Expression and analysis of heparin-binding regions of the amyloid precursor protein of Alzheimer's disease

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Abstract Deletion mutagenesis studies have suggested that there are two domains within APP which bind heparan sulphate. These domains have been cloned and expressed in the yeast *Pichia pastoris*. Both recombinant proteins bound to heparin. One domain (APP316–447) was further characterised by binding studies with peptides encompassing this region. Peptides homologous to APP316–346 and APP416–447 were found to bind heparin. Circular dichroism studies show that APP416–447 shifted towards an α-helical conformation in the presence of heparin. This study suggests that heparin-binding domains may lie within regions high in α-helical structure.

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Key words: APP; Heparin binding; Alzheimer's disease; Peptide; Secondary structure

1. Introduction

Alzheimer's disease (AD) is characterised by the presence of amyloid plaques in the brain. A major component of these plaques is Aβ, a polypeptide derived from a larger 110–130 kDa amyloid precursor protein (APP) [1–3]. The physiological role of APP is unclear. APP may play a role in neurite outgrowth [4–8]. APP has been shown to bind heparan sulphate proteoglycans (HSPGs), heparin and extracellular matrix macromolecules [8–12]. We have shown that the HSPGs glypican and perlecan inhibit the ability of APP to stimulate neurite outgrowth suggesting that the binding of APP to HSPGs is functionally important [13].

Deletion mutagenesis studies suggest that APP contains two domains responsible for binding to heparan sulphate [14]. The cysteine-rich domain, (CRD), between residues 18 and 194 includes a putative heparin-binding sequence HBD1 [8]. A putative α-helix-rich domain (HRD) which lies between residues 311 and 493 [14] includes another heparin-binding sequence, HBD2 [10].

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Abbreviations: Aβ, the amyloid protein obtained from APP cleavage; AD, Alzheimer's disease; APP, amyloid precursor protein; APP695, the 695 amino acid isoform of the amyloid precursor protein; HSPG(s), heparan sulphate proteoglycan(s); PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraaceticacid disodium salt; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

The aim of the present study was to map regions of APP which bind heparin. To accomplish this, both CRD and HRD were cloned and expressed in the methylotrophic yeast *Pichia pastoris*, and the expressed proteins examined for heparin binding. To localise specific heparin-binding regions within HRD, peptides spanning residues 316–447 of APP695 were synthesised and analysed by heparin affinity chromatography and circular dichroism. Our studies show that the regions rich in secondary structure within APP are both capable of heparin binding.

2. Materials and methods

2.1. Materials

Econo-Pac heparin cartridges (5 ml) were from Bio-Rad (North Ryde, NSW, Australia). Amidated and acetylated peptides encompassing residues 316–346, 347–381, 382–412 and 413–447 of APP695 were commercially synthesised by Chiron-Mimotopes (Clayton, Victoria, Australia). Peptides were purified by reversed-phase high performance liquid chromatography on a 5 μm Brownlee Spheri5 RP-18 column (100×4.6 mm), using an Applied Biosystems 400 Solvent Delivery System as described by LePage et al. [15]. The pPIC-9 expression vector was purchased from Invitrogen (San Diego, CA) and the oligonucleotide primers from Life Technologies (Mulgrave, Victoria, Australia). HiTrap chelating affinity columns were from AMRAD Pharmacia Biotech (Boronia, Victoria, Australia). Heparin (porcine intestinal mucosa), ethylenediaminetetraaceticacid disodium salt (EDTA) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). The anti-FLAG-M2 monoclonal antibody was from Eastman Kodak (New Haven, CT) and the anti-APP monoclonal antibody 22C11 was from Boehringer Mannheim (Germany). Goat anti-mouse immunoglobulins conjugated to alkaline phosphatase were from Promega Corporation (Madison, WI). The bicinchoninic acid (BCA) protein assay kit was from Pierce Biochemicals (Rockford, IL).

2.2. Expression of recombinant CRD and HRD of human APP in P. pastoris

2.2.1. Cysteine-rich domain (CRD).

The cysteine-rich domain (CRD) from residues 18–194 was amplified from a vector containing the APP695 cDNA sequence [16] with primer 1 (5'-CCCCGGGATG-CTGGAACCCACTGATGG-3') and primer 2 (5'-CCCCGGGCTA-GTCACTTTCTTCAGCCAGTGGG-3'). The 551 base pair fragment was digested with *Sma*I and cloned into the pPIC-9 vector digested with *Sna*BI. The expression vector was digested with *Bgl*II prior to transfection into *P. pastoris* (GS115) to allow for recombination of the expression cassette into the alcohol oxidase site [17]. Cultures were grown for 48 h and induced with 3% (v/v) methanol for 24 h as previously described [18]. Since the pPIC-9 vector directs secreted expression of the recombinant protein, cells were removed from the medium by centrifugation (10 000×g, 4°C, 30 min), the medium was passed through a 0.45 μm filter and PMSF added to a final concentration of 0.4 mM. The medium was then applied on to a 5 ml HiTrap

affinity column equilibrated with 0.02 M Na_2HPO_4, 0.5 M NaCl, pH 7.2 (start buffer) and charged with 0.1 M $CuSO_4$. The column was washed with 25 ml start buffer and eluted with start buffer containing 0.05 M EDTA. The eluate was then applied on to a Sephadex G-25 (PD-10) column to desalt and exchange the buffer into 50 mM Tris-HCl pH 7.4. Yields of the recombinant protein were 24 mg purified protein/l.

2.2.2. α-Helix-rich domain (HRD). The putative α-helix-rich domain (HRD) was amplified from APP_{695} cDNA as described above with primer 1 (5'-CTCGAGGAA TTCGACTACAAGGACGACG-ATGACAAGTTCCAGAAAGCCAAAGAG-3') and primer 2 (5'-CAGGGAGAATTCTTAATTCATGCGCTCATAAATGACACG G-3'). Primer 1 also contained the eight-amino acid FLAG epitope (5'-GACTACAAGGAC GACGATGACAAG-3') for immunodetection of the recombinant protein. The PCR fragment was digested with *Eco*RI and cloned into *Eco*RI digested pPIC-9. The expression vector was digested as described above. Recombinant protein expression was induced for 5 days using 1% (v/v) methanol as previously described [19]. The medium was collected as described above and protease inhibitors added (0.4 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 2 mM EDTA, 5 μM E-64). The supernatant was diluted 50-fold for heparin-binding analysis. Yields of the recombinant protein were approximately 100 μg/l.

2.3. SDS-polyacrylamide gel electrophoresis and Western blotting

Aliquots of culture supernatant from *P. pastoris* were subjected to electrophoresis on 15% mini SDS-polyacrylamide gels [20]. Gels were electrophoresed at 40 mA each, proteins were transferred for 3 h at a current of 1 Amp onto nitrocellulose membranes. Membranes were blocked in 5% (w/v) skim milk powder in 20 mM Tris-HCl pH 7.4 containing 0.15 M NaCl (TBS). CRD was detected with 22C11, a monoclonal antibody which recognises an epitope near the N-terminus of APP. HRD was detected with the anti-FLAG-M2 monoclonal antibody. Membranes were rinsed in TBS, incubated with a secondary antibody conjugated to alkaline phosphatase and developed with Fast Red/Naphthol AS-MX substrate.

2.4. Heparin affinity chromatography

Recombinant protein (100 μg) or purified peptides (1 mg) were applied on to a 5 ml Econo-Pac heparin cartridge pre-equilibrated in 20 mM Tris-HCl pH 7.4 (Tris buffer) using a Bio-Rad Econo System. After an initial wash phase of 20 min with Tris buffer, the column was eluted with a linear gradient of 0–1 M NaCl at a flow rate of 1.0 ml/min over 60 min. Fractions of 1.0 ml were collected. To detect the recombinant proteins, 300 μl fractions were applied on to nitrocellulose membranes using a Bio-dot Microfiltration apparatus (Bio-Rad). Membranes were blocked in 5% (w/v) skim milk powder in TBS and detected with the appropriate monoclonal antibodies as described above. Images were digitised and staining intensity quantified by densitometry using the NIH Image software (V1.57, W. Rasband, NIH, Bethesda, MD). The amount of protein was determined using the BCA protein assay kit from Pierce with bovine serum albumin as a standard [21]. Eluted peptides were detected spectrophotometrically by measuring the absorbance of each fraction at 214 nm.

2.5. Circular dichroism

Circular dichroism (CD) spectra were recorded at 20°C in a 1 mm cell using an AVIV 62 DS spectrometer with an electronic temperature controlling device (Lakewood, NJ). Control CD spectra were generated for the 40 mM sodium phosphate buffer pH 7.4 and for heparin alone to correct for any background influence. The fraction of α-helical content was calculated according to the method of Wu et al. [22] using the observed mean residue ellipticity at 222 nm and the method of Greenfield and Fasman [23] using the mean residue ellipticity at 208 nm.

3. Results

3.1. Expression of CRD and HRD in P. pastoris

Deletion mutagenesis studies by Clarris et al. [14] suggested that at least two regions in APP, the CRD between residues 18–194 and a putative HRD between residues 311–493 may bind heparin. To confirm whether these regions contain hep-
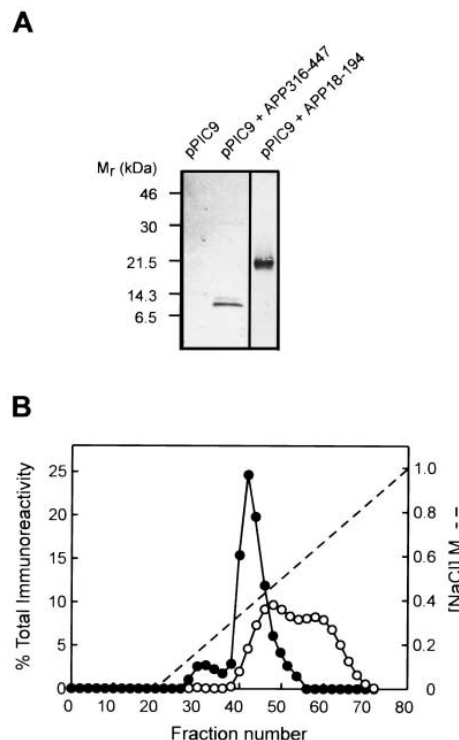

Fig. 1. Analysis of the heparin-binding domains of APP expressed in *P. pastoris*. A: Western blot of HRD and CRD secreted into the culture medium. Culture medium from the pPIC-9 vector alone showed no immunoreactivity. HRD was detected with the anti-FLAG monoclonal antibody. Three bands (13 kDa, 10 kDa and 34 kDa) were observed on the Western blot. CRD was detected with the monoclonal antibody 22C11. A 20 kDa band was observed. B: Heparin affinity chromatography of CRD (●) and HRD (○). 100 μg of recombinant protein was applied on to a heparin-sepharose affinity column and eluted with a linear gradient of 0–1 M NaCl in 20 mM Tris-HCl pH 7.4. CRD eluted at ~0.35 M NaCl and HRD between 0.4–0.7 M NaCl. Results are expressed as a percentage of the total immunoreactivity for each recombinant protein.

arin-binding domains, both regions were cloned and expressed in *P. pastoris*.

As the pPIC-9 expression vector directs extracellular expression of the recombinant protein, the culture supernatant was analysed by Western blotting. CRD was detected with the monoclonal antibody 22C11. A single 20 kDa band was observed, corresponding to the calculated molecular mass of 20.9 kDa (Fig. 1A). HRD was detected with an anti-FLAG monoclonal antibody [24]. Two major bands migrating at 13 kDa and 10 kDa were detected (Fig. 1B), lower than expected from the predicted molecular mass of the recombinant protein (16.9 kDa). A very faint band migrating at 34 kDa was also observed. As this band was approximately twice the size expected for the recombinant fragment, it may correspond to a dimeric form.

3.2. Heparin affinity chromatography of CRD and HRD

The ability of CRD and HRD to bind heparin was examined. The supernatant obtained from the *P. pastoris* cultures was diluted 1:10 with Tris buffer and applied to a heparin column. Aliquots of fractions eluting from the column were applied on to nitrocellulose membranes and immunoreactive proteins detected with 22C11 for CRD or the anti-FLAG-M2
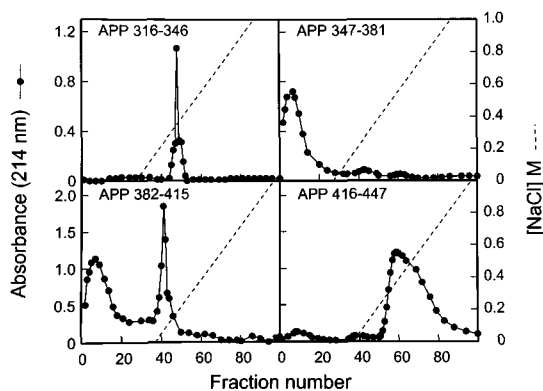
Fig. 2. Heparin affinity chromatography of peptides encompassing APP316–447. Each peptide (1 mg) was applied to a 5 ml heparin affinity column, and eluted with a linear gradient of NaCl from 0–1 M. APP316–346 and APP416–447 bound strongly to the column, APP382–415 bound weakly, while APP347–381 did not bind.

Fig. 4. Helical wheel representation of the orientation of residues in a putative α-helical model of APP416–447. Basic amino acid residues predominantly segregate to one side of the helix, which may favour heparin binding.

monoclonal antibody for HRD. Both recombinant proteins bound to the column. The CRD protein eluted off the column as a sharp peak at ∼0.35 M NaCl (Fig. 1B). The HRD protein eluted off the column in a broad peak between 0.4 and 0.7 M NaCl (Fig. 1B).

3.3. Heparin affinity chromatography of peptides encompassing APP316–447

To examine the heparin binding of specific regions within HRD further, four peptides of similar length homologous to APP316–346, APP347–381, APP382–415 and APP416–447 of APP_{695} were synthesised. To assess the ability of these peptides to bind heparin, 1 mg of each peptide was applied to the heparin affinity column and eluted with a linear gradient of NaCl from 0–1 M. Fractions (1.0 ml) were collected and the absorbance measured spectrophotometrically at 214 nm (Fig. 2). APP316–346 and APP416–447 bound strongly to the column, eluting at 0.38 M and 0.34 M NaCl respectively.
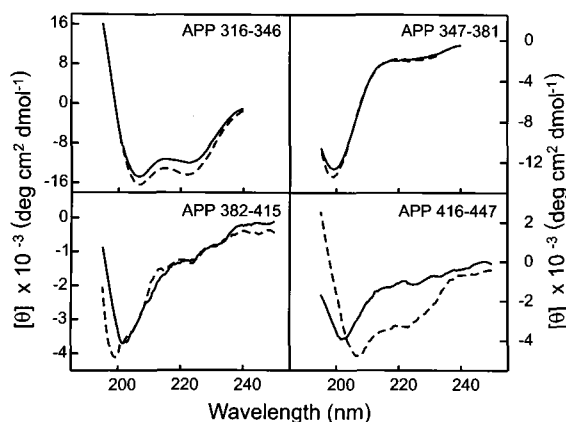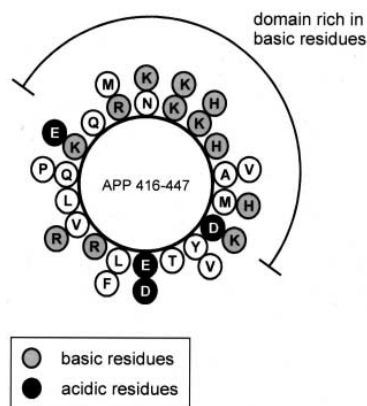

Fig. 3. CD spectra of peptides encompassing APP316–447. Each peptide (1 mg) was subjected to CD in the absence (—) or presence (- - -) of 150 µg/ml heparin. APP316–346 exhibited a spectrum representative of an α-helix both with and without heparin. APP347–381 and APP382–415 were representative of random coil. APP416–447 was random in the absence of heparin, but underwent a conformational change to more α-helical character when heparin was added.

APP382–415 eluted at 0.27 M NaCl while APP347–381 did not bind.

3.4. Circular dichroism

To study the peptide-heparin interactions further, each peptide was analysed by circular dichroism spectroscopy in the presence or absence of heparin (Fig. 3). The CD spectrum for APP316–346 in the absence (—) of heparin showed α-helical characteristics, represented by the double minima at 208 nm and 222 nm and a maximum at ∼190 nm. The percentage of α-helix was calculated to be 33% by the method of Wu et al. [22] and 36% by the Greenfield and Fasman method [23]. In the presence of 150 µg/ml heparin the relative α-helical content increased by 24% as calculated by the method of Wu et al. [22] and by 17% by the method of Greenfield and Fasman [23]. APP347–381 and APP382–415 both demonstrated random coil characteristics with a single large negative peak at 199–200 nm and a very weak peak at 220 nm both in the absence and presence of heparin. In the absence of heparin, APP416–447 demonstrated characteristics of a random coil form. In the presence of heparin, the relative α-helical content increased by 26%, evident by an increase in mean residue ellipticity at 208 nm and 222 nm.

4. Discussion

Deletion mutagenesis studies suggest that there may be two regions within APP which bind heparin [14]. Both these domains, the CRD (APP18–194), and a putative HRD (APP311–493) have also been predicted to be rich in secondary structure [8,14]. In the present study, these domains were cloned and expressed in *P. pastoris* and found to bind heparin.

Western blotting analysis demonstrated that the CRD expressed in *P. pastoris* had a molecular mass of 20 kDa, which agrees with the calculated molecular mass. The 13 kDa product observed for the HRD would be the expected recombinant fragment, possibly C-terminally truncated (either intracellularly or during secretion). The 10 kDa product may be due to further C-terminal proteolysis. Proteolytic degradation in the *P. pastoris* expression system can occur and has been

documented [18]. The identity of the recombinant protein was confirmed by the observation that the anti-FLAG antibody still detected the 13 kDa and 10 kDa products but no immunoreactivity was detected in the untransfected culture supernatant.

Four peptides encompassing HRD were synthesised and used to further characterise the heparin-binding capability and structural conformation of specific regions within this domain. The entire region between residues 316–447 contains a high concentration of basic amino acids. The peptides APP316–346, APP382–415 and APP416–447 all bound to heparin, while APP347–381 did not bind. The fact that APP347–381 had a concentration of basic amino acids similar to the other peptides on either side of this region, suggested that a high concentration of basic amino acids alone may be insufficient to predict heparin binding. APP316–346 and APP382–415 had a net charge of +3, APP416–447 had a net charge of +11, and APP347–381 had a net negative charge of −1. Thus, the overall net charge on these peptides correlated with their heparin-binding capability. However, the binding of ligands to heparin is unlikely to involve solely a simple electrostatic interaction, but is more likely to involve a specific conformational structure as well.

CD studies showed that APP316–346 has α-helical characteristics both in the absence and presence of heparin. APP416–447 underwent a conformational change from random coil to α-helix in the presence of heparin, demonstrated by the double minima at 208 nm and 222 nm. The CD spectrum for APP347–381 and APP382–415 suggested that both these peptides adopt a random conformation in solution. APP382–415 bound to heparin, although with a lower affinity than APP316–346 and APP415–447. Heparin binding of this peptide may fail to provide electrostatic stabilisation of the peptide which could favour formation of α-helix.

Our previous studies [14] predicted that the region of APP between residues 316 and 447 is predominantly α-helix. Our current studies with peptides further defines regions which regions within this domain may be involved in heparin binding due to their secondary structure. Since APP416–447 demonstrated a clear shift from random form to α-helix in the presence of heparin, a helical wheel representation of this peptide was constructed (Fig. 4). In this model, basic residues were found to segregate on one side of the helical surface. Helical wheel determination of the other peptides did not show this segregation of basic residues. α-Helix may thus provide a good structure for binding heparin as it presents a rigid linear array of residues in which spacing of the residues may be appropriate for binding of the negative charges in heparin.

The binding of many proteins to heparin is often through regions containing α-helix [25,26]. Proteins such as acidic and basic fibroblast growth factor are induced to adopt a higher degree of α-helical structure upon binding to heparin [27,28]. APP peptides which did not have demonstrable secondary structure did not bind heparin. Several other studies have shown that heparin-binding domains are often associated with regions rich in secondary and tertiary structure [25,26,29].

The significance of heparin-binding domains for APP is still unclear. APP may play a role in neurite outgrowth, possibly through an interaction with HSPGs on the cell surface [13,30]. The RERMS sequence present within APP316–447 may be associated with the promotion of neurite outgrowth [7]

although recent studies [30] suggest that the effect of this sequence on neurite outgrowth is not modulated via an HSPG. The N-terminal heparin-binding region may be involved in the trophic function of APP [8]. A better understanding of the heparin-binding interactions of APP will shed more light on the function of APP and its role in AD pathogenesis.

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