The intracellular cytoplasmic domain of the Alzheimer's disease amyloid precursor protein interacts with phosphotyrosine-binding domain proteins in the yeast two-hybrid system

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Abstract We have used the yeast two-hybrid system to screen for proteins that interact with the carboxy-terminal domain of APP. Six different clones were isolated and sequence analyses revealed that they encoded domains of a previously described neuronal protein Fe65, a homologue of Fe65 and a homologue of protein X11. All of these proteins contain one or more phosphotyrosine binding (PTB) domains. PTB domain proteins bind to the sequence Asn-Pro-X-Tyr when the Tyr is phosphorylated and are believed to function in signal transduction. APP contains such a motif. These results are consistent with a role for APP in signal transduction mechanisms.

Key words: APP; Alzheimer's disease; Fe65; X11; Phosphotyrosine-binding domain

1. Introduction

Deposits of β-amyloid in neuritic plaques and blood vessel walls are a principal pathological feature in the brains of patients with Alzheimer's disease. These amyloid deposits contain the 39–43 amino acid β-amyloid peptide which is derived by proteolytic cleavage from its much larger precursor, the amyloid precursor protein (APP). β-amyloid is a normal physiological product of APP metabolism. However, it is thought that aberrant processing of APP to produce either increased amounts of β-amyloid or longer (42/43 amino acid) and hence more amyloidogenic β-amyloid isoforms, is a primary pathogenic event leading to amyloid deposition in Alzheimer's disease (see [1–3] for reviews).

APP gene mRNA transcripts are alternatively spliced so as to generate a number of different APP molecules; the principal isoforms comprising 695, 751 and 770 amino acids. The longer isoforms contain a domain that displays homology to the Kunitz class of protease inhibitors [4]. APP695 is the predominant isoform found in brain.

APP is a membrane-spanning protein which crosses the plasma membrane once and contains a large extracellular domain (which in APP751 and APP770 contains the Kunitz protease inhibitor domain) and a smaller 47 amino acid intracellular domain. β-amyloid is derived from sequences contained within and just external to the membrane spanning domain (see [1–3] for reviews).

Although secreted extracellular domains of APP containing the Kunitz protease inhibitor domain may act as extracellular serine protease inhibitors, [5,6] the function of membrane-bound APP is not fully understood. However, APP has some structural features of cell surface receptors [7] and the intracellular domain of APP has been shown to bind G_o [8] which suggests that it may be involved in signal transduction processes. In common with a number of cell surface receptors, membrane-bound APP has been shown to be re-internalised into lysosomes where intact β-amyloid containing fragments are produced and this is believed to be one route for the production of β-amyloid [9–12]. The carboxy terminal intracellular domain of APP contains the motif Asn-Pro-Thr-Tyr which is a sequence known to mediate re-internalisation via clathrin-coated pits [13]. If the tyrosine in this sequence is phosphorylated, then this motif is also a consensus sequence for binding to phosphotyrosine binding (PTB) domains [14–16] (and see [17] for review). To understand further the function of APP and the mechanism(s) of β-amyloid production, we have used the yeast two-hybrid system to identify proteins that interact with the intracellular carboxy-terminal portion of APP. Here we demonstrate that this domain of APP interacts with a number of PTB domain containing proteins.

2. Materials and methods

To generate a GAL-4 DNA binding domain-APP fusion 'bait' plasmid, sequences encoding the intracellular carboxy-terminal 47 amino acids of APP (APPc) were amplified by PCR and cloned into the GAL-4 DNA binding domain yeast shuttle vector pY1 [18] so as to produce pY1APPc. Clones were sequenced to check that no PCR errors had been introduced and that APP sequences were in-frame with GAL-4 encoding sequences. pY1APPc was used to screen a human brain cDNA library containing the GAL-4 transactivation domain in pGAD10 (Clontech) by co-transformation of both plasmids into yeast Y190. Yeast transformations were performed using lithium acetate/heat shock as described (Clontech). Y190 possesses both lacZ and His GAL reporters. Transformants were grown for 8 days at 30°C on synthetic selection medium lacking tryptophan, leucine and histidine and containing 25 mM 3-amino triazole. Large (>2 mm) colonies surviving selection were picked and assayed for β-galactosidase activity using a freeze-fracture assay. Plasmids from colonies positive on both the nutrient (His) and β-galactosidase assays were rescued into *Escherichia coli* HB101. To check that positive plasmids only transactivated in the presence of pY1APPc, yeast Y190 were re-transformed with the candidate plasmids either alone, or with pY1 or pLAM5′ (Clontech) that contains the GAL-4 DNA binding domain fused to an extraneous fragment of laminin and colonies again assayed for β-galactosidase activity. Positive controls included transformation of Y190 with pCL1 (that contains full-length GAL-4) and co-transformation with pVA3 and pTD1 (that contain respectively the GAL-4 DNA binding domain fused to p53 encoding sequences, and GAL-4 transactivation domain fused to SV40 large T antigen encoding sequences). pCL1, pVA3 and pTD1 were obtained from Clontech.

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Plasmids from positive clones which transactivated only in the presence of pY1APPc were sequenced and homology searches performed using the BLAST network service.

3. Results and discussion

Approximately 10^6 independent library colonies were screened with pY1APPc using the yeast two-hybrid system and six positive clones identified. Analyses of the open reading frames of these clones revealed that three contained identical inserts which encoded the carboxy-terminal 422 amino acids of the human homologue (HFe65) of a previously described rat neuronal protein, Fe65 [19]. A fourth clone encoded the carboxy-terminal 648 amino acids of HFe65. The fifth clone contained sequences that encoded an open reading frame of 150 amino acids that were 64% identical to HFe65 at the amino acid sequence level. This suggests that clone 5 might contain a homologue of HFe65 (HFe65-like) and that there may be a family of Fe65 proteins. No in-frame start codon with surrounding Kozak sequence or stop codon were present in the clone 5 insert which indicates that it lacks both the amino- and carboxy-terminal encoding DNA sequences of this protein. The sixth clone contained an open reading frame encoding 441 amino acids that bore greatest similarity to a mouse homologue of protein X11 [20] (99% homology at the amino acid sequence level) but which displayed less homology to human protein X11 [21] (83% homology at the amino acid sequence level). Further searches revealed that overlapping but identical sequences to the clone 6 insert had previously been deposited in the database (Accession numbers R89683, R13101, R18654 and T16098). Clone 6 contains an in-frame stop codon but no in-frame ATG with surrounding Kozak sequence and so may well code for a truncated carboxy-terminal fragment. To simplify the terminology in this report, we have termed the protein encoded by clone 6, protein X11-like.

Analyses of the proteins encoded by the six clones revealed that they all contain at least one phosphotyrosine binding (PTB) domain. The HFe65 clones contain two PTB domains; the HFe65-like clone contains one PTB domain (indeed, the amino acid sequences encoded in the clone represent a single PTB domain); and the protein X11-like clone contains one PTB domain. The PTB domains of HFe65, HFe65-like and protein X11-like are shown and aligned in Figs. 1 and 2. It has previously been noted that both Fe65 and protein X11 have PTB domains [22,23].

Fiore et al. [23] have shown that the rat Fe65 PTB domains

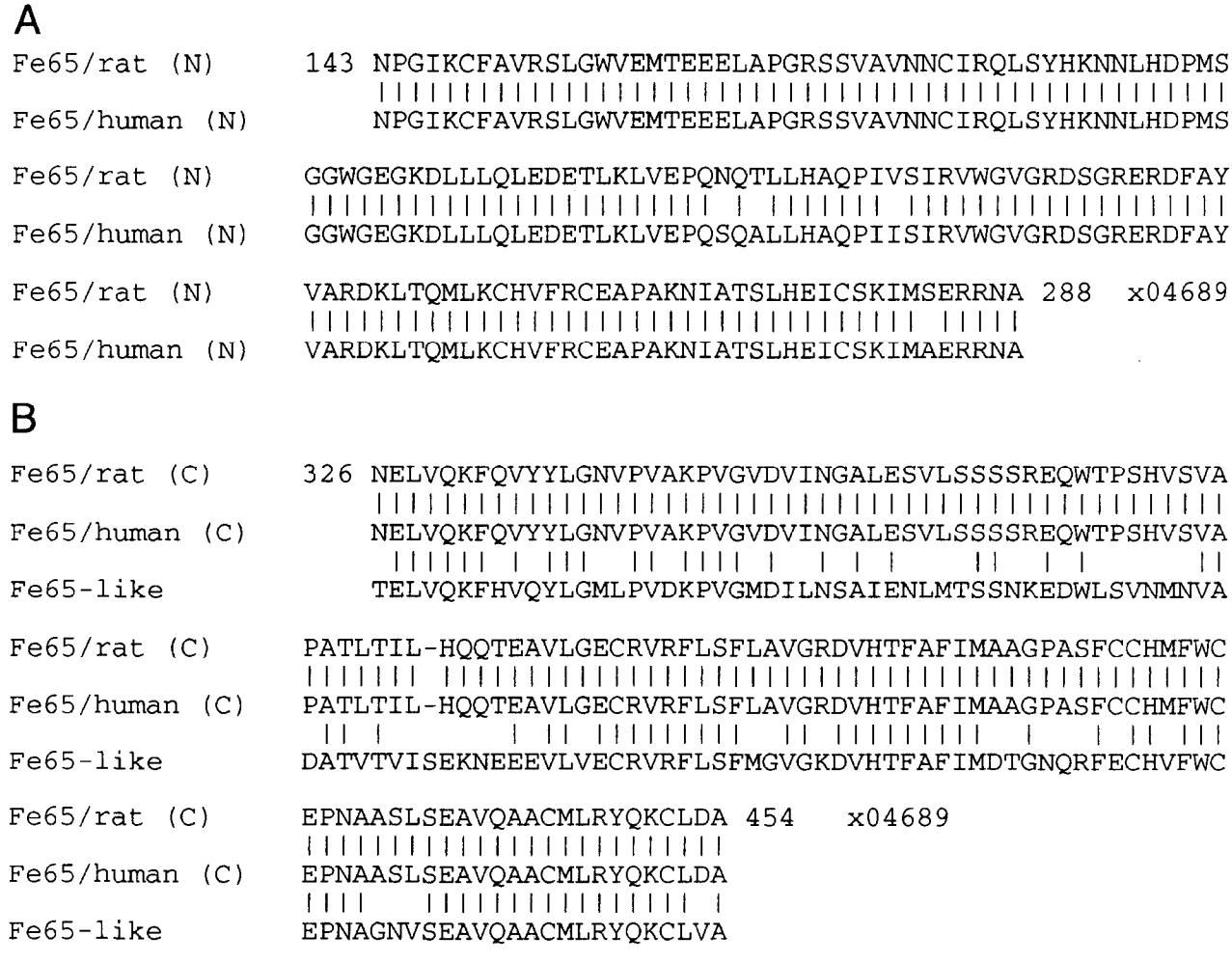

Fig. 1. Alignments of the PTB domain amino acid sequences from rat and human Fe65 and human Fe65-like protein. A: Alignment of the amino-terminal (N) PTB domains of human and rat Fe65. B: Alignment of the carboxy-terminal (C) PTB domains of rat and human Fe65 with the PTB domain identified in human Fe65-like protein. Dashed line indicates a gap in the alignment. The accession numbers for the individual proteins are listed at the end of relevant sequences.

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X11-like            LIDGIIFAANYLGSTQLLSERNPSKNIRMMQAQEAVSRVKRMQKAAKIKK
                    |||||||||||||||||||||| |||||||||||| ||||||||||||||
X11/mouse      298  LIDGIIFAANYLGSTQLLSERTPSKNIRMMQAQEAASRVKRMQKAAKIKK
                    |||||||||||||||||||| ||||| |||||||| || |  || ||  |
X11            326  LIDGIIFAANYLGSTQLLSDKTPSKNVRMMQAQEAVSRIKMAQKLAKSRK

X11-like            KANSEGDAQTLTEVDLFISTQRIKVLNADTQETMMDHALRTISYIADIGNIVVLM
                    |||||||||||||||||||||||||||||||||||||||||||||||||||||||
X11/mouse           KANSEGDAQTLTEVDLFISTQRIKVLNADTQETMMDHALRTISYIADIGNIVVLM
                    ||  ||  |  ||||||| |||||||||||||||| ||||||||||||||||
X11                 KA-PEGESQPMTEVDLFILTQRIKVLNADTQETMMDHPLRTISYIADIGNIVVLM

X11-like            ARRRMPRSASQDCIETTPGAQEGKKQYKMICHVFESEDAQLIAQSIGQAFSVAYQ
                    |||||||||||||||||||||||||||||||||||||||||||||||||||||||
X11/mouse           ARRRMPRSASQDCIETTPGAQEGKKQYKMICHVFESEDAQLIAQSIGQAFSVAYQ
                    |||| ||| ||   |     | || |||||||||||||||||||||||||||||
X11                 ARRRIPRSNSQENVEASHPSQDGKRQYKMICHVFESEDAQLIAQSIGQAFSVAYQ

X11-like            EFLRA
                    |||||
X11/mouse           EFLRA  462   L34676
                    |||||
X11                 EFLRA  489   L04953
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Fig. 2. Alignments of the PTB domain sequences from the amino acid sequences of the mouse and human versions of the X11 protein plus the PTB domain sequence of the human X11-like protein.

interact with the carboxy-terminus of APP in the yeast two-hybrid system and in co-precipitation experiments with Glutathione-S transferase-Fe65 fusion proteins. However, their approach involved utilising Fe65 sequences as 'bait' to screen a library rather than APP sequences. Our findings that the intracellular domain of APP interacts with human Fe65 when APP is used as 'bait' thus confirms and extends their findings.

In addition, we demonstrate that two other PTB domain proteins, HFe65-like (a homologue of Fe65) and protein X11-like (a homologue of protein X11), all interact with the intracellular carboxy-terminal domain of APP in the yeast two-hybrid system. Protein X11 was originally identified as a candidate for the Friedreich's ataxia disease gene [21]. A mouse homologue of protein X11 has been cloned [20] and the sequences we have isolated for protein X11-like protein display high and greater homology to this murine homologue than to protein X11 (protein X11-like is 99% identical to the mouse protein X11 but 83% identical to human protein X11) which suggests that there might be a family of these proteins. Thus both the mouse and human genomes contain a protein X11-like gene. In situ hybridisation studies with mouse protein X11-like probes reveal that its transcripts are widely expressed throughout the nervous system including the cortex and hippocampus, two of the regions affected in Alzheimer's disease [21].

The Fe65-like sequences we have isolated encode for 150 amino acids and this appears to essentially comprise a single PTB domain (see [22] for alignment of some PTB domains). Thus, it appears that an isolated PTB domain from Fe65-like protein is sufficient for binding to the carboxy-terminal intracellular domain of APP.

The PTB domain was originally identified in the Src homology 2 (SH2) containing protein Shc [24,25]. Like SH2 domains, PTB domains appear to bind tyrosine phosphorylated proteins although SH2 and PTB domains are structurally unrelated. It is believed that PTB domains impart on proteins an ability to bind the sequence motif Asn-Pro-X-Tyr when the Tyr is phosphorylated and that PTB domain containing proteins function in mechanisms for transducing signals from the cell surface [15,17,24,25]. The intracellular portion of APP used here as bait contains one Asn-Pro-X-Tyr motif.

APP has previously been shown to interact with G_o and to have cell signalling functions in vitro via its carboxy-terminal sequences [8,26]. In addition, APP has recently been reported to interact with N-Pak, a neural-specific p21 activated kinase [27]. Such observations suggest that APP may have a role in signal transduction. In this context, it is striking that we find that three different PTB domain containing proteins will interact with APP in the yeast two-hybrid system; proteins containing PTB domains are believed to function in signal transduction [17]. Thus our findings further implicate APP (either directly or indirectly) in such cell signalling mechanisms.

The Asn-Pro-X-Tyr motif is also believed to mediate re-internalisation of receptors from the cell surface via clathrin-coated pits [13]. Such a re-internalisation of APP into lysosomes is one route for the generation of secreted β-amyloid and mutation of the Asn-Pro-X-Tyr motif is inhibitory to β-amyloid production [12]. In this respect, it will be interesting to determine how expression of the three PTB domain proteins described here influence APP processing and β-amyloid production. Such studies are currently underway in this laboratory.

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References

[1] Ashall, F. and Goate, A.M. (1994) Trends Biochem. Sci. 19, 42–46.
[2] Selkoe, D.J. (1994) Annu. Rev. Neurosci. 17, 489–517.
[3] Octave, J.-N. (1995) Rev. Neurosci. 6, 287–316.
[4] Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shirohiri, S. and Ito, H. (1988) Nature 331, 385–396.
[5] Oltersdorf, T., Fritz, L.C., Schenk, D.B., Lieberberg, I., Johnson-Wood, K.L., Beattie, E.C., Ward, P.J., Blacher, R.W., Dovey, H.F. and Sinha, S. (1989) Nature 341, 144–147.
[6] Van Nostrand, W.E., Wagner, S.L., Suzuki, M., Choi, B.H., Farrow, J.S., Geddes, J.W., Cotman, C.W. and Cunningham, D.D. (1989) Nature 341, 549.
[7] Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K. and Muller-Hill, B. (1987) Nature 325, 733–736.
[8] Nishimoto, I., Okamoto, T., Matsuura, Y., Takahashi, S., Murayama, Y. and Ogata, E. (1993) Nature 362, 75–79.
[9] Cole, G.M., Bell, L., Truong, Q.B. and Saitoh, T. (1992) Ann. NY Acad. Sci. 674, 103–117.
[10] Golde, T.E., Estus, S., Younkin, L.H., Selkoe, D.J. and Younkin, S.G. (1992) Science 255, 728–730.
[11] Haass, C., Koo, E.H., Mellon, A., Hung, A.Y. and Selkoe, D.J. (1992) Nature 357, 500–503.
[12] Koo, E.H. and Squazzo, S.L. (1994) J. Biol. Chem. 269, 17386–17389.
[13] Chen, W.-J., Goldstein, J.L. and Brown, M.S. (1990) J. Biol. Chem. 265, 3116–3123.
[14] Campbell, K.S., Ogris, E., Burke, B., Su, W., Auger, K.R., Druker, B.J., Schaffhausen, B.S., Roberts, T.M. and Pallas, D.C. (1994) Proc. Natl. Acad. Sci. USA 91, 6344–6348.
[15] Songyang, Z., Margolis, B., Chaudhuri, M., Shoelson, S.E. and Cantley, L.C. (1995) J. Biol. Chem. 270, 14863–14866.
[16] Stephens, R.M., Loeb, D.M., Copeland, T.D., Pawson, T., Greene, L.A. and Kaplan, D.R. (1994) Neuron 12, 691–705.
[17] Van der Geer, P. and Pawson, T. (1995) Trends Biochem. Sci. 20, 277–280.
[18] Sadowski, I., Bell, B., Broad, P. and Hollis, M. (1992) Gene 118, 137–141.
[19] Duilio, A., Zambrano, N., Mogavero, A.R., Ammendola, R., Cimino, F. and Russo, T. (1991) Nucl. Acids Res. 19, 5269–5274.
[20] Duclos, F. and Koenig, M. (1995) Mamm. Genome. 6, 57–58.
[21] Duclos, F., Boschert, U., Sirugo, G., Mandel, J.-L., Hen, R. and Koenig, M. (1993) Proc. Natl. Acad. Sci. USA 90, 109–113.
[22] Bork, P. and Margolis, B. (1995) Cell 80, 693–694.
[23] Fiore, F., Zambrano, N., Minopoli, G., Donini, V., Duilio, A. and Russo, T. (1995) J. Biol. Chem. 270, 30853–30856.
[24] Kavanaugh, W.M. and Williams, L.T. (1994) Science 266, 1862–1865.
[25] Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V. and Margolis, B. (1994) J. Biol. Chem. 269, 32031–32034.
[26] Okamoto, T., Takeda, S., Giambarella, U., Murayama, Y., Matsui, T., Katada, T., Matsuura, Y. and Nishimoto, I. (1996) EMBO J. 15, 3769–3777.
[27] Neve, R. and McPhie, D. (1996) Neurobiol. Aging 17, S190.