtained after centrifuging the lysate at 10000 *g* for 15 min at 4 °C. The column was eluted serially with 8 M urea/0.1 M NaH₂PO₄/0.01 M Tris at pH 8.0, then 6.3, and finally with 5 column vol. at pH 4.5. The last eluate was found to contain most of the C109 applied to the column, and was dialysed against either distilled water or PBS, with frequent changes of dialysis fluid. Under these conditions, recombinant protein was found to precipitate spontaneously and was stored in aliquots at 4 °C. Typically C109 constituted 5–20 % of total bacterial protein, depending on conditions of induction.

C109 isolated by immobilized metal-chelate affinity chromatography was further purified by h.p.l.c. Protein lyophilized after dialysis against distilled water was biotinylated with sulpho-NHS-biotin (Pierce) essentially as described in [29,30] and the resulting labelled peptide purified on a μ Bondapak C18 column (0.38 cm $\times$ 30 cm) (Waters) using a linear gradient of 0–100 % acetonitrile in 0.1 % trifluoroacetic acid at 1 ml/min. The column effluent was monitored by absorbance at 214 nm, and fractions were characterized by SDS/17% PAGE as Coomassie Blue-stained bands or by immunoblot analysis. N-terminal sequencing was carried out on purified fractions or individual bands from gels electroeluted on to poly(vinylidene difluoride) membranes as described [30].

In vitro mutagenesis

Constructs corresponding to two recently-described variant APP molecules associated with early-onset familial AD were generated by *in vitro* mutagenesis. C109 (642) changes a T to G at codon 642, thereby converting valine into glycine [31]; C109 (595/596) is a double mutation that converts G into T at codon 595 and A to C at codon 596, thereby converting lysine into asparagine and methionine to leucine respectively [32]. Site-directed mutagenesis (pAlter-1 kit; Promega) was carried out using a mutated 30-mer positive-strand oligonucleotide (ATCTCTGAAGTGAATCTG-GATGCAGAAATTC) for C109 (595/596), and an 18-mer (CAGTGATCGGCATCACC) for C109 (642). Once in pSelect, DNA was transformed into JM109 cells and sequenced (Sequenase; USB) to verify each mutation. Mutated DNA was amplified with the same PCR primers as used for C109 digested with *BstEII* and *NdeI*, cloned into pT7Ad23K13, and expressed as described above.

Biosynthetic labelling of expressed protein

Induced and uninduced cells carrying the C109 construct were grown in minimal M9 medium supplemented with 0.4 % glucose, 0.001 % thiamine, 1 mM MgSO₄, with carbenicillin (100 μ g/ml final concn.) and left at 30 °C overnight. Cells were then diluted 1:1000 and cultured with shaking at 30 °C overnight, after which

Figure 1 Schematic representation of the pT7-C109 construct, indicating orientation of insert (hatched) and histidine hexamer tail (PolyHIS)

ϕ 10-S10 represents the promoter for bacteriophage T7 RNA polymerase and the ribosome binding and translational start site for the T7 gene 10. Also indicated are the ampicillin-resistance gene (*AmpR*), the origin of replication (*ori*) and selected unique restriction sites.

Figure 2 Amino acid sequence of the C109 construct, including the initiation methionine residue and the histidine tail

Underlined are residues 2–110 (above), corresponding to the C-terminal end of APP. The β -amyloid sequence ($A\beta$) comprises residues 1–42 (below). The horizontal bars indicate the location of the peptides used to develop antibodies or, in the case of anti-SP41 and anti-SP28 [35], the specific epitopes to which these antibodies have been mapped. Abbreviation: mab, monoclonal antibody.

the temperature was shifted to 37 °C. At an A_{600} of ~ 0.4 , cultures were induced with IPTG (0.4 mM final concn.). Cells were grown with and without rifampicin (Sigma), added 15 min after induction, at a concentration of 200 $\mu\text{g}/\text{ml}$. [^{35}S]Methionine (0.02 $\mu\text{Ci}/\mu\text{l}$ final concn.) was added to 50 μl aliquots of induced cells at various time points, and then incubated for 2 min at 37 °C. Labelling was stopped by addition of 50 μl of 2 $\times$ sample buffer [100 mM Tris/HCl (pH 6.8)/200 mM dithiothreitol/4% SDS/0.2% Bromophenol Blue/20% glycerol]. Samples were boiled for 5 min and separated by SDS/18%-PAGE [33] or Tris/Tricine [34] gel electrophoresis. Gels were vacuum-dried and exposed for autoradiography overnight.

Immunoblot analysis

Conditions for protein fractionation, transfer to poly(vinylidene difluoride) membranes, blocking reactions and incubation with primary and secondary antibodies were as previously described [35]. Monoclonal antibody F12 is specific for the C-terminal 20 residues of APP₆₉₅ (676–695) [35]. Anti-SP-28 and anti-SP-41 are polyclonal antibodies raised to synthetic peptides corresponding to amino acids 597–624 and 584–624 of APP₆₉₅; the precise epitopes recognized by these antibodies have been mapped to residues 617–624 and 602–607 of βAPP respectively [36]. Monoclonal antibody 6E10 (kindly provided by Dr. H. M. Wisniewski) reacts with residues 1–17 of $A\beta$ [37], and polyclonal antiserum 369 (generously supplied by Dr. S. E. Gandy) was raised to a synthetic peptide corresponding to APP₆₉₅ residues 645–694 (i.e. cytoplasmic domain) [38]. Anti-C109 is a mouse polyclonal antiserum raised to the 16–18 kDa C109 Coomassie-Blue stained band cut out of an SDS/polyacrylamide gel. Antibodies and epitopes are summarized in Figure 2.

Immunogold labelling and electron microscopy

Formvar-coated, 300-mesh nickel or copper grids (Electron Microscopy Sciences) were floated on drops of the C109 preparation for 30 min and then washed by floating on five successive drops of PBS with 0.5% BSA (PBS/BSA) (5 min/drop). Grids were incubated on drops of monoclonal antibody (MAb) F12 or normal mouse IgG, diluted to 0.1 mg/ml with PBS/BSA, for 30 min, washed as above, then incubated for 30 min with a biotin-conjugated rabbit anti-mouse IgG secondary antiserum, diluted to 0.1 mg/ml with PBS/BSA. They were then washed again, and incubated with 10 nm streptavidin-conjugated gold particles, diluted 1:10 from the stock concentration (Amersham)

with PBS/BSA. All grids were again washed as described above, followed by five additional washes on drops of distilled deionized water and finally negatively stained with a 2% aqueous solution of uranyl acetate. Samples were examined on a JEOL JEM 1200ex scanning transmission electron microscope with a Tracor-Northern 5500 X-ray microanalysis system.

RESULTS

Expression of C109

To express the C-terminal 109 amino acids encoded by the major APP transcripts, this portion of a full-length cDNA was PCR-amplified, and cloned into a plasmid vector, pT7Ad23K13 [27], under control of the $\phi 10$ promoter for bacteriophage T7 RNA polymerase (Figure 1).

Primers were designed to introduce an *Nde*I restriction site in front of the C109 sequence and to create a *Bst*EII site to link it to a sequence encoding a histidine hexamer tail to facilitate affinity purification of recombinant protein. An initial transformation was carried out by cloning into DH α to establish stable recombinant plasmids. These plasmids were then transferred to the expression strain BL-21 (DE-3)/pLys S, in which T7 RNA polymerase transcription is under the control of the lac UV₅ promoter, inducible by IPTG. In this system, the presence of pLys S, which includes the promoter for T7 RNA polymerase immediately after the lysozyme gene, has the effect of increasing the tolerance of the expression vector for toxic target plasmids [28].

On induction, plasmid-C109-containing cells were found to have an additional 16–18 kDa Coomassie Blue-stainable band when SDS/PAGE was carried out on induced-cell lysates (Figure 3). This band could not be seen on uninduced or control lysates and could not be solubilized from IPTG-induced C109 cells that were treated with Triton X-100 or PBS (Figure 4). A 3 h induction was found to be optimal for expression, and was not improved by longer incubation periods; indeed, electron microscopy of cell pellets obtained after longer incubation periods showed significant lysis and cell death compared with controls (results not shown). Although the apparent molecular mass of this band exceeded the predicted value of 13.6 kDa, similarly aberrant migration has been noted by others both in cell-free and

Figure 3 Expression of C109 by BL21(DE3)/pLys S cells carrying the C109 plasmid grown to mid-exponential phase uninduced (lane a) and after induction with IPTG (lane b)

Cell lysates were fractionated by SDS/15%-PAGE and revealed by staining with Coomassie Blue. Abbreviation used: M, molecular mass.

Figure 4 IPTG-induced BL21(DE3)/C109 pLys S cells were extracted serially with PBS (lane c), Triton X-100 (lane b) and then boiled in 4% SDS sample buffer (lane a)

After centrifugation, equal concentrations of supernatant were separated by SDS/PAGE and blotted to poly(vinylidene difluoride) paper for antibody binding with mAb F12 at a dilution of 1:500. Abbreviation: *M*, molecular mass.

Figure 5 SDS/PAGE patterns of C109 recombinant protein after elution from a Ni-NTA-agarose column (lane 1), after dialysis against water, freeze-drying and resuspension in SDS sample buffer (lane 2), and after biotinylation (lane 3)

Lanes 4 and 5 are replicate Western blots developed with anti-SP28 (1:200 dilution) and mAb F12 respectively. Biotinylated reactants were developed with streptavidin-peroxidase; proteins were analysed on a 17%-polyacrylamide gel. Abbreviation: *M*, molecular-mass (*M*) markers.

eukaryotic expression systems [17,18], and presumably reflects an extended configuration of the protein when size-fractionated under reducing and dissociating conditions. The isofocusing point of this protein assessed by two-dimensional gel electrophoresis (results not shown) was in good agreement with the predicted pI of 6.3. Moreover, N-terminal sequence analysis of a narrow portion of the top edge of this broad band yielded a single sequence corresponding to the first 14 residues of the construct, MKTEEISEVKMDAE, when subjected to Edman degradation. On Western-blot analysis, this band was diffusely reactive with an anti-APP MAb specific for the last 20 residues of the cytoplasmic tail; also apparent on Western blots of SDS-solubilized lysates was binding to a ladder of bands extending to the top of the gel, corresponding to dimers and polymers of the 16–18 kDa band (Figure 4). Immunoblot analysis of crude lysates with anti-SP28, an antibody specific for an epitope in the domain

defined by residues 20–28 of the A β sequence [36] (Figure 2), revealed a 4–5 kDa reactive species that was not detected in uninduced control lysates; this species could *not* be detected as a distinct Coomassie Blue-stained band when induced and un-induced lysates were compared by SDS/PAGE (Figure 3).

Purification of C109 and related peptides

C109 was then purified by metal-chelate affinity chromatography on an Ni-NTA-agarose column. Bacteria were solubilized in 6 M guanidine, and the column was eluted with 7 M urea. Immunoblot analysis of serial eluates showed that all F12-reactive material could be retrieved when the pH was lowered to 4.5, and was not eluted at higher pH values. The purified C109 fraction obtained after elution from the Ni column gave a major band at 16–18 kDa when run on an SDS/17%-polyacrylamide gel and stained with Coomassie Blue, the appearance of which was unchanged if the sample was exhaustively dialysed against distilled water, freeze-dried and resuspended in SDS sample buffer (Figure 5, lanes 1 and 2). However, if purified C109 was biotinylated before size fractionation and allowed to react with streptavidin-peroxidase, a band at 4–5 kDa could be seen (lane 3) that was not seen with standard protein stains. All gels contained higher-molecular-mass bands that were F12-reactive by immunoblot analysis; however, the 4–5 kDa material did not bind F12 on Western blots (lane 5). By contrast, anti-SP28 bound well to the 4–5 kDa band and only weakly to the 16–18 kDa F12-reactive species (lane 4). Both antisera reacted with a ladder of bands with higher apparent molecular masses, though with differing binding patterns.

C109-related peptides were further resolved by reverse-phase h.p.l.c. of affinity-purified material. The elution profile is shown in Figure 6, which resolved six peaks (A–F) that were analysed by SDS/PAGE. Peaks A and B, which represented ~1–2% of the total material loaded on the column, contained the 4–5 kDa band revealed by immunoblot analysis and after biotinylation, whereas peaks C–F were free of this band. The latter resolved two bands in the range 16–18 kDa, which could also be seen in Tris/Tricine gels (results not shown), and had similar higher-molecular-mass banding patterns.

Peaks A and E were separately pooled and subjected to N-terminal microsequencing. Peak A yielded a major sequence MKTEEIXEVK, corresponding to the N-terminus of C109; also present was a minor sequence, PXIKTVRGA, matching the N-terminus of *E. coli* ribosomal protein A [39]. Peak E also yielded a major sequence beginning at position one of C109; however, the lower band (Figure 6, inset) proved to be a degradation product beginning at residue 9 (valine), with additional minor sequences beginning at positions 7 (serine) and 13 (alanine). Thus the heterogeneity of the 14–16 kDa band seen in crude lysates was likely due to proteolysis at the N-terminal end of the molecule.

Western-blot analysis

Immunoreactivity of recombinant C109 was further defined with additional antibodies directed to A β epitopes, or specific for the cytoplasmic tail of full-length transcripts. As seen in Figure 7, the 4–5 kDa species bound anti-SP-41, which reacts with an epitope at the N-terminus of A β [31], mAb 6E10 a monoclonal antibody raised to a synthetic peptide corresponding to residues 1–17 of A β [32], and a polyclonal mouse antiserum raised to purified C109 eluted from gels. However, this band was *not* reactive with F12, and also did not bind a second C-terminal antibody, 369 [33]. By contrast, the 16–18 kDa bands bound all antisera, though with varying intensity, and only weakly to anti-SP-28.

Figure 6 H.p.l.c. profile of 1.5–2.0 mg of purified C109 protein separated on a C18 μ Bondapak analytical column using a 0–100% acetonitrile gradient in 0.1% trifluoroacetic acid

The inset shows fractions A–F separated by SDS/17%-PAGE and stained with Coomassie Blue. Abbreviation: M, molecular-mass (*M*) markers.

Figure 7 C109 purified from Ni-NTA agarose column before h.p.l.c. purification

This fraction was separated on a Tris/Tricine gel, blotted on to poly(vinylidene difluoride) paper, and reacted with (a) polyclonal mouse antibody to the 16–18 kDa band, (b) anti-SP28, (c) anti-SP41, (d) mAb 6E10 and (e) mAb F12.

Figure 8 Autoradiogram (overnight exposure), showing incorporation of [35 S]methionine in uninduced cells (lane 1) and after 10, 20, 30, 60 and 120 min of induction (lanes 2–6)

An SDS/18%-polyacrylamide gel was used. Abbreviation: *M*, molecular mass.

Biosynthetic labelling of C109 and mutagenized constructs

Two mutant C109 constructs expressing a single mutation of T to A at codon 642 and a double mutation of G to T/A to C at codons 595/596 were obtained by site-directed mutagenesis. Both mutations were confirmed by direct sequence analysis of DNA PCR-amplified from the vector; both resulted in yields of recombinant protein resembling that obtained with unmutated C109 when expressed in *E. coli* induced with IPTG, and both resulted in a major species at 16–18 kDa on purification by metal-chelate exchange chromatography, and a minor species at 4–5 kDa that could be revealed by Western-blot analysis with anti-SP28 or after biotinylation of purified protein.

Labelling experiments were performed to determine whether the 4–5 kDa fragment resulted from premature termination of transcription or from degradation by bacterial proteinases after synthesis (Figure 8). Endogenous synthesis of bacterial proteins was inhibited with rifampicin in order to visualize better the recombinant proteins by Coomassie Blue staining of gels in addition to radioautography. These studies demonstrated that

the 4–5 kDa species could not be attributed to aberrant ribosomal binding, and also showed fall-off of label after 2–3 h of induction, implying some toxicity to *E. coli* with long induction periods, as was apparent by direct ultrastructural examination of cells.

In vitro fibrillogenesis

When C109 eluate from the Ni-NTA column was exhaustively dialysed against distilled water or PBS, a precipitate formed on standing. Electron microscopy of this material revealed only amorphous aggregates. Additional aliquots were studied at 48 h, 2, 12 and 20 weeks later, stored at 4 °C with 0.2% Na₂S₂O₃. Again, amorphous material was apparent which, however, bound F12 specifically, as demonstrated by biotinylated antimouse antibody and streptavidin-conjugated gold particles (Figure 9a). At 20 weeks, however, both PBS and water samples were found to have

Figure 9 Electron micrographs of (a) amorphous C109 in distilled water, pH 4.5 at 12 weeks post-isolation (magnification $\times 75\,000$), (b) fibril mass at 20 weeks ($\times 75\,000$), (c) individual fibrils in a looping configuration with occasional twists ($\times 60\,000$) and (d) an individual fibril at higher magnification ($\times 75\,000$)

(a), (b), and (d) were immunogold-labelled with mAb F12 as described in the Materials and methods section.

developed a minor ($< 5\%$) fibrillar component on ultrastructural analysis (Figure 9b). Fibrils were approx. 10 nm in diameter and had a long curved morphology, with occasional twists (Figure 9c), and bound MAb F12 (Figure 9d). This material was not birefringent when stained with alkaline Congo Red.

DISCUSSION

We used the RNA polymerase of bacteriophage T7 to direct the expression in *E. coli* of an APP construct corresponding to the C-terminal 109 residues of full-length transcripts (C109). The T7 system has been widely used for the high-level expression of

both prokaryotic and eukaryotic genes, with our constructs constituting 5–20% of total bacterial protein. Specific features of the vector include a downstream $\phi 10$ -S10 promoter segment, and an in-frame histidine hexamer tail for purification purposes. In addition, the BL21 (pLys S) host used for induction incorporates the gene for T7 lysozyme, which inhibits RNA polymerase under basal conditions and facilitates extraction of induced cells. These cells are also deficient in lon and ompT proteinases, thus minimizing degradation of recombinant protein by bacterial proteinases [28].

Only limited efforts have been reported for the expression of APP constructs in bacteria. In an early study, Selkoe et al. [40]

expressed a construct encoding the 400 C-terminal residues of APP and obtained proteins of 50–58 kDa, as well as three or four bands of lower apparent molecular mass that they attributed to proteolysis [40]. More recently, Roch et al. [41] reported the expression in *E. coli* and purification of a series of peptide fragments corresponding to parts of the secreted form of APP 695, which they used to map the growth regulatory activity of this protein to a 40-amino-acid domain C-terminal to the Kunitz-type proteinase inhibitor (KPI) insert cassette. In their system, in which constructs were generated by PCR, APP constituted 3–5% of total protein in T4 phage-infected bacteria. Recombinant protein was purified by heparin affinity chromatography and size-exclusion h.p.l.c. [41]. Since the heparin-binding domain of APP has been mapped to a segment more proximal to the KPI domain, however, this protocol would not be suitable for our, and related, constructs. Others have used the baculovirus system for the expression of full-length, deletion and mutagenized constructs of different APP transcripts in insect cells [42–45]. Processing of APP in this system has been of interest, as the host cell is assumed to duplicate some (if not all) post-translational modifications known for mammalian cells, and because expressed full-length transcripts associate with membrane fractions and undergo cleavage to release electrophoretically heterogeneous C-terminal fragments. In particular, a 17 kDa fragment had the size of C100 by SDS/PAGE, and bound an antibody directed against a major epitope in the region of APP₆₉₅ residues 597–601 [46].

C100 constructs have been overexpressed in cultured mammalian cells (COS, P19, PC12, 293, CV-1) [18–21,47] to yield major products of ~16 kDa apparent molecular mass, though smaller fragments have also been noted in some systems [18,21]. In PC12 cells, levels of a 16.3 kDa fragment were found to be increased by treatment with chloroquine [48] or phorbol esters, with the latter effect augmented by okadaic acid [38]. In 293 cells, a complex set of C-terminally reactive APP fragments have been identified on Tris/Tricine gels at a molecular mass of 8–12 kDa [11]. Similarly to our C109 construct, these apparent molecular masses exceed the predicted size of 11 kDa and reflect anomalous migration on gels first noted by Dyrks et al. in an *in vitro* translation system [17]. In COS and CV-1 cells, expressed product formed randomly distributed intracellular Congo Red-negative amyloid deposit-like structures or perinuclear inclusions containing 8–22 nm fibrils respectively [19,49]. Thus dissociation between APP polymerization and the property of Congo Red metachromasia with typical apple-green birefringence has now been identified in (a) preamyloid deposits of patients with AD or Down's syndrome [50], (b) fibrillar material formed directly from C109, (c) transfected cell lines [19], and for (d) a fibrillogenic non- $A\beta$ C-terminal APP peptide [14].

In addition to full-length C109, Edman degradation revealed that our purified expressed protein had undergone some minor proteolysis at the N-terminus. Whether this was due to aberrant initiation of synthesis or co-purification with a bacterial exopeptidase is unclear. Also of interest was the presence of small (~1%) amounts of a C109 fragment of 4–5 kDa that had the N-terminus of the construct by microsequencing, but presumably lacks the cytoplasmic and histidine hexamer tails. This fragment corresponds in size and immunoreactivity to the soluble forms of $A\beta$ that have recently been identified in medium of cultured cells and in body fluids [7,8]. Co-purification of this fragment with C109 may reflect denaturation and/or oxidation during purification [23], or an intrinsic property to self-aggregate [51] or bind to APP, possibilities which are currently under investigation. If the latter should prove to be the case, this would provide an alternative explanation to local processing for the presence of N- and C-terminal epitopes of full-length APP that have been

observed by immunohistochemistry in cerebral amyloid deposits [15,16] of patients with AD.

Two C109 constructs bearing two mutations associated with early-onset AD [31,32] have also been successfully expressed in high yield using the T7 system, and both recombinant proteins appear to be processed to a minor 4–5 kDa species. Study of the intrinsic fibrillogenic potential of these proteins, and their processing in a prokaryotic expression system, may help to clarify current information that some APP mutations appear to have no effect on processing in transfected cell lines [52], whereas others (notably the Swedish familial AD double mutation) significantly increase the production of soluble $A\beta$ [53,54]. Such studies may help to extend analysis of the effect of these mutations on *in vitro* fibrillogenesis of peptides corresponding to parts of the APP or $A\beta$ sequence [55,56].

Our serendipitous observation of *in vitro* fibrillogenesis by expressed purified C109 after a 20-week lag period suggests at least two testable mechanisms for polymerization of unmutated construct. One possibility is that the full-length C109 has an intrinsic potential to form fibrils apart from the $A\beta$ sequence. The recent observation of fibrillogenic sequences among peptides with sequence identity to parts of the APP cytoplasmic tail, and the lack of Congo Red binding by these structures [14] is consistent with this possibility. Alternatively, very small amounts of fragments corresponding to $A\beta$ might provide a nucleation mechanism for aggregation, as has been recently shown for fibrils composed of denatured transthyretin monomer [57].

In summary, we have successfully expressed and characterized a C109 construct in bacteria. In a previous report, this 16–18 kDa fragment was shown to correspond in size and immunoreactivity to a C-terminal, potentially amyloidogenic, APP fragment identified in the walls of cerebral microvessels, and to be functionally active in binding to an RHDS receptor on a cultured cell line [58]. This construct has an intrinsic potential to form fibrils and provides an *in vitro* system for examining the kinetics of this process, as well as the effect of APP mutations known to be associated with cerebral amyloid angiopathy [54,55] or early-onset familial AD [31,32].

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