MOLECULAR CLONING AND SEQUENCE ANALYSIS OF A HUMAN BRAIN cDNA OF AN ALZHEIMER AMYLOID PRECURSOR (APP) INTERACTING PROTEIN

BY

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A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree of
Master of Science

Department of Biochemistry and Medical Genetic

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Francis Amara, for providing me the opportunity to study in his laboratory and for his guidance and inspiration.

I would also like to extend my appreciation to my academic advisory committee, Dr. Grant Hatch, Department of Pharmacology, and Dr. Asad Junaid, Department of Internal Medicine, for their time and valuable comments during the review of my thesis. My special thanks to Dr. Patrick Choy and Dr. Yenwen Gong for their help during my study. Also, I like to thank to Ken Asham, Teralee Burton, and Ken Anyadike for their help in correcting my thesis.

Finally, my deepest thanks to my wife, Manna, for her support and encouragement.

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LIST OF ABBREVIATION

AA	amino acid
ABP	APP binding protein
AD	Alzheimer disease
'AD'	activation domain
APP	Alzheimer amyloid precursor protein
APP_{COOH}	carboxyl-terminal region of APP
APP-BP1	APP-binding protein 1
β-AP	beta-amyloid peptide
BD	binding domain
BLAST	Basic Local Alignment Search Tool
cAMP	cyclic adenosine 5'-monophosphate
CDC (CDK)	cyclin-dependent protein
CP2/LSF	CATTT-binding protein 2/Late SV40 transcription factor
CK2	casein kinase II
CRE	cAMP response element
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA-BD	DNA binding domain
DNA-AD	DNA activation domain
DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol bis (2-aminoethyl-ether) tetraacetic acid
ER	endoplasmic reticulum
ERAB	endoplasmic reticulum associated binding protein
FAD	familial Alzheimer disease
g	gram
G_0	guanosine 5'-triphosphote-binding protein 0

HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
His	Histidine
-His, -Leu, -Trp	requires histidine (His), or leucine (Leu), or tryptophan (Trp) in the medium or on agar plate to grow
HSP	high-scoring segment pair
Kda	kilodalton
IR	insulin receptor
LacZ	LacZ report gene
Leu	Leucine
LiAc	lithium acetate
M	molar
MAP	mitogen-activated protein
MCS	multiple cloning site
Mena	the mammalian homologue of the drosophila enabled protein
ml	milliliter
mm	millimeter
mM	millimolar
MT	microtubule
MTT	3-(4,5, -simethylthiazol-2-yl)-2,5, -dimethylthiazolium bromide
MW	molecular weight
NaOH	sodium hydroxide
NFT	neurofibrillary tangle
ODC	ornithine decarboxylase
PBS	phosphate-buffered saline
PDZ	postsynaptic density-95/Drosophila discs large tumor suppressor/Zonula occludens-1
PEG	polyethylene glycol
PHF	paired helical filament
PID	phosphotyrosine interaction domain
PKA	protein kinase A
PKC	protein kinase C

PS1	presenilin 1
PS2	presenilin 2
PTB	phosphotyrosine binding domain
RAGE	receptor for advanced glycation end products
ROS	reactive oxygen species
sAPP	the secreted form of APP
SD	yeast minimal synthetic dropout medium
SDS	sodium dodecylsulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SR	scavenger receptor
SSC	sodium citrate transfer buffer
TAE	Tris-acetate-EDTA solution
TBS	Tris-buffered saline
TBS-T	TBS with Tween 20
TE	Tris-EDTA solution
TEMED	N, N, N', N'-tetramethyl ethylene diamine
Trp	Trptophan
UAS	upstream activation site
YPD	complete yeast medium
Y190	the yeast strain used in the experiments

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Abstract

Alzheimer disease (AD) is the most common form of dementia. It is characterized by the accumulation of beta-amyloid peptides (β-AP) derived from Alzheimer amyloid precursor protein (APP) and neurofibrillary tangles in the human brain. Knowledge of the normal functions of APP will provide clues to our understanding of the pathogenesis of Alzheimer disease. The location of APP (membrane-bound) and its structural features are characteristics of plasma membrane receptors such as integrins and epidermal growth factor receptors. Analysis of a protein interaction network from its intracellular carboxyl-terminal domain (APP_{COOH}) indicated that APP might be involved in signal transduction pathways. In order to understand the functions of APP, it is important to identify its effector binding proteins.

To address this question, the yeast-two hybrid system was employed. This system is based on a Gal-4 transcriptional assay for detecting specific protein-protein interactions in yeast. This can be used to screen libraries for genes encoding proteins that interact with a known "bait" protein. We constructed the APP "bait" fusion protein used to screen for a "match" in a normal human brain expression library. After screening, 11 beta-galactosidase putative positive clones were selected for further analysis. Subsequently, clone#4 was identified as an APP binding protein (ABP), which specifically interacts with the carboxyl-terminal domain of APP. Sequence analysis of ABP showed a 1432 bp cDNA fragment with a ATG start codon (methionine) at amino acid position +1, an upstream Kozak

consensus sequence (GACGGC) at nucleotide –6 to -2, a stop codon at amino acid position +193 and a polyadenylation signal at nucleotide 964-969. Translation of the cDNA sequence using the Swiss Prot program revealed an open reading frame of 192 amino acids with a putative molecular mass of 21.46 kDa. Swiss-Prot data base search did not reveal any other sequence or a known protein with significant homology to the ABP. Search for motifs predicted protein kinase K (PKC)(amino acid 132-134, 183-185), cAMP-dependent protein kinase (amino acid 55-58), casein kinase II phosphorylation (CK2)(amino acid 142-145, 183-186) and N-myristylation (amino acid 11-16, 98-103, 128-133) sites in the putative protein ABP. This indicates that ABP may be a membrane protein and involved in the signal transduction pathway of APP.

Co-immunoprecipitation, which was performed on a human neuroblastoma cell line, SK-N-SH, showed that ABP could be found in the complex immunoprecipitated with anti-APP antibodies and likewise, APP could be found in the complex immunoprecipitated with ABP fusion protein. This indicates that ABP interacts with APP in mammalian cells. Northern blots revealed that ABP corresponds to a widely expressed 1.5 Kb mRNA transcript in human tissues.

In summary, we have identified a human brain cDNA, which encodes an APP_{COOH} binding protein, ABP. It appears to be a general membrane protein that interacts with APP_{COOH} in mammalian cells and may be involved in a signal transduction pathway mediating the effect of APP. The interaction between APP and ABP may lead to dysfunctions associated with AD. Further experiments will be required to ascertain the role played by ABP, if any in the pathogenesis of AD.

INTRODUCTION

1.0.Alzheimer disease

1.0.1.Alzheimer disease

In 1907, Alois Alzheimer described a novel brain disease that principally affects the aged population and results in progressive cognitive decline. This disorder was later called Alzheimer disease (AD), the most common cause of dementia. It affects 5-10% of all people over 65 years of age, leaving individuals confused and incompetent to care for themselves (Hurtley, 1998).

AD is characterized by the triad of amyloid plaques, neurofibrillary tangles and dementia, defined by Alois Alzheimer. Today, these terms are commonly accepted and used. In AD, the major clinical symptoms (e.g. psychiatric and behavioral) implicate the cerebral cortex as a major site of pathological changes. In the autopsy of AD brains, most of the cerebral cortex and many subcortical areas contain large numbers of plaques and tangles (Arnold *et al*, 1991; Berg *et al*, 1993; Geula, 1998). The amyloid plaque (50 um in diameter) is a complex lesion of the cortical neuropil. The core of the plaque is composed of extracellular amyloid fibrils surrounded by dystrophic neurites (both dendrites and axon terminals) and by activated microglia and reactive astrocytes. The main protein components consist of the 40-43 amino acid residue β-AP, proteoglycans, serum amyloid pentraxin, apolipoprotein E, α-1-antichymotrypsin and transforming growth factor beta (TGF-beta)(Abraham *et al*, 1988; Snow and Molouf, 1993; Tennent *et al*,

1995; Peress and Perillo, 1995; Yankner, 1996; Igeta *et al*, 1997; Hartmann *et al*, 1997). Among them, the major one is β-AP, which is thought to play a key role in the pathogenesis of AD. Other components of the plaque may contribute directly to the formation or toxicity of β-AP. It has been discovered since 1968 that the density of senile plaques found in the brain post mortem shows a significant correlation with severity of clinical dementia measured in life (Blessed *et al*, 1968).

The neurofibrillary tangles (NFTs) are found in nerve cell bodies and apical dentrites. Each tangle contains paired helical filaments (PHFs) as major components, whose main protein subunit is the microtubule-associated protein, tau. It was found that tau was abnormally hyperphosphorylated in AD (Grunke-Iqbal *et al*, 1986a; Grunke-Iqbal *et al*, 1986 b; Goedert *et al*, 1993; Zemlan, 1996; Reynolds *et al*, 1997; Hoffmann *et al*, 1997; Friedhoff and Mandelkow, 1998). These studies showed that the sites Thr181, Ser199, Ser202, Thr205, Thr231, Ser235, Ser396, Ser404 and Ser 422 are phosphorylated in PHF-tau but not in normal adult tau. Several kinases have been implicated in the formation of PHF-tau in AD (Lee, 1995). These include microtubule associated protein (MAP) kinase, cyclin-dependent protein 5,2 (CDK5, CDC2) kinase as well as a proline-directed kinase and the protein phosphatase 2A, 2B. Since PHF-tau loses the ability to bind to microtubules (MTs), the accumulations of PHF-tau may lead to the depolymerization of MTs, the disruption of axonal transport and dysfunction and/or degeneration of neurons in AD (Merrick *et al*, 1997). It has been reported that neurons with

PHFs degenerate, and the degree of this degeneration directly correlates with the degree of dementia in the affected individuals (Alafuzoff *et al*, 1987; Arriagada *et al*, 1992; Hock *et al*, 1995; Terry, 1996). Therefore, the presence of such "neuritic plaques" together with numerous neurofibrillary tangles continues to serve as the basis for a definitive pathological diagnosis of AD.

The relationship between the formation of NFTs and the amyloid plaque is poorly understood. Some data indicated that NFTs do not cause plaques because the latter is absent in other neurodegenerative diseases with NFTs. However, it was found that in most cases, amyloid plaques and NFTs coexist (Beyreuther and Masters, 1996). Recently, a report provides clues to the mechanisms linking amyloid filament formation with neurofibrillary degeneration in AD. It suggests that the assembly of tau into NFTs requires the presence of an additional factor, glycosaminoglycans, which has been shown to be associated with both amyloid plaques and the NFTs component of tau (Goedert *et al*, 1996).

1.0.2. Amyloid Precursor Protein

β-AP is derived from the amyloid precursor protein (APP). Over 10 isoforms of APP have been identified. The three major forms are APP-770, 751 and 695, which differ only in the presence or absence of the Kunitz-type protease inhibitor and 'OX-2' antigen domain. APP-770 (contain the Kunitz-inhibitor and 'OX-2' antigen domains) and APP-751 (contain the Kunitz-inhibitor domain) are expressed in both neural and non-neural tissues

(Kitagchi *et al*, 1988; Ponte *et al*, 1988). APP-695 is expressed at high levels in neurons (Yamazaki *et al*, 1995). The APP gene was cloned in 1987 (Kang *et al*, 1987). APP has structural features characteristic of cell surface receptors such as integrins and epidermal growth factor receptors. It has a long extracellular domain, a single transmembrane region and a relatively short cytoplasmic tail. There is a region rich in cysteines and three lysine residues immediately downstream from the transmembrane domain, which may interact with the phospholipid head groups in the membrane. In addition to these features, the carboxyl–terminal domain of APP shares a highly conserved stretch of five amino acids (ENPTY) with tyrosine in the middle with many known plasma membranes receptors including integrins. Tyrosine is autophosphorylated in the epidermal growth factor receptor (Downward *et al*, 1984;Tamkun *et al*, 1986) (Figure 1). The β-AP portion just crosses the membrane with a 28 extracellular AA domain and another 12-14 transmembrane AA domain. The cleavage of APP by an 'α-secretase' enzyme occurs within the β-AP sequence and results in the secretion of about 100 KDa N-terminal fragment (sAPP). On the other hand, intact β-AP is derived from APP by cellular processing pathways. This pathway involves the excision of β-AP portion by the sequential action of "β-" and "γ-secretase" enzyme (Haass and Selkoe, 1993) (Figure 2).

The normal function of APP is poorly understood. The secreted form of APP (sAPP) has been implicated as an autocrine factor to stimulate cell proliferation through cell-surface binding (Jin *et al*, 1994). It acts as a

mediator for promoting cell-substratum adhesion, probably through an interaction with laminin (Schubert *et al*, 1989;Kibbey *et al*, 1993) and as a regulator of intraneuronal calcium to protect the neuron against hypoglycemic damage (Mattson *et al*, 1993). sAPP also appears to promote neurite outgrowth as a substrate interacting with extracellular matrix (Koo *et al*, 1993). Recently, the biological functions of APP *in vivo* have been examined in homozygous APP-knockout mice (Zheng *et al*, 1995). Homozygous APP-deficient mice were viable and fertile, raising the possibility that the APP deficit may have been compensated. However, mutant animals weighed 15-20% less than age-matched wild-type controls and exhibited a decreased locomotor activity, forelimb grip strength and reactive gliosis in the brain, indicating an impaired neuronal function as a result of the APP-null mutation. In another mouse model, the exon 2 of APP gene was disrupted. These mice showed a severely impaired behavioral phenotype and incidence of agenesis of the corpus callosum substantially above that of wild-type littermates, suggesting a new dominant phenotype of mutant APP (Muller *et al*, 1994).

1.1.The Role of Alzheimer Precursor Protein in the Pathogene -sis of Alzheimer Disease

The deposition of amyloid in neuritic plaques is detected only in related conditions of AD, such as Down's syndrome and to a much lesser degree in normal aging (Horsburgh and Saitoh, 1994). This implicates that neuritic plaques appear to represent an early pathologic event. Although the mechanism of AD is complex, the most attention has been focused on the

role of beta amyloid. Molecular genetic and cellular biological studies have implicated APP in a key role in the pathogenesis of AD. There are several lines of evidence supporting this hypothesis (Yankner , 1996).

The hypothesis was first supported in the patients with trisomy 21 (Down's syndrome). These patients developed neuropathological changes similar to those of AD, especially amyloid plaques at an early age. Because APP is located on human chromosome 21 (Kang *et al*, 1987), the presence of an extra copy of APP gene in Down's syndrome patients strongly suggests that the overexpressing of APP may lead to typical AD by increasing the processing of APP into β-AP (Haass and Selkoe, 1993).

The application of genetic linkage analysis to large pedigrees having an autosomal dominant form of AD has provided further evidence about the crucial role of APP in the pathogenesis of AD. It was found that all of the inherited APP mutations identified in the above families occur in proximity to the β-AP domain (Figure 3). The first mutation was identified at APP codon 717, proximal to β-AP domain (Goate *et al*, 1991). The second APP mutation is APP-770 codon 670/671 (double mutations) (Mullan *et al*, 1992). The other mutations include APP-770 codon 693 for hereditary cerebral hemorrhage with amyloidosis of the Dutch-type (HCHWA-D) (Levy *et al*, 1990) and APP-770 codon 692 associated with severe cerebral amyloid angiopathy (CAA) and pre-senile dementia (Hendricks *et al*, 1992)(Figure 3). All these mutations result in an increasing production of β-APs. Although mutations in the APP gene represent only 1-5% of AD cases (Tanzi *et al*,

1992), the effects of inherited APP mutations provide support for the idea that increasing β-AP generation may lead to the development of AD. Recently, the development of a transgenic mouse model of β-AP plaque formation also sheds light on the role of APP in the pathogenesis of AD. This was first achieved by an overexpression of APP containing one of the mutations (APP-717) associated with familial AD (FAD) (Games *et al*, 1995). These transgenic mice develop large numbers of amyloid plaques in the cortex. Importantly, the age dependence and neuroanatomical pattern of β-AP deposition is similar that which occurs in AD. Also, the amyloid plaques are directly associated with reactive gliosis, dystrophic neurites, and apoptotic cells, suggesting that plaques induce neurodegenerative changes. Studies have also demonstrated that the APP derivative, β-AP fibrils are neurotoxic. β-APs have been shown to induce apoptosis in cultured hippocampal neurons, cortical neurons, lateral spetial neurons, cerebellar granule cells and neurotypic SH-SY5Y cells, and appear to be the primary cause of neuron loss in AD (Carette *et al*, 1993; Forloni *et al*, 1993; Watt *et al*, 1994; Copani *et al*, 1995; Lockhart *et al*, 1994; Li *et al*, 1996). The mechanisms of neurotoxicity of β-APs have been investigated. On exposing rodent primary cortical neurons and phenochromocytoma cell line (PC12) to β-AP1-42s, the inhibition of 3 - (4,5 -dimethylthiazol – 2 – yl) - 2,5 - dimethylthiazolium bromide (MTT) reduction was found (Shearman *et al*, 1994; 1995). Since MTT reduction in cells is carried out mainly by redox enzymes at mitochondria inner membrane, it suggests that β-AP1-42s inhibit

the functions of mitochondria by disrupting redox enzymes. On the other hand, it was reported that β-AP1-42s form Ca^{2+}-permeable channels and thus may disrupt ionic homeostasis and result in the activation of cellular proteases and nucleases, losing the balance of protein phosphorylation/dephosphorylation (Rhee *et al*, 1998). β-AP1-42s has also been shown to generate reactive oxygen species (ROS) production in rat primary cortical neurons, which increases the oxidative stress of the brain and is directly toxic to neurons (Behl *et al*, 1994). Recently, two targets of β-AP have been identified, which are the receptor for advanced glycation end products (RAGE) and scavenger receptor (SR). It was found that both RAGE and SR specifically bound to β-AP and mediated β-AP-induced oxidant stress (Yan *et al*, 1996; El Khoury *et al*, 1996). In animal models of neuronal degeneration, it was also confirmed that β-AP is toxic to neurons and induces neurodegeneration (Kowall *et al*, 1991). The primate models of AD developed by injecting the insoluble, fribrillar form of β-APs into the brain displayed the pathological characteristic of the human disease (Kowall *et al*, 1991; Hendricks *et al*, 1992; Jin *et al*, 1994;Games *et al*, 1995; Geula, 1998).

In 1995, linkage analysis followed by positional cloning led to the identification of two other highly homolous familiar AD genes, presenilin 1 (PS1) and 2 (PS2) (Sherrington *et al*, 1995; Levy-Lahad *et al*, 1995). Around 50 missense mutations in PS1 and PS2 have been found in families with early-onset AD (Selkoe, 1998). Interestingly, recent studies in the mutated PS transfected cells, transgenic mice, plasma, skin fibroblast media, and

human brain tissues carrying PS mutations demonstrated a selective and highly significant increase in the levels of β-AP1-42s as a direct consequence of expressing mutant PS (Duff *et al*, 1996; Scheuner *et al*, 1996; Lemere *et al*, 1996; Citron *et al*, 1997; Tomita *et al*, 1997). The investigation of the mechanism by which PS mutations selectively increase β-AP1-42 production has revealed that PS proteins interact with APP, predominantly in the endoplasmic reticulum (ER) where PS and APP are co-localized and selectively alter APP processing (Xia *et al*, 1997). It indicates a common pathogenic mechanism involved in AD that the increased β-AP1-42 is a unifying pathological feature of AD and a primary cause in the pathogenesis of AD.

In fact, the discovery of another gene encoding an endoplasmic reticulum associated binding protein (ERAB) (Yan *et al*, 1997) underscores the crucial role played by APP in the pathogenesis of AD. ERAB is highly expressed in neurons and mainly localized in the ER where β-AP was thought to be produced. *In vitro* and *vivo* studies have shown that ERAB interact with β-APs and are thus regarded as intracellular targets for β-AP1-42s. The overexpression of ERABs, which was found to occur in AD, appears to potentiate cellular perturbation such as supression of MTT reduction and enhanced apoptosis due to β-AP1-42s

Take together, there is no doubt that the increasing data support the idea that APP plays a key role in the pathogenesis of AD. Therefore, In order

to understand the molecular events leading to the development of AD, it is necessary to elucidate the biological functions of APP.

1.2.Focus on protein-protein interaction network around the carboxyl-terminal region of APP

The hypothesis that APP functions like a cell-surface receptor has been proposed for over 10 years (Kang *et al*, 1987). However, no ligands for the extracellular domain of APP has been clearly demonstrated. In contrast, research data have documented the existence of a protein-protein interaction network around the intracellular carboxyl-terminal domain of APP (APP_{COOH}). This opens a stimulating new research area that will provide clues to define the functions of APP. So far, four proteins have been identified in this network, which is centered on the APP_{COOH}.

The first type of protein is the brain guanosine 5'-triphosphate (GTP)-binding protein G_0 (Nishimoto *et al*, 1993). G_0 binds to the APP_{COOH} sequence (His 657-Lys676) which shows a specific G_0-activating function and is necessary for APP-G_0 complex formation (Figure 1 and 4). On the other hand, G_0 protein treated with GTP-$\gamma\beta$ loses the ability to associate with APP. Since G_0 is a member of the heterometric G protein family that serves as a signal transducer of cell surface receptors, these findings strongly suggest that APP is a G_0-linked receptor. This notion was confirmed by subsequent findings that APP activated G_0 through His 657-Lys676 in response to anti-APP monoclonal antibody in reconstituted vesicles (Okamoto *et al*, 1995), indicating APP has a receptor function.

In addition, the role of G_0 linked to APP, which is involved in AD, has been investigated (Okamoto *et al*, 1996; Yamatsuji *et al*, 1996; Giambarella *et al*, 1997). The results showed that the FAD-associated V642 mutants of APP, V642I, V642F and V642G constitutively and selectively activate G_0 and induce apoptosis in NK1 cells. It is very interesting that cAMP response element (CRE) activity, which is an established mediator of long-term memory formation, has been shown to be suppressed (Horsburgh and Saitoh, 1994). This research data are consistent with the hypothesis that APP functions as a cell-surface receptor by coupling to the G_0 protein.

Fe65, the second identified protein in this network, interacts with the last 33 carboxyl-terminal residues of APP (Fiore *et al*, 1995) (Figure 4). It contains two phosphotyrosine interaction (PID) or phosphotyrosine binding (PTB) domains and one WW domain of which PTB1 is responsible for Fe65 binding to APP. Since PTB is mainly identified in Shc family proteins which recognize phophorylated NPXY motif of insulin receptor (IR) and epidermal growth factor (EGF) receptor that are involved in signal transduction (Pelicci *et al*, 1992; Fiore *et al*, 1995; Gustafson *et al*, 1995). It was proposed that Fe65 is an adaptor protein used to transduce the signal from APP to downstream targets. The possible role of the Fe65 adaptor protein was confirmed by the finding that several proteins interact with Fe65 through it's PID1 and WW domains (Ermekova *et al*, 1997; Zambrano *et al*, 1998).

One of these ligands is Mena, the mammalian homologue of the drosophila enabled protein. Mena belongs to the enabled family of proteins which are strongly enriched in focal contacts, and in cellular sites where active actin remodeling occurs, such as lamellipodia (Gertler *et al*, 1996). Mena also binds to profilin, a protein interacting with G-actin (Gertler *et al*, 1996). These findings suggest that F65 could function as an adaptor protein to transduce signals involving APP to cytoskeleton dynamics through Mena and its downstream regulators. On the other hand, Mena was also found in the growth core of P19 cells induced to differentiate to neuron, which started to appear during the period of active neurotic development (Gertler *et al*, 1996). This indicated that Mena might be involved in neuritic outgrowth (Figure 5).

The other ligand for the PID1 domain of Fe65 is the human alpha-globin transcription factor, (CAATT-binding protein 2/Late SV40 transcription factor (CP2/LSF)), previously described as a transcription factor involved in the regulation of several genes (Zambrano *et al*, 1998) (Figure 5). At least, two targets of CP2/LSF, c-fos and ornithine decarboxylase (ODC) promoters were identified to be activated in early stages following mitogenic stimuli. The phosphorylation of CP2/LSF by mitogen-activated protein kinase (MAP Kinase) regulates the translocation of CP2/LSF to the nuclear fraction (Volker *et al*, 1997). Collectively, the above observations implicate that Fe65 connects APP to the cytoskeleton through Mena which in turn interacts with profilin–actin complex to regulate axonal targeting through the cell-matrix and

cell-cell interaction. They also indicate that the interaction between Fe65 and APP activates CP2/LSF, the downstream target of a MAP kinase signal transduction cascade and regulates immediate early and /or early genes, which could play an important role in neuronal molecular differentiation related to synaptic plasticity and in the learning process (Russo *et al*, 1998). Meanwhile, the role for Fe65 in the pathogenesis of AD was also investigated. It was demonstrated that mutant APP shows an altered *in vivo* interaction with Fe65 (Zambrano *et al*, 1997).

The third protein interacting with the APP_{COOH} is X11 (McLoughlin *et al*, 1996). This protein shares similar features of being an adaptor protein with F65. It interacts with APP through a PID/PTB domain similar to those found in Fe65, and has a postsynaptic density-95/Drosophila discs large tumor suppressor/Zonula occludens-1 (PDZ) domain (Figure 5). Unfortunately, the ligand for PDZ domain is not known. The sequence of APP is critical for X11 binding and contains a NPTY motif identical to signals responsible for APP targeting to lysosoma compartments (Haass *et al*, 1992). It was hypothesized that X11 could be involved in the regulation of APP processing.

APP-BP1 is another ligand binding to the carboxyl-terminal region of APP (Chow *et al*, 1996). This is a novel protein, whose MW is around 59 Kda (Chow *et al*, 1996). It is not known which region of APP_{COOH} is responsible for APP-BP1 binding (Figure 5). Sequence analysis of APP-BP1 indicates that it is 61% similar to a protein encoded by the Arabidopsis AXR1 gene,

which is required for a normal response to the hormone, auxin (Chow *et al*, 1996). Mutations in the AXR1gene result in morphological defects due to reduced auxin sensitivity (Lincoln *et al*, 1990). The fact that APP-BP1 is a human homologue of ARX1 implies that APP may function as a membrane receptor and play a role in transducing APP-mediated signals into cells.

1.3.Objectives

All the information from this protein-protein interaction network provides an important contribution to the understanding of the biological functions of APP. Therefore, it is evident that defining these functions would be greatly enhanced by identifying the APP effector binding proteins in this network and investigating their roles in signaling pathways.

The purpose of my project was to clone and analyze the sequence of the APP_{COOH} interacting protein.

2. Rationale and Experimental Approach

To address this problem, the yeast two-hybrid system was used to clone the putative APP adaptor protein. The yeast two-hybrid system is commonly used to clone genes. There are three advantages in this system:

(1). Sensitivity: This system is very sensitive, and can detect relatively weak and transient protein-protein interaction. Such interaction may not be biochemically detectable, but may be critical for the proper functions of complex biological systems (Guarente, 1993; Estojak *et al*, 1995).

(2). Accuracy: The two-hybrid assay is performed *in vivo*. In this case, proteins are more likely to be in their native conformations, which may lead to increased accuracy of detection.

(3). Simplicity: It is easy to clone genes. There are two constituted yeast-cloning vectors in this system, which encode "bait" fusion protein and "library" fusion protein, respectively. When an interaction between these two proteins is detected, the cDNA of the gene, which you are interested in, is in one of the yeast expression vectors.

The principle of the two-hybrid system is based on the fact that many eukaryotic trans-acting transcription factors are composed of physically separable, functionally independent domains. Such regulators often contain a DNA-binding domain (DNA-BD) that binds to a specific enhancer-like sequence. In yeast, it is referred to as an upstream activation site (UAS). One or more activation domains ('AD') direct the RNA polymerase II complex to transcribe the gene downstream of UAS. This UAS together with TATA regions can be switched to create a novel promoter, which activates reporter genes in yeast (Clontech Matchmaker Gal4 Two-Hybrid User Manual).

In our experiments, the Gal4-based two-hybrid system was chosen. The DNA-BD and DNA-'AD' are both derived from the yeast *cis*-acting transcription element, Gal4. The native Gal1 UAS provides the binding site for Gal4 DNA-BD. Two different cloning vectors are used to generate fusions of these domains to genes encoding proteins that potentially interact with each other. The recombinant hybrid proteins are coexpressed in yeast. An

interaction between a bait protein (fused to the DNA-BD) and a library protein (fused to the 'AD') creates a novel transcriptional activator with binding affinity for a Gal4-responsive UAS. This factor then activates reporter genes having Gal4-responsive UAS in their promoter and this process makes the protein-protein interaction phenotypically detectable. The cDNAs of interest used to generate the 'AD' fusion protein library can be easily identified (Figure 6).

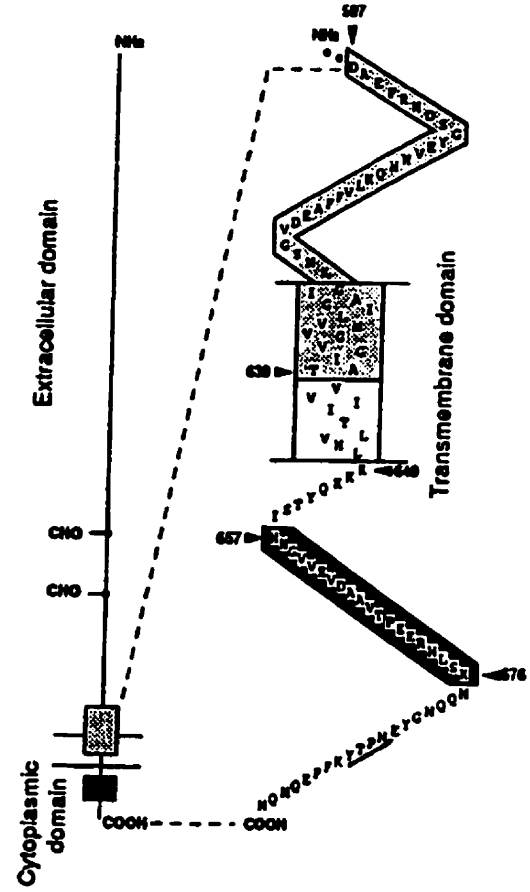


Figure 1. The proposed domain structure of APP-695. Hatched box: β-AP protein. Black box: peptide Histinine657-Lysine676. Filled circles: N-glycosylation sites. Numbering represents sequence position. Underline indicates NPTY motif (Adapted from Nishimoto et al, 1993).

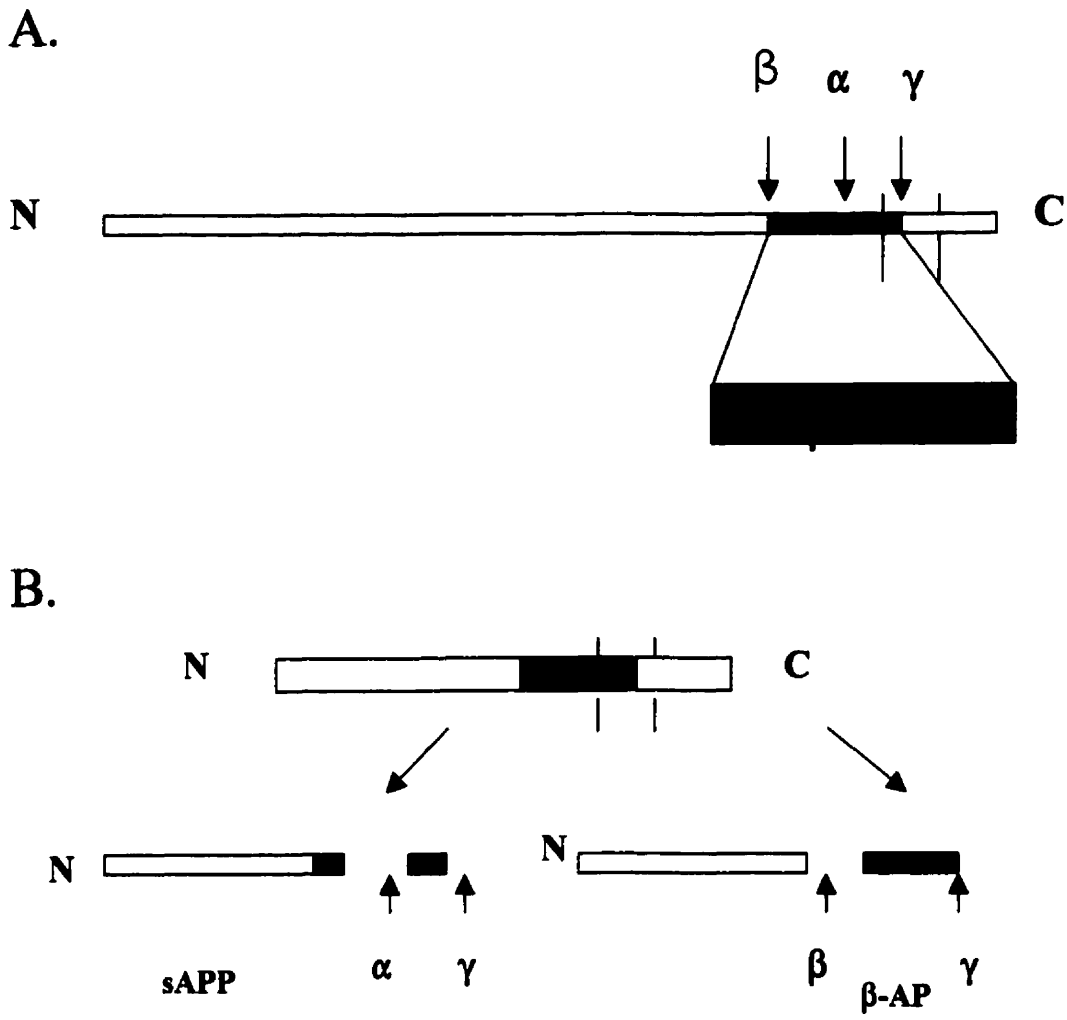


Figure 2. A: Secretase cleavage of APP. APP is cleaved inside the β-AP sequence by α-secretase. β- and γ-secretase cleave APP on either side of the β-AP sequence, respectively. B: APP is processed by via two alternative pathways, resulting in cleavage by α- or β-secretase yielding sAPP or β-AP.

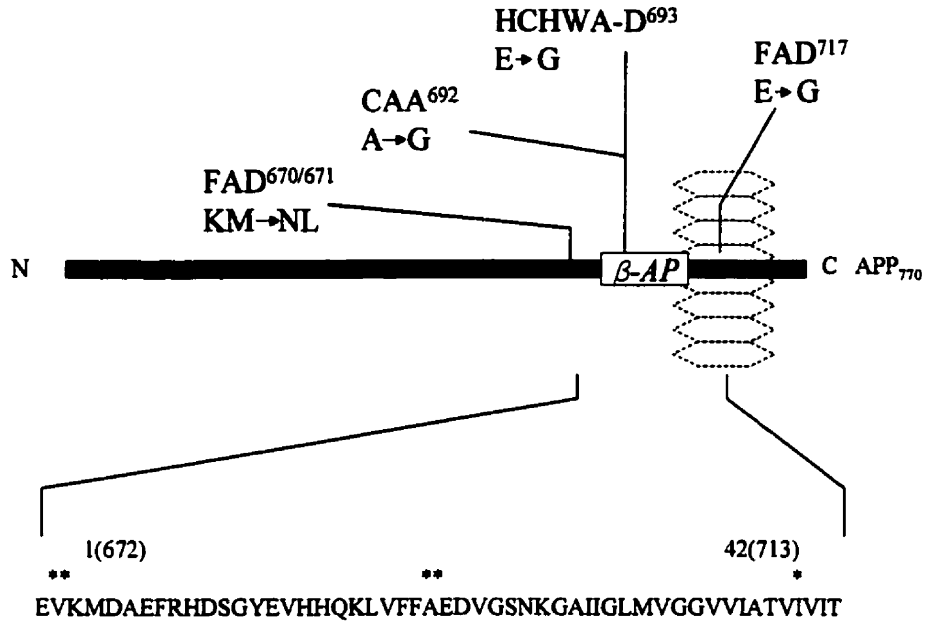


Figure 3. APP missense mutations associated with familial AD or closely related beta amyloid disorders. A box indicates β-AP domain. A number of point mutations in APP molecule have been identified in vicinity of the β-AP. FAD: early-onset familial AD; CAA: cerebral amyloid angiopathy; HCHWA-D: hereditary cerebral haemorrhage with amyloidosis of the Dutch-type (adapted from Leslie et al, 1995).

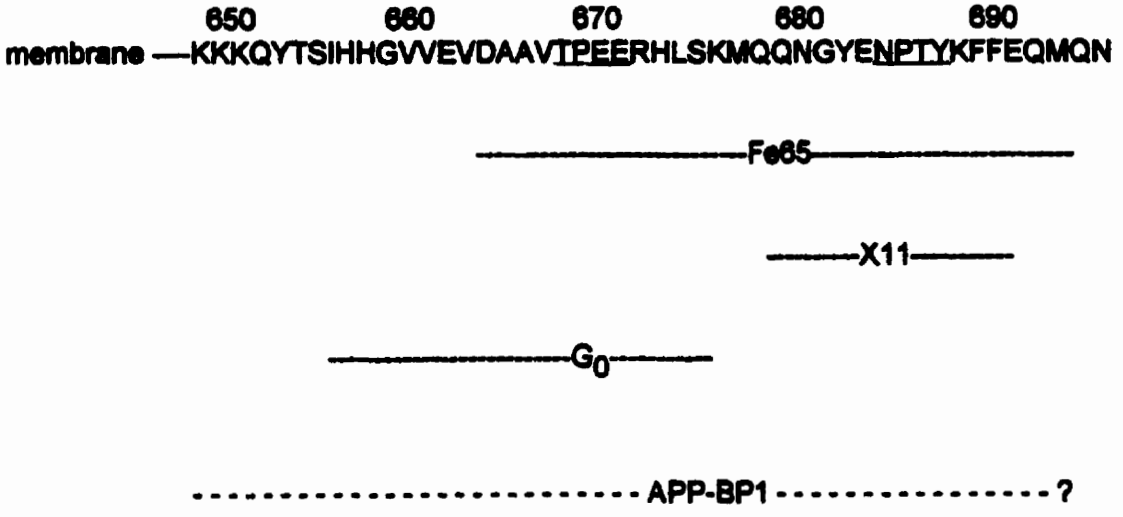


Figure 4. The minimal regions of APP involved in the interaction with Fe65, X11, G_0 and APP-BP1 (adapted from Russo et al, 1998).

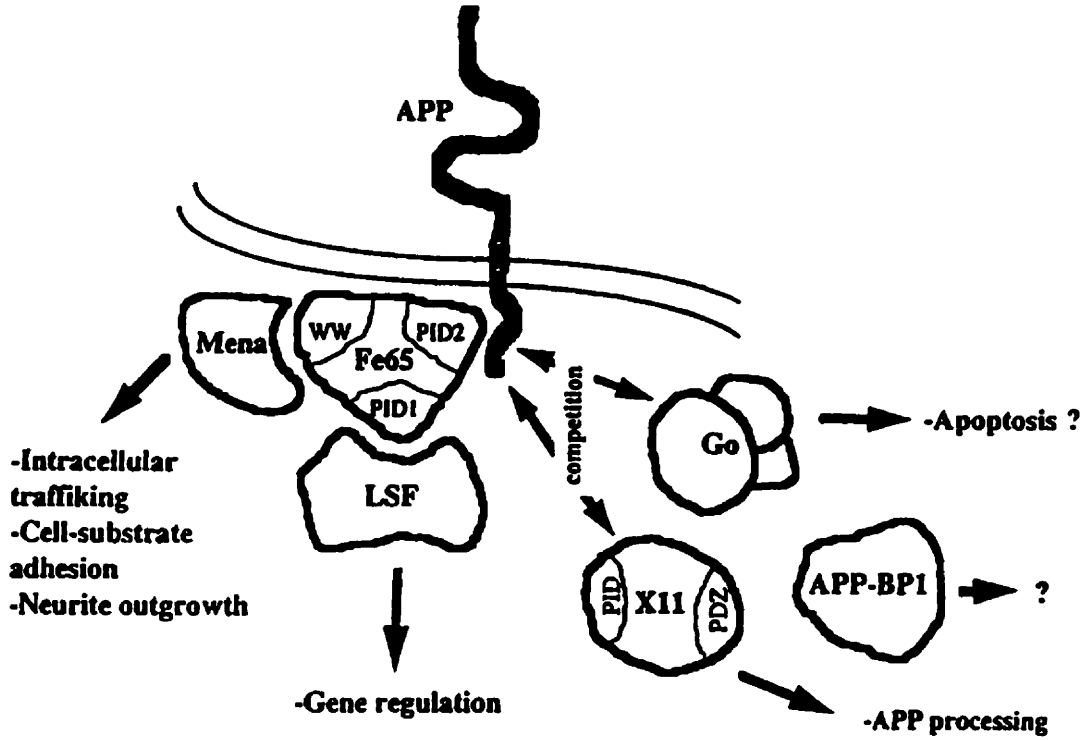
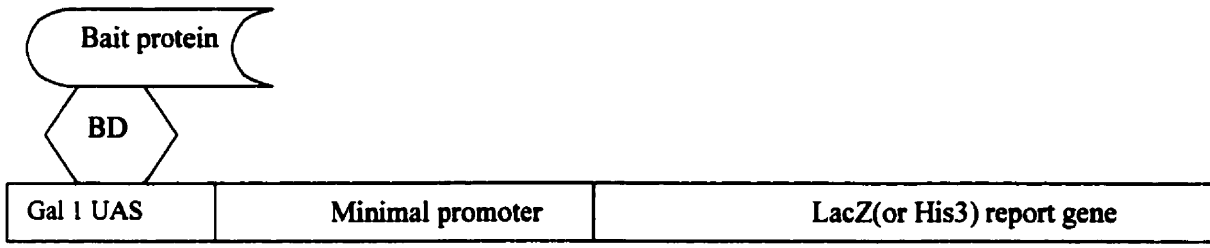
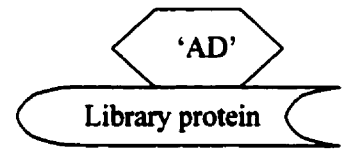


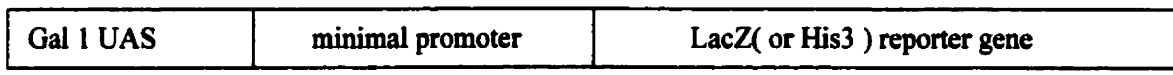
Figure 5. Schematic representation of the protein protein interaction network centered to the APP cytodomain. Some possible downstream targets of the network are indicated (adapted from Russo et al, 1998).

A The DNA-BD/protein X (bait) hybrid binds to the GAL1 UAS but cannot activate transcription without the activation domain ('AD').



B In the absence of bait protein, the 'AD'/library fusion protein can not bind to the GAL1 UAS and thus not activate transcription.





C Interaction between the bait and library proteins *in vivo* activates transcription of the reporter gene.

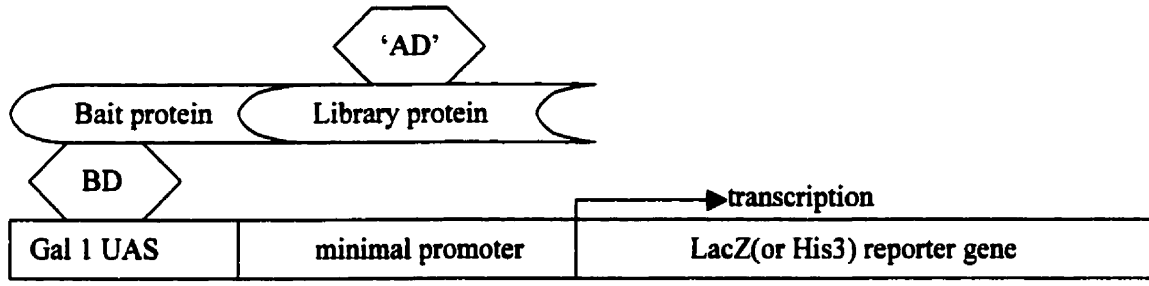


Figure 6. Gal4-based two-hybrid system. The DNA-BD is a.a. 1-147 of the yeast Gal4 protein, which binds to the Gal 1 UAS upstream of the reporter gene. The *LaZ* and *His3* reporter genes are separate constructs integrated in the yeast genome. The 'AD' is a.a. 786-881 of the Gal4 protein and has transcriptional activation function. BD, binding domain; 'AD', activation domain; UAS, upstream activation site.

3.MATERIALS and METHODS

3.1.MATERIALS AND SUPPLIES

The following reagents were purchased from specific manufacturers and used according to the manufacturer's instructions, unless specifically annotated.

Gibco Life Technology, Inc:	Neuroblasal A-Medium
	TRIZOL Reagent
Promega Inc:	Restriction Enzyme
Roche Diagnostic, Inc:	Restriction Enzyme
	Fugene ^{TM}-6
	Transfection Reagent
	Random Primer DNA Labeling
	Kit
Difco Laboratories, Inc:	LB agar and medium
Clontech Laboratories, Inc:	Gal4 DNA-binding domain (BD)
	cloning vector pAS2-1
	Gal4 activation domain (AD)
	cloning vector pACT2
	Yeast Strain Y190, Yeast YPD
	Yeast agar
	Minimal Synthetic dropout (SD)
	Base and agar base,
	Yeast dropout supplement

	Herring test carrier DNA
	Gal4 AD monoclonal antibody
Bio-Rad Laboratories, Inc:	Zeta probe blotting membrane
	DNA purification kit
Invitrogen, Inc:	pcDNA 3.1 vector
American Type Culture Collection:	Neuroblastoma cell line SK-N-SH
Qiagen, Inc:	Plasmid Mini-preparation kit
	Plasmid Midi-preparation kit
	Plasmid Maxi-preparation kit
Zymed Laboratories, Inc:	Mouse amyloid precursor protein antibody
Amersham Pharmica Biotech:	Protein G-sepharose 4 fast flow

3.2 METHODS

3.2.1.Culturing and handling of bacteria

E. *coli*, DH5α™ was transformed by plasmids, then added to 1 ml LB broth, incubated at 37^{0}C for one hour. Then, the solution volume was brought down to 100 ul, which was plated out on LB agar plate with appropriate antibiotics and the plate was incubated overnight at 37^{0}C.

Steriloops were used to pick up well-defined colonies from the plate and suspended in the different culture tubes containing 3ml LB broth with the appropriate antibiotics. The cultures were grown overnight in a 37^{0}C shaking incubator (250 rpm).

Two milliliters of the subculture was used for isolation of plasmids. Isolated plamids were subjected to analysis. Appropriate plasmid with the correct orientation was selected. The 1ml subculture containing the interested plasmids was further expanded for preparation of plasmids.

Alternatively, 1 ml of the overnight bacteria culture was combined with 0.3 ml of sterile, 50 % glycerol and stored at –70^{0}C. For recovery of the stored bacteria, a sterile inoculating loop was used to recover bacteria and replating on LB agar plates with selected antibiotics.

3.2.2.Large-scale preparation of plasmid

Large-scale amount of plasmids (100 - 500 μg) was prepared according to the manufacturer's instruction (Qiagen) with slight modifications.

Briefly, a single colony was picked up from a freshly streaked selective plate and incubated in a starter culture of 2ml LB medium containing the

appropriate selective antibiotics. The culture was incubated for four hours at 37^{0}C with vigorous shaking (250rpm). Then, the culture was diluted into 100 ml selective LB medium, and grown at 37^{0}C for 12-16 hours with shaking. The amplified bacteria cells were harvested by centrifugation at 6000 rpm (Beckman JA-10 rotor) for 15 minutes. Ten milliliters of buffer P1 (50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 100 ug/ml RNase A) was added to the pellets, prior to suspension by vortexing. The solution was then combined with 10 ml of buffer P2 (200 mM NaOH, 1% SDS), mixed gently but thoroughly by inverting 4-6 times, and incubated at room temperature for five minutes. 10 ml of chilled buffer P3 (3.0M potassium acetate, pH 5.5) was added and contents were mixed immediately by inverting 4-6 times, and incubated on ice for 30 minutes. The solution became cloudy as the protein denatured. Samples were then centrifuged for 30 minutes at 14,000 rpm at 4^{0}C. The supernatants containing plasmid DNA were transferred to a new 50ml centrifuge tube and recentrifuged as the same as above for 15 minutes. The supernatants were removed and transferred to the pre-equilibrated QIAGEN-tip 500 column and allowed to enter the resin by gravity flow. QIAGEN-tip 500 was then washed by 2x30 ml buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol). Plasmid DNA was eluted with 15 ml buffer QF (1.2M NaCl; 50mM Tris-Cl, pH 8.5; 15% isopropanol). Ten point to five milliliters (0.7 volume) of room temperature isopropanol was added into this buffer QF containing the eluted plasmid DNA. The DNA was collected by centrifugation at 9,500 rpm (Beckman JS-13 rotor) for 30 minutes at 4^{0}C. The pellet was dissolved in 0.2ml deionized water, and

the concentration of DNA was measured.

3.2.3.Restriction enzyme digestions

For analysis of plasmid DNA, a 30ul volume restriction enzyme digest reaction was used.

The reaction contained 1.0 ug of plasmid DNA; 3 ul of 10X restriction enzyme buffer and 1 ul of restriction enzyme. The mixtures were incubated at an appropriate temperature in a water bath for 2 hours. The samples were then analyzed by agar gel electrophoresis.

For the isolation of the DNA fragments in preparation for construction of fusion vector, a 200 ul reaction volume was used. The reaction consists of 40-50 ug of DNA; 20 ul of 10X restriction enzyme buffer and 80-100 u of restriction enzyme. The mixtures were incubated overnight at an appropriate temperature and DNA fragments were isolated by agar gel electrophoresis (see below).

3.2.4.Agarose gel electrophoresis

One gram agarose was mixed with 100 ml 1X TAE (0.04 M Tris base; 0.02M glacial acetic acid; 1mM disodium EDTA, pH 7.2). The solution was boiled in a microwave oven for 2 minutes and cooled to approximately 55^0C. Four microliters of ethidium bromide (10 mg/ml) was added to the solution.

The solution was then poured into a gel tray with combs for making a gel slab. 30 ul sample and 5 ul of 6X loading buffer (0.125% bromophenol blue; 0.125% xylene cyanole; 12.5% ficoll) were mixed and loaded per well. The samples were subjected to electrophoresis in 1X TAE buffer at 90-110V for 1.5-2.0 hours. DNA bands were visualized by an ultraviolet light transilluminator

(260nm).

3.2.5.Recovery and Purification of DNA

DNA purification kit (Bio-Rad Laboratories, Inc) was used in experiments. After agar gel electrophoresis, the DNA bands of interest were excised and placed into a 1.5 ml microcentrifuge tube and centrifuged for several seconds to bring down the gel slice. The gel slice volume was then estimated by weighing the gel slice and converted to ml (for conversion of gs to mls, 1g of a gel slice is equal to 1 ml) and the amount of pre-A-Gene matrix required to bind the DNA was also estimated. The capacity of the Prep-A-Gene matrix is 0.2 ng DNA per ml of completely resuspended matrix. Based on the volume of the gel slice plus the amount of Prep-A-Gene matris required for total DNA binding, 3 volumes of Prep-A-Gene matrix purification kits binding buffer (6M sodium perchlorate; 50mM Tris, pH 8.0; 10mM EDTA, pH 8.0) was added to the gel slice and agitated gently to dissolve at 50^0C for several minutes. The predetermined amount of Prep-A-Gene matrix was then added and the contents were mixed gently by vortexing, followed by incubation at room temperature. The DNA containing Prep-A-Gene matrix was pelleted by centrifugation for 30 seconds. The pellet was washed by resuspending it in an amount of DNA purification kit binding buffer equivalent to 25 times the amount of added matrix. The matrix was collected again and washed two times with a 25 X matrix volume of prepared washing buffer (400mM NaCl; 20mM Tris, pH 7.5; 20mM EDTA, pH 7.5; 50% ethanol v/v). After the second wash and centrifugation, the matrix was pelleted and the bound DNA was eluted by resuspending it in at

least 1 pellet volume of distilled water and incubation at 50^{0}C for five minutes.

3.2.6.Ligation

A Rapid DNA ligation kit was used for DNA ligation reactions according to manufacture's instruction (Boehringer Mannheim).

The pre-prepared vector and insert DNA were dissolved in 10 ul 1 × Conc. DNA dilution buffer. The total DNA was 1.0~2.0ug. The molar ratio of vector DNA to insert DNA was 1 to 3 or 1 to 5.The solution was mixed with10 ul T4 DNA ligation buffer (2x Conc) and the contents were mixed thoroughly. 1 ul T4 DNA ligase was then added and mixed gently but thoroughly. The ligation reaction mixture was incubated at room temperature for 15 minutes and used directly for the transformation of competent cells.

3.2.7.The transformation of competent cells

DH5α competent cells were removed from the -70^{0}C freezer and thawed on ice. After mixing, 100ul of competent cells were transfered to a 1.5 ml pre-cooled microcentrifuge tube. The ligation reaction mixture was then added and mixed by gently taping tube. Cells were incubated on ice for 30 minutes and heat-shocked at 37^{0}C for 45 seconds. Cells were then placed on ice for two minutes and dissolved in 0.95 ml LB broth at room temperature and shaken at 250 rpm for one hour at 37^{0}C. Cells were collected by centrifugation and resuspended in 100 ul LB medium. Cells were then plated on LB agar plates with appropriate selective antibiotics and incubated overnight at 37^{0}C.

3.2.8.Small-Scale preparation of plasmd

A Wizard™ Plus Minipreps DNA purification system was used to prepare plasmid DNA for analysis. Two milliliters of bacterial culture was pelleted by centrifugation for five minutes at 7,000 rpm. The cell pellet was resuspended in 200 µl of cell resuspension solution (50 mM Tris, pH 7.5; 10mM EDTA; 0.1 mg/ml RNase A) and transferred to a 1.5 ml microcentrifuge tube. 0.2 ml of cell lysis solution (0.2M NAOH; 1%SDS) was then added and the contents were mixed by inverting the tube 4–6 times until the solution became clear. 0.2 ml of neutralization solution (1.32 M potassium acetate) was added to the tube and mixed well. The lysate was collected by centrifugation at 10,500 rpm for five minutes. One milliliter of the resuspended resin was pipetted into a barrel of the Minicolumn/Syringe assembly. All of the clear lysate was removed from the miniprep and transferred to the Minicolumn/Syringe assembly. A vacuum was applied to pull the resin/lysate mix into the Minicolumn until the entire sample completely passed through the column. Two milliliters of the column solution (80 mM potassium acetate; 8.3 mM Tris-HCL, pH 7.5; 40µM EDTA; 55% ethanol) was then added to the Syringe Barrel and a vacuum was reapplied to draw the solution through the Minicolumn. The vacuum continued for 30 seconds after the solution had been pulled through the column. The Syringe Barrel was removed and the Minicolumn was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 10,500 rpm for 2 minutes. The Minicolumn was then transferred to a new microcentrifuge tube and 50 µl of 65^0 C water was applied to the Minicolumn and allowed to absorb in the matrix for

one minute. DNA was eluted by centrifugation at 10,500 rpm for 20 seconds.

3.2.9.Culturing and Handling of Yeast Cells

Yeast strain Y190 was applied in the experiments. Y190 has two reporter genes, Histidine (His) and LacZ, but not Tryptophan (Trp) and Leucine (Leu) genes. The former two genes provide double selections for interacting proteins. While the latter two genes allow the transformed yeast by expression vector expressing Trp or Leu to grow on yeast minimal synthetic dropout medium without Trp or Leu (SD/-Trp or SD/-Leu). Trp or Leu thus is regarded as the transformation markers to screen the transformed Yeast and reduce the false positive results. Briefly, Y190 was stored in YPD medium with 25% glycerol at –70°C. To recover the frozen strain and prepare a working stock plate, a small portion of the frozen glycerol stock was streaked onto a YPD (or appropriate SD) agar plate and incubated at 30°C until yeast colonies reached 2 mm in diameter. These colonies were used as working stock and plates were sealed with parafilm and stored at 4°C for up to two months. To prepare liquid overnight culture, a fresh colony was picked up from the stock plate and suspended in 50 ml of medium. The yeasts were incubated at 30°C for 16-18 hrs with shaking at 250 rpm to stationary phage (OD600> 1.5). To prepare a glycerol stock cultures of new yeast transformants, an isolated colony was scraped from the agar plate and resuspended in 500 µl of YPD medium (or appropriate SD) in a microcentrifuge tube. The tube was vortexed vigorously to thoroughly disperse the cells. Sterile 50% glycerol was then added to a final concentration of 25%. The tube was shaken and stored at –70°C.

3.2.10.Preparation of APP-770 cDNA

APP-770 was chosen as a bait protein in the experiments. APP-770 was shown to express ubiquitously and has the same APP_{COOH} as other APP isoforms. The available full-length APP-770 (3593 bp) was subcloned into pGEM-9Zf (-) vector (Figure 7). The sequence of APP-770 starts at –15 and stops at + 3578 which contains the whole open reading frame of APP-770 encoding 770 amino acids. The pGEM-9Zf (-) containing APP-770 was first checked by restriction enzyme (*EcoR1*) analysis.

To isolate the full-length APP-770, pGEM-9Zf (-) containing APP-770 was digested with *SpeI*. After gel electrophoresis, 2.0 kb size band was identified that contains the whole open reading frame of APP-770. The other 4.0kb size band represents the pGEM-9Zf (-) vector plus the carboxyl-terminal region of APP-770 sequence subcloned in pGEM-9Zf (-) vector. 2.0 kb band was excised and extracted from the agarose gel. The extracted APP-770 fragments were ready for the subcloning experiments.

3.2.11.Construction of pAS2-1/APP-770 Fusion Vector

Yeast expression vector pAS2-1 was used in the Gal4 two-hybrid library screening. pAS2-1 expresses the Gal4 DNA binding domain (BD) (the yeast Gal4 protein, amino acid 1-147). There is a multiple cloning site immediately downstream of Gal4 DNA-BD domain sequence, which was used to generate bait fusion protein with Gal4 DNA-SD for screening a match interacting protein from a cDNA library. pAS2-1 also encodes Leu, which is a selective transformation marker to allow Y190 transformed by pAS2-1 to grow on SD/-

Leu agar plate. The bait protein used in the experiments was APP-770. To prepare the full-length APP-770, *SpeI* fragments were first inserted into pGEM-5Zf (+/-) vector, which consists of restrition enzyme *NcoI* and *Sal I* site surrounding the *SpeI* site. APP-770 *Nco I / Sal I* fragments in the correct orientation were subcloned into the yeast expression vector pAS2-1. The constructed pAS2-1 (BD)/APP-770 was examined by *Nco I/ Sal I* digestion and the *Nco I/ Sal I* fragment containing full-length APP-770 was identified. Therefore, pAS2-1 (BD)/APP-770 fusion yeast expression vector was constructed, which encodes a Gal4 DNA-BD/APP-770 fusion protein and are ready for library screening (Figure7).

3.2.12.Small-Scale LiAc transformation of Yeast Cells

One milliliter of YPD was inoculated with a colony from a stock plate and vigorously vortexed to disperse cells. The cells were then transferred to a flask containing 50 ml of YPD and incubated at 30^0C for 16-18 hrs with shaking at 250 rpm to stationary phase. 30 ml of overnight culture was transferred to a flask containing 300 ml of YPD and incubated at 30^0C for three hours with shaking (230 rpm). Cells were placed in 50 ml tubes and centrifuged at 1,000 g for five minutes at room temperature. The cell pellets were dissolved in 25 ml of distilled water by vortexing and pooled, followed by centrifugation at 1,000 g for five minutes at room temperature. The cell pellet was resuspended in 1.5 ml of fresh, sterile 1X TE/LiAc. 0.1 µg of pAS2-1/APP-770 and 0.1 mg of herring testes carrier DNA was added to the tube and mixed. 0.1ml of yeast competent cells and 0.6 ml of sterile PEG/LiAc was then added, respectively. The mixture

was incubated at 30^{0}C for 30 minutes with shaking at 200 rpm. 70µl of dimethyl sulfoxide (DMSO) was added and mixed by gentle inversion of the tube. The cells were heat shocked for 15 minutes in a 42^{0}C water bath and chilled on ice for 2 minutes. The cells were pelleted by centrifugation for five seconds at 14,000 rpm at room temperature and resuspended in 0.5 ml of sterile 1X TE buffer. The cells were plated on a 100 mm plate containing SD/-Leu that will select for the desired transformants. The plate was incubated at 30^{0}C until colonies appeared (around +14 days).

3.2.13.Library-Scale LiAc transformation of Yeast Cells

Human brain MATCHMAKER cDNA library was used in this experiment. This cDNA library is derived from a normal, whole brain from a 37-years-old Caucasian male. The cDNA library is preconstructed in yeast cloning vector pACT2 at cloning site *XhoI*/*EcoR*I. While pACT2 contains Gal4 DNA-AD sequence immediately upstream of the inserted cDNA library. Therefore, these preconstructed pACT2s encode Gal4 DNA-AD/library protein fusion proteins, which is thus used for screening human brain cDNA library. To conduct library-scale LiAc transformation of yeast by pACT2s preconstructed with human brain cDNA library, several colonies were picked up from the SD/-Leu plate with the growth of the yeast transformed by the pAS2-1 (BD)/APP-770 fusion vectors (small-scale LiAc transformation) and inoculated in 1 ml of YPD medium with vigorous vortexing to disperse the cells. The cells were transferred to a flask containing 150 ml of SD/-Leu medium and incubated at 30^{0}C for 18 hrs with shaking at 250 rpm. The overnight culture was then transferred into 1 L of SD/-

Leu medium and then incubated at 30^{0}C with shaking at 250rpm. The mixture was placed in 50 ml tubes and the cells were centrifuged at 1, 000 g for five minutes at room temperature. The cells were centrifuged and resuspended in 50 ml of sterile water. The cells were pooled in one tube and pelleted by centrifugation at 1,000 g for five minutes at room temperature. The pellets were resuspended in 8 ml of freshly prepared sterile 1x TE/Lithium acetate (LiAc) solution, 0.8 mg pACT2/cDNA library fusion and 20 mg Herring tests carrier DNA was added and mixed well. The mixture was combined with 8 ml of sterile polyethylene glycol (PEG)/LiAc solution with vortexing at high speed and incubated at 30^{0}C for 30 minutes with shaking at 250 rpm. Seven milliliters of (DMSO) was then added and mixed well by gentle inversion. The cells were heat-shocked for 15 minutes in a 42^{0}C water bath and chilled on ice for two minutes .The cells were pelleted by centrifugation for 5 minutes at 1,000 rpm at room temperature and then resuspended in 10 ml of 1x TE buffer. One hundred microliters of cell suspension was plated on an agar plate (150 mm in a diameter). The plates were incubated at 30^{0}C until colonies appeared. The colonies were used for analysis of β-Galactosidase assay as described later.

3.2.14.β-Galactosidase assay

A colony-lift filter assay was applied for identification of putative positive clones. A sterile Whatman #5 filter was first presoaked in 2.5 ml of β-galactosidase assay transfer buffer (Z buffer)/X-gal solution in a clean 150-mm plate for later use. A clean, dry filter paper was placed over the surface of the plate of colonies to be assayed. Holes were made through the filter into the

agar in three asymmetric locations to orient the filter to the agar. When the filter became wet, it was lifted carefully off the agar plate with forceps and transferred to a pool of liquid nitrogen. The filter was completely submerged for ten seconds. After the filter became frozen, it was removed from the liquid nitrogen and allowed to thaw at room temperature. The filter with colony side up was placed carefully on the presoaked filter to avoid trapping air bubbles under or between the filters .The filters were incubated at 30^0C and checked periodically for the appearance of blue colonies. The β-Galactosidase-producing colonies were identified by aligning the filter to the agar plate using markerd holes.

3.2.15.pACT2/ABPcNDA vectors isolation from yeast Cells

A 2 mm patch of yeast was spread onto SD/-Trp agar plate to keep pressure on the pACT2/ABP cDNA vectors. The plate was incubated at 30^0C for 3-4 days until the patch showed abundant yeast growth. A portion of the patch (~10 mm^2) was scraped up and transferred to a 1.5 ml microcentrifuge tube. After addition of 30 µl of suspension buffer (10 mM Tris-Cl, pH 8.0; 1mM EDTA; 4.5 units/µl Lyticase) to each tube, the mixture was pipetted up and down repeatedly to thoroughly resuspend the colony and incubated at 37^0C for 30 minutes. One hundred and seventy microliters of lysis buffer (2% v/v Triton-100; 1% v/v SDS; 100mM NaCl; 10mM Tris-Cl, pH 8.0; 1mM EDTA) was added to each tube and mixed well. The solution was transferred to a microcentrifuge tube containing 200 µl of glass beads. After adding 200 µl of phenol: chloroform: isoamyl alcohol (25:24:1), the tube was subjected to vortexing at the highest speed for five minutes and centrifuged at 14,000 rpm for 10 minutes.

The aqueous (upper) phase was transferred to a fresh tube with consequent addition of 8 μl of 10 mM ammonium acetate and 500 μl of 95-100% ethanol and placed on ice for one hour. The tube was centrifuged at 14,000 rpm for 10 minutes and the supernatant was discarded. The pellet was left dried and resuspended in 20 μl of water. The pACT2/ABP cDNA vectors were ready for further analysis.

3.2.16.Isolation of total RNA

Total RNA was isolated from human organs (brain, lung, kidney and heart). Briefly, 0.5 g of tissue was homogenized with TRIZOL reagent for one minute. The homogenates were poured into a 50 ml teflon tube and incubated at room temperature for ten minutes to dissociate nucleoprotein complexes following addition of 3 ml of chloroform and shaking for 30 seconds. Samples were spun at 4^0C, 10,000×g for 15 minutes (8000 rpm in a beckman JS 13.1 rotor). After centrifugation, the upper aqueous phase was transferred to a sterile 30 ml centrifuge tube followed by addition of an equal volume of ice-cold isopropanol. The solution was mixed well and stored at room temperature for five minutes. Samples were centrifuged again as same as the above to pellet the RNA. The pellet was washed with 15 ml of 75% ice cold ethanol and RNA was finally collected by centrifugation and the RNA was resuspended in 0.6 ml of diethyl pyrocarbonate (DEPC) treated water.

Ten microliters of total RNA was removed and diluted with 990 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0) for measuring the amount of RNA recovered at OD_{260}.

3.2.17.Radioactive labeling of ABPcNDA probes

A Random Primer DNA Labeling Kit (Boehringer Mannheim, Inc) was used for labeling ABP cDNA. ABP cDNA was prepared by the restriction enzyme *XhoI/EcoRI* digestion of pACT2/ABP cDNA fusion vector and isolation of the *XhoI/EcoRI* fragment. The purified ABP cDNA (500ng) was denatured by heating for ten minutes at 100^0C and cooling on ice, followed by addition of 3 µl of dATP, dGTP, dTTP mixture (ratio 1:1:1); 2 µl reaction buffer; 5 µl 50µCi (^{32}P) dCTP (3,000Ci/mmol) aqueous solution; 1 µl Klenow enzyme. The contents were incubated for 30 minutes at 37^0C. The reaction was stopped by adding 2 µl 0.2M EDTA (pH 8.0). The labeling mixture was then passed through a spin column.

Ten microliters of reaction solution was taken from the eluted labeling mixture to measure the specific activity of ABP cDNA probes in LS 650 Multi-Purpose Scintillation Counter (Beckman)

3.2.18.Northern blot

Northern blot hybridization was performed according to the manufacture's instruction (Zeta probe blotting membrane, Bio-Rad) with some modifications. Briefly, 20 µg of total RNA was incubated with 10 µl loading buffer at 65^0C for 0.5 hr. Samples were then loaded onto the prepared gel (1% agarose; 2.2 mol/L formaldehyde) and subjected to electrophoresis in 1x formaldehyde gel-running buffer at 100 V for one hour. RNA was transferred to Zeta probe nylon membranes (Bio-Rad) overnight in 10 x sodium citrate transfer buffer (10XSSC). Membranes were cross-linked by exposure to ultravoilet light.

After fixation, membranes were first prehybridized in 10 ml prehybridization solution at 43^0C for 15 minutes followed by hybridization with ^{32}P-labelled ABP cDNA probes (> 1×10^7 cpm) overnight at 43^0C.

Following hybridization, the membranes were washed twice for 15 minutes at room temperature in 2 x SSC and 0.1% SDS, once in 0.5 x SSC and 0.1% SDS and again in 0.1 x SSC and 0.1% SDS at 55^0C. After washing, the blotted membranes were subjected to autoradiography with Kodak BioMax film at -70^0C for a day and the film was developed.

3.2.19.Culturing neuroblastoma cells, SK-N-SH

The neuroblastoma cell line SK-N-SH was used in the experiments. SK-N-SN cells are derived from a human female, 4 years old. The APP metabolism of these cells has been characterized and shown to mimic that of neurons. SK-N-SH cells were cultured in neurobasal^{TM}-A medium supplemented with 2 mM L-glutamine, and 10% fetal bovine serum in a constant environment of 37^0C, 5% CO_2 in a humidity controlled incubator. SK-N-SH cells were examined every day and the neurobasal^{TM}-A Medium was changed every two days. The cells that approached confluence were passaged and subcultivated at a ratio of 1:4.

3.2.20.Construction of pcDNA 3.1/ABPcDNA fusion vector

The common mammalian expression vector pcDNA3.1 (Invitrogen) was selected as the carrier vector of the ABP gene for expressing ABP in mammalian cells. The ABP *Hind III* fragments with 'AD' from pACT2 ('AD')/ABPcDNA were subcloned into pcDNA3.1 vector at MCS downstream of PCMV promoter (Figure 9). The reconstructed pcDNA 3.1/ABP-'AD' fusion

vector will express an ABP-'AD' fusion protein, which can be detected by using an antibody against AD.

3.2.21.Stable transfection of SK-N-SH cells

FuGENE™-6 Transfection reagent was applied in transfection reactions according to the manufacture's instruction (Boehinger Mannheim, Inc).

SK-N-SH cells were harvested by trypsinization. 3% 10^5 cells were plated in a 35-mm dish and incubated until 50-80% confluence was achieved. In a small sterile tube, 6 µl FuGENE™-6 transfection reagent was diluted with Neurobasal-A Medium into a 200 µl volume and incubated for 15 minutes at room temperature. 2.0 µg pcDNA 3.1 plasmid with the neomycin resistant gene and ABP sequence was then added to the tube and the tube was incubated for 30 minutes at room temperature. The mixture was added to the SK-N-SK cells and evenly dispersed before returning to the incubator for 24 hours.

To select the transfected cells, the cells were harvested and transferred to a flask with 10 ml Neurobasal-A Medium supplemented with neomycin (0.8g/ml) and recultured. The cells were checked every day and medium was changed until the cell culture became confluent. The cells were either collected for immediate use or resuspended in Neurobasal-A Medium (contain 5% DMSO) and stored at -70^0C.

3.2.22.Preparation of cell lysate

Cell lysate was prepared as described previously (Vander heiden, 1997). Briefly, cells were collected from three 75 cm^2 flasks (each: 10 ml medium) and transferred to a 50 ml centrifuge tube following trypsinization and pelleted by

centrifugation for five minutes at 2,500 rpm at room temperature. The pellet was washed with sterile 3 ml PBS buffer twice and resuspended in a 1.5 ml microcentrifuge tube with 1.5 ml PBS. The cells were pelleted by centrifugation for ten minutes at 14,000 rpm at 4^0C.

Five hundred microliters of lysis buffer (Sucrose, 250 mM; HEPES, 20 mM; HCL, 10 mM; $MgCl_2$, 1.5 mM; EDTA, 1.0 mM; EGTA, 1.0 mM; DTT, 1.0 mM) was added to the tube and sonicated for 20 minutes with periodic vortexing. The tube was centrifuged for ten minutes at 3,000 rpm at 4^0C. The supernatant was removed and transferred to a clear 1.5 ml microcentrifuge tube and centrifuged for 25 minutes at 14,000 rpm at 4^0C. The supernatant was collected and transferred to a new 1.5 ml tube.

Fifty microliters was taken for determining the concentration of protein and the other was immediately used or stored at -70^0C.

3.2.23.SDS-PAGE

The resolving gel (8% or 10%) was freshly made and poured into a vertical gel casting apparatus. Polymerization was initiated by the addition of ammonium persulphate and TEMED and 100% ethanol was applied for covering the resolving gel. Polymerization was allowed to last at least 15 minutes.

After polymerization, ethanol was poured out and gel was dried for two minutes. The 5% stacking gel (freshly prepared) was then poured on top of polymerized resolving gel. A ten-well Teflon comb was inserted in the gel. The stacking gel was allowed to polymerize for another 15 minutes before the comb

was removed.

The prepared cell lysate containing ABP was mixed with 5 µl 4% loading buffer and heated for five minutes in boiling water, centrifuged briefly and cooled on ice immediately prior to loading. Up to 30 µl of lysate was loaded in each well. Meanwhile, 5ul of protein standard (Amersham Life Techology) in 5 µl of SDS sample buffer was also placed in boiling water as described above and loaded in a well. The protein sample was subjected to electrophoresis in 1%gel running buffer at 100 V for 2-2.5 hours at room temperature until the proteins were separated.

3.2.24. Western blotting

The transfer sandwich was assembled in 1% transfer buffer in the following order: clear plate; Whatman paper; Hybond ECL nitrocellulose membrane (Amersham Life Science); SDS gel; Whatman paper; black plate. The sandwich was placed in western blotting apparatus with the black plate facing the cathode and the clear plate facing the anode and an ice pack was placed on the black side. The protein was transferred at 30V overnight at 4^0C and membrane was then separated from the SDS gel, and air dried. The protein marker position was indicated with a waterproof pen. Membranes were blocked for one hour at room temperature in Tris buffered saline (TBS) containing 5% skin milk with gentle shaking. After washing in TBS, the membrane was incubated with the first antibody (anti-APP, concentration: 1 µg/µl; anti-AD, concentration; 2 µg/µl) in 2 ml TBS-T containing 5% skim milk in a capped tube at 4^0C with shaking overnight. The membrane was then washed with TBS-

Tween 20 and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-mouse IgG (Amersham) diluted 1:500 in TBS-T with 5% skim milk. After the final wash in TBS-T, the membrane was processed with a chemiluminescence kit (Amersham) for visualization of immunoreactive protein bands.

3.2.25.Co-immunoprecipitation

Fifty microliters of the cell lysate containing 150 μg protein was incubated with 2 μg anti-APP antibody or 4 μg anti-'AD' antibody on ice for one hour. The prepared protein G-sephorase beads in 10 μl lysis buffer was then added to the mixture and incubated on ice for 40 minutes. The beads were pelleted by centrifugation at 5,500 rpm for one minute and washed with lysis buffer for several times. The beads were pelleted again, resuspended in 50 μl of SDS loading buffer, and heated in boiling water for five minutes. The supernatant was collected by centrifugation (> 10,000 g) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) for analysis.

Western blot was applied to analyze the immunocomplexes. For APP immunocomplexes, western blot was analyzed with an antibody to Gal4-'AD' tagged protein, and the blot was restripped and reprobed with APP antibody. Likewise, the Gal4-'AD' tagged protein immunocomplexes were analyzed by the APP antibody, restripped and reprobed for Gal4-'AD' tagged protein.

3.2.26.Sequencing and sequence analysis of ABPcNDA

After large scale preparation of pACT2/ABPcDNA fusion vectors, the vectors were sent away for sequencing. The sequencing was performed using the Perken Elmer Prism automated florescent instrument at the DNA Core Services Facility of Queen's University, Kingston, Ontario and the University of Calgary, Alberta, Canada. The translation of the ABP sequence and the prediction of the molecular weight of ABP was then carried out using Translate Tool and Swiss-Prot Program at ExPASy Molecular Biology World Wide Web (WWW) Server of the Swiss Institute of Bioinformatics (SIB). Translate Tool allows the translation of a nucleotide (DNA/RNA) sequence to a protein sequence. The translated protein sequences are presented in six frames, 5'3'frame1-3 and 3'5'frame1-3. The right one can be determined according to information of the predicted open reading frames, which is late subjected to Swiss-Prot Program. Swiss-Prot is an annotated protein sequence database, which annotates function(s) of the protein, post-translational modifications, domains and sites, secondary structure, similarities to other proteins, and provides a quick summary of protein sequence (SQ) content following the submission of protein sequence. The format of the SQ is described as the length of the sequence in amino acids (AA) followed by the molecular weight (MW) rounded to the nearest mass unit (Dalton). Therefore, when the protein sequence (ABP) was identified, its molecular weight was thus predicted.

To search the ABP amino acid sequence homologues from the entire available AA sequence database, the Basic Local Alignment Search Tool

(BLAST) was applied in the National Center for Biotechnology Information (NCBI). BLAST is a set of similarity search programs designed to explore all of the available sequence databases of protein or DNA. It uses a heuristic algorithm, which seeks local as opposed to global alignments and is therefore able to detect relationships among sequences, which share only isolated regions of similarity. But the programs are not generally useful for motif-style searching. The fundamental unit of BLAST algorithm output is the High- Scoring Segment Pair (HSP). An HSP consists of two sequences, a query and database sequence fragments of arbitrary but equal length whose alignment is locally maximal and for which the alignment score meets or exceeds a threshold. A Maximal-Scoring Segment Pair (MSP) is the highest scoring of all possible segment pairs that can be produced from the two sequences. The approach to similarity searching taken by the BLAST programs is first to look for similar HSPs between the query sequence and a database sequence, then to evaluate the statistical significance of any matches that were found, and finally to report only those matches, that satisfy a user-selectable threshold of significance. There are five programs in BLAST. We used Blastp, which compares an amino acid query sequence (ABP) against a protein sequence database. The homologues of ABP were identified according to the reported matches and statistical significance.

Analysis of motifs was finally conducted by using the Prosite Program at ExPASy Molecular Biology WWW Service of SIB. Prosite is a method of determining the function of uncharacterized proteins translated from cDNA

sequences, which contains a database of biologically significant sites and patterns (motifs). If the sequence of an unknown protein is too distantly related to any protein of known structure, it can be identified by the existence of motifs. These motifs are required on the structure of specific region(s) of a protein, which may be important for its binding properties or enzymatic activity. The AA sequence of ABP was input into Prosite Program and the motifs identified were given by the short (not more than four or five residues long) conserved sequences in certain positions. The functions of ABP were therefore predicted.

4. RESULTS

4.1.Gal4 two-hybrid library screening

The yeast stain Y190 was used in the yeast two-hybrid screening. Y190 has no expression of Typtophan (Trp), Leucine (Leu) and Histidine (His), and these characteristics can be used as selective markers for identifying positive clones. Using LiAc transformation procedure, Y190 was transformed with 2μg of the prepared pAS2-1 (BD)/APP-770 fusion vectors. These were grown on SD/-Leu agar plates at 30^0C. After several days, a number of pink color colonies (conferred by the ade2-101 mutation for identification of Y190) appeared. These colonies were picked up and restreaked on the same selection SD agar plate. Numerous pink color colonies reappeared. The result indicated that the pAS2-1 (BD)/APP-770 fusion vector containing leucine gene had been transformed into Y190 and leucine was expressed in Y190, which enabled Y190 to grow on SD /-Leu.

$1x10^6$ clones of yeast expression vectors, pACT2s constructed with human brain cNDA library, encoding Gal4 transcription activation domains, were transformed into Y190 pretransformed by pAS2-1 (BD)/APP-770 fusion vector. Positive colonies were selected on 50 of 25 mM 3-amino-1, 2,4-triazole (3-AT) SD/-Leu/-Trp/-His agar plates at 30^0C. 3-AT was used to depress the weak His expression of Y190 and thus reduced false positive clones. After 14 days of incubation, colonies were visible. These colonies were further subjected to a colony-lift filter β-galactosidase assay. After eight hours, four colonies were positive (blue) for β-galactosidase assay (Figure 10). These four corresponding

colonies were restreaked on SD/-Leu/-Trp/-His agar plates, incubated at 30^{0}C. They were grown on SD/-Leu/-Trp/-His agar plates, and reassayed for β-galactosidase activity. Positive clones were isolated from Y190 for further analysis.

4.2.Analysis of positive clones

To verify that these clones encoded proteins capable of interacting with APP-770, they were co-transformed into Y190 with pAS2-1 (BD)/APP-770 fusion vectors. The transformed Y190 was grown on SD/-Leu/-Trp/-His agar plates and the resultant colonies were subjected to β-galactosidase assay. Blue colonies were identified, which confirmed the interaction (in Yeast) between proteins encoded by the positive clones and APP-770 (Figure 11).

In order to find out which clones encoded the proteins that interact with the carboxyl-terminal region of APP-770, five partial sequence fragments of APP-770 were prepared by restriction with *PstI, BamHI, EcoRI* and subcloned into pAS2-1 (Figure 12). These partial APP-770 sequence/pAS2-1 (BD) fusion vectors were co-transformed with each of the four positive clones into Y190. Protein-protein interaction was indicated by positive β-galactosidase activity. The results showed that only clone #4, designed as an APP binding protein (ABP) was capable of interacting with the carboxyl-terminal region of APP-770 (Figure 13). ABP was isolated and sent to the University of Calgary and Queen's University for automated sequencing. Sequencing results from both centers were consistent. The results showed that the sequence of ABP contained a start codon at amino acid position +1, an upstream Kozak

consensus sequence (GACGGC) at nucleotide -6 to –2, a stop codon at amino acid position +193 and a poly A signal at nucleotide 964 to 969. This suggested that the ABP sequence might represent a full-length open reading frame (Figure 14).

The characteristics of ABP cDNA were predicted by aligning its sequence and translating it using Swiss Prot Translation Tool followed by a data search at NCBI with the BLAST program. According to the sequence of ABP cDNA, there were three open reading frames translated from the sequence. However, there was only one that was in frame with 'AD' sequence. The open reading frame contained 192 amino acids with a molecular weight of 21.46 KD (Figure 15). After the data base search, it was found that ABP did not resemble any known protein. The result indicated that ABP might represent a novel protein. In order to implicate the functions of ABP, a motif data base search was conduced by using the PROSITE program at ExPASy. The search revealed four motifs: protein kinase C (PKC)(a.a.132-134, 183-185), cAMP-dependent protein kinase (a.a.55-58), casein kinase II (CK2)(a.a.142-145, 183-186) phosphorylation and N-myristylation (a.a.11-16, 98-103, 128-133) sites (Figure 15). Therefore, it was suggested that ABP might be a membrane protein, which could be involved in mechanisms controlling cellular processes such as the growth and transmission of regulatory signals.

4.3.Analysis of ABP expression in human Tissues

APP-770 has been showed to be expressed ubiquitously (Ponte *et al*, 1988). The expression of ABP may provide clues to understanding the role of

ABP in mechanisms of APP-770 in humans, especially if ABP interacts with APP-770 in AD patients.

To address where ABP expresses, total RNA from adult human tissues was isolated by Tri-Pure reagents, transferred to Zeta probe nylon membrane, and hybridized with ^{32}P-labled ABP cDNA. After washing the blot, it was exposed for one day. A transcript corresponding to 1.5 Kb, which is consistent with size of full-length ABP cDNA, was detected in all tissues examined, such as heart, lungs, kidneys, and liver (Figure 16). This result suggests that ABP is a ubiquitous protein in humans.

4.4.ABP interacts with APP-770 in mammalian cells

The neuroblastoma cell line SK-N-SH was used for studying protein-protein interaction in mammalian cells. SK-N-SH was chosen because it highly expresses endogenous APP. Also, the metabolism of APP in SK-N-SH has been widely studied and characterized (Bourbonniere *et al*, 1996; kim *et al*, 1999).

The reconstructed pcDNA3.1/ABPcDNA was stably transformed into SK-N-SH cells. The transformed SK-N-SH cells were selected by noemycin (0.8g/ml). Co-immunoprecipitation was then carried out as described previously. The cell lysates were first incubated with Mouse amyloid precursor protein antibody (1.0 μg/ml) against N-terminal region of APP.

The APP immunocomplexes were isolated by protein G-sepharose beads and analyzed by western blots with Gal4-'AD' monoclonal antibodies (1.0 μg/ml) to Gal4 'AD'-tagged ABP. The blot was restripped and reprobed with

APP antibody (Figure 17 B). Likewise, the Gal4 'AD'-tagged ABP immuncomplexes were pulled down and analyzed by the APP antibody, restripped and reprobed for Gal4 'AD'-tagged ABP (Figure 17 C). The Gal4 'AD'-tagged ABP could be found only in the complex immunoprecipitated with anti-APP antibodies from SK-N-SH transfectants (Figure 17B, Lane 2), but not from the control (Figure 17B, Lane1). APP could be found in the complex immunoprecipitated with anti-'AD' (Figure 17C). The data indicate that ABP may interact with APP in mammalian cells.

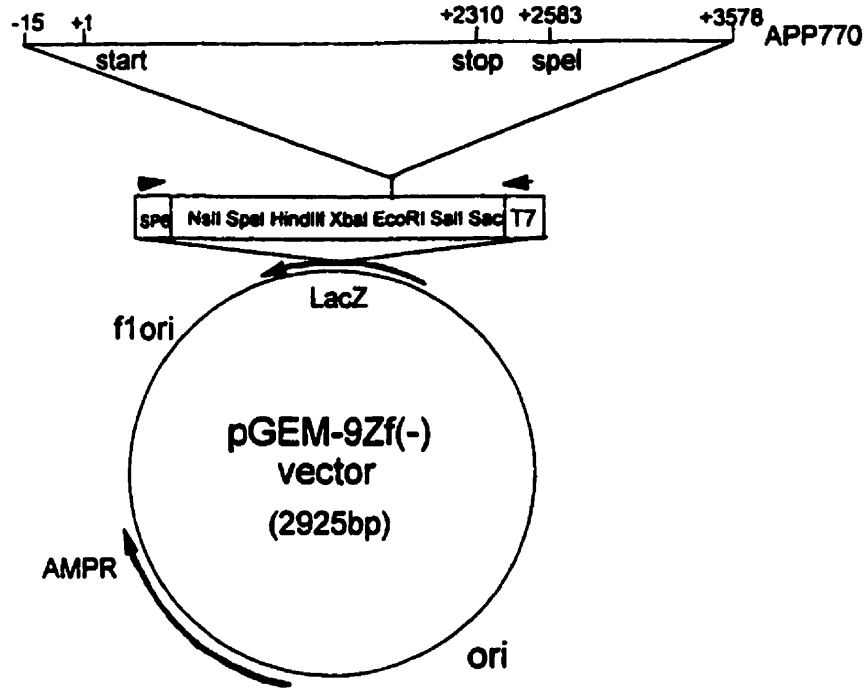
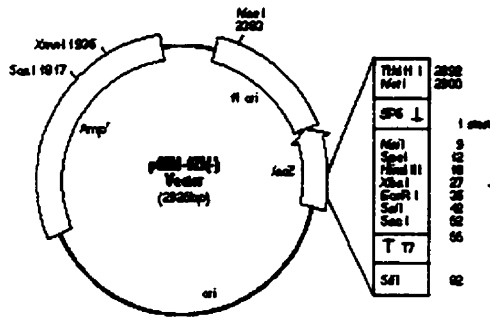


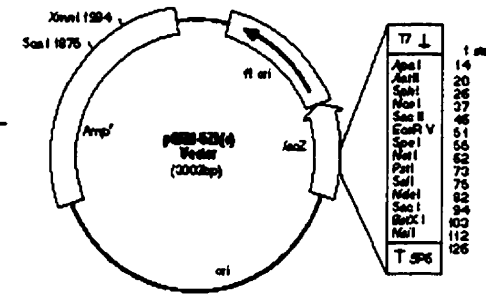
Figure 7. Schematic of the pGEM-9Zf (-) vector containing the full-length APP-770. Multiple cloning sites are shown. Full-length APP-770 is subcloned into pGEM-9Zf (-) vector at *EcoR1* site. The size of inserted APP-770 is 3593 bp that contains the whole open reading frame of APP-770 encoding 770 amino acids. The positions of start and stop codons and *SpeI* sites are shown.

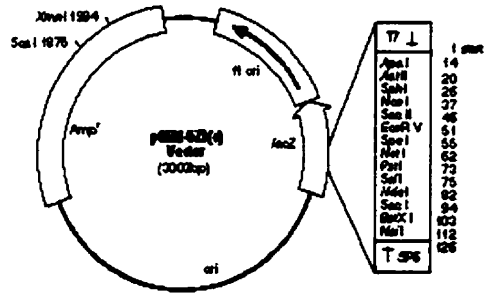


APP-770 (-15 to +3578)

Digested by
restriction enzyme
Spe I

Digested by restriction
enzyme Spe I





APP-770 (-15 to +2583)

Digested by
Restriction enzyme
Nco I/Sal I

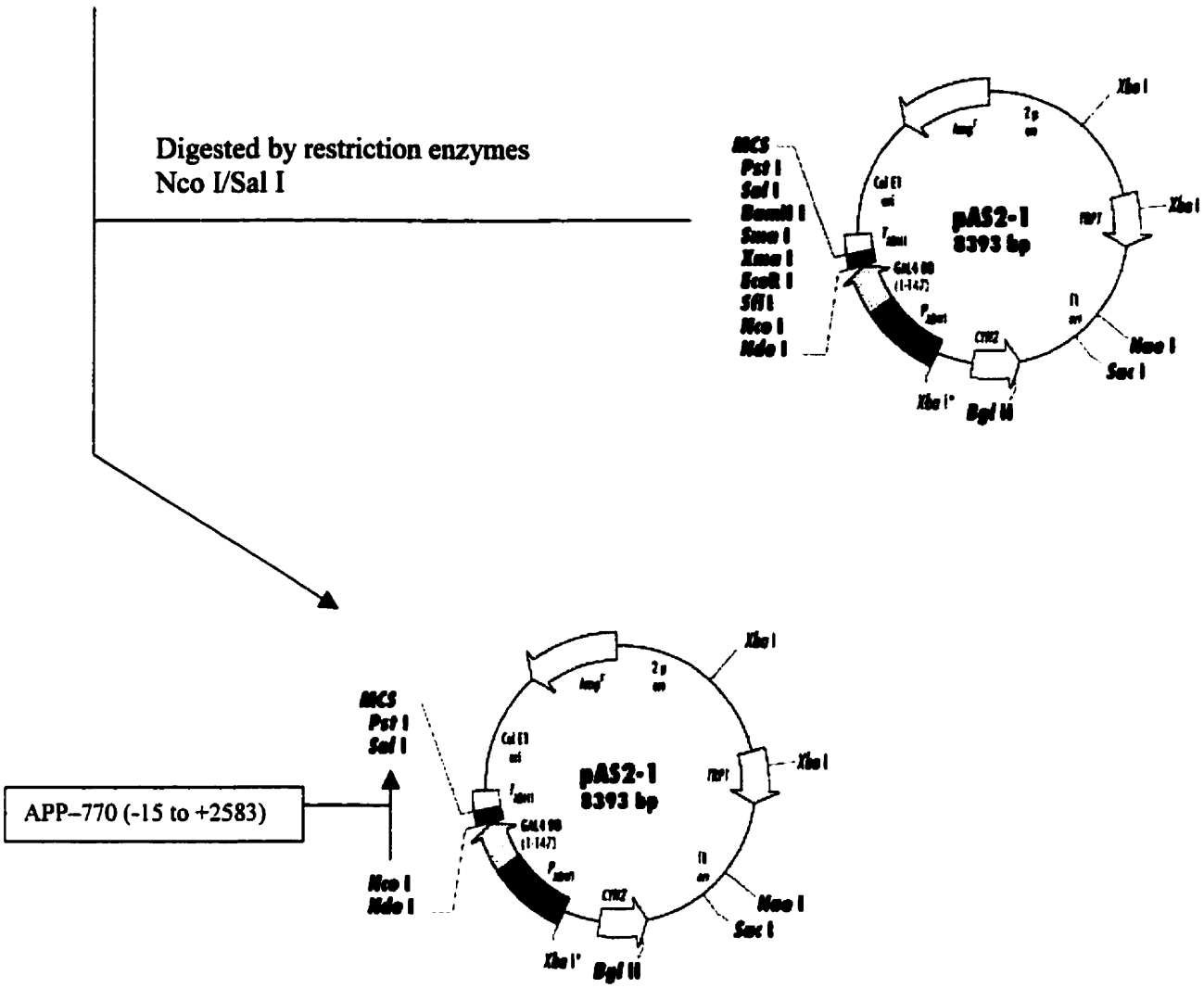
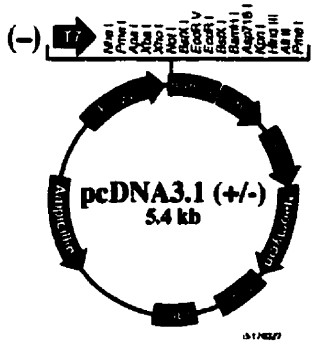
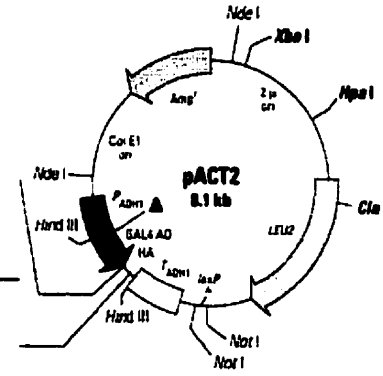


Figure 8. The construction of yeast pAS2-1/APP-770 fusion vector. The pGEM-9Zf(-) vector containing full-length APP-770 was first digested by *SpeI*. The *SpeI* fragment was then subcloned into pGEM-5Zf(+/-). The pGEM-5Zf(+/-) with the *SpeI* fragment insert was treated with restriction enzymes *Nco I/Sal I* and *Nco I/Sal I* fragment was finally inserted into yeast pAS2-1 vector at a site just down stream of BD sequence.



Digested by restriction
Enzyme Hind III

Digested by restriction enzyme
Hind III



Clone #4 cDNA

Clone #4 cDNA + AD

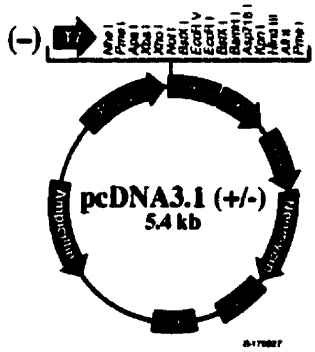


Figure 9.The construction of pcDNA 3.1/ABPcDNA fusion vector. Yeast pACT2 vector with ABP cDNA was digested by *Hind III*. The *Hind III* fragment contained the start codon of 'AD' and downstream ABP cDNA sequence. The *Hind III* fragment was then subcloned into pcDNA 3.1 vector at MSC. The constructed pcDNA 3.1 was found to express 'AD' and ABP fusion protein. Therefore, ABP could be detected by antibodies against AD.

Figure 10. Positive colonies identified in yeast two-hybrid library screening. Yeast expression vectors with APP-770/BD fusion sequence or with cDNA library sequence were sequentially transformed into Y190s. Positive colonies were selected by growing the transformed Y190s on SD/-Leu/-Trp/-His agar plates and later on subjected to β–galactosidase assay. In the end, 4 colonies became blue. A typical positive colony is depicted.

Figure 11. The interaction between the ABP and APP$_{COOH}$-770. pACT2/ABPcDNA and pAS2-1 (BD)/ APP$_{COOH}$-770 fusion vectors were co-transformed into Y190s. Y190s were plated on SD/-Leu/-Trp/-His agar plates .The appeared colonies were subjected to β–galactosidase assay. The blue colonies indicate the interaction between ABP and APP$_{COOH}$-770.

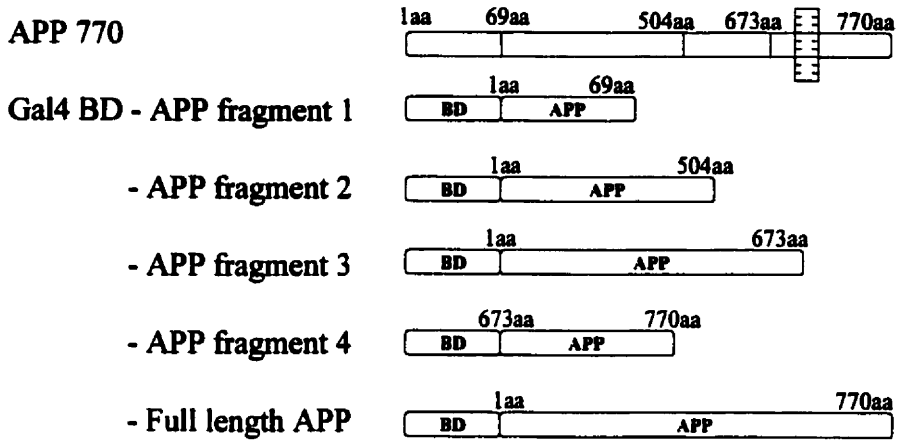


Figure 12. The construction of partial sequence APP-770/pAS2-1 (BD) fusion vector. Full-length APP-770 was digested by *Pst I, BamH I* and *EcoR I,* and these fragments were subcloned into pAS2-1. Full-length APP-770 was also subcloned into pAS2-1 as control. The amino acids encoded by these fragments are shown.

Bait	Library protein	AD-clone #1	AD-clone #2	AD-clone #3	AD-clone #4
BD-APP Fragment1		+	-	-	-
BD-APP Fragment 2		+	-	-	-
BD-APP Fragment 3		+	+	+	-
BD-APP Fragment 4		-	-	-	+
BD-full length APP		+	+	+	+

Figure 13. Detection of the interaction between APP fragment and 4 positive library proteins. " +": The protein-protein interaction. " –": No protein-protein interaction.

```
                                                          ******
-35                    CAACG  GCTGGGACTG  ACCCCCGCTC  CCCCGACGGC
  1 ATGTGGCTCC  AGCCCCGGCC  TGGCCGCCAG  GGAGCGCCAC  TGGCTTCCCG

 51 CCACCCGAAG  GGAGCTCTGG  ACCCTCAGAG  CCCCTTGCAG  AGGACGGATC

101 TAGCTCCTGT  ATATATATCC  TGTATATATA  TTTTATTGCA  TGCACGACCT

151 TGGGGGGAGA  ACAGAAGGGG  GACGACGCCC  CCGCACCTCC  TGCGATCACC

201 TCCTGCGATC  TGGCTGGCTT  GGATCTCGTT  TTTAACCCGT  TCCTGCCCCA

251 CCTGCCCTAT  AGTGAGTCGT  ATTAGGATGG  AGGATTCAGA  AGGTGGCCCT

301 GGATCTCGAC  CCCTATGTGA  AGAAGCTACT  CAATGCCCGG  CGACGCGTTG

351 TCTTGGTCAC  CAACATTCTA  CAGAATGCTC  AGGAACGACT  GAGACGGCTA

401 AACCACAGTG  TTGCCAAGGA  AACAGCCCGC  AGGAGGAGCA  ATGCTGGATT

451 CGGGAATTTA  CCCCCCTGGC  TCCCCAGGCA  AATAACAGAC  GAGCCTATGG

501 AACTCAGGAG  CACAAGTACT  GTTCCCCAGC  TGCCTTGTTT  CAACAGACAT

551 GCAAAGATCC  TAGGAGACAG  TCCCCATAGA  CCTTCAGACA  TTAAAAAGGG

601 AGCCGTACAG  TTTGTTTGAA  GCACTTCGTC  TTACCCATTT  ATGTATGGGG
651 GCCCCCAGGA  AAACCTACAC  ACAGCCCAAA  TGAGGTTCCC  CAAGGACTTT
701 ACATTAATTA  TGGCTCTTGC  TTCCTTTCAC  AAATGAGCTT  GAGGCCTCTA
751 CTTTTTTTTT  TTTTAAAGCT  GCATACTTGA  GGGCTTACCT  TCCTTCAGAA
801 CTAGGTTAAC  CAGAGGGGCT  TCCTTTGTAT  GTTACATGCC  TGGTTACAGG
851 GGCCTGGACA  GGAAGGGGGC  AGGCAAAGTC  AAAGTGAATG  AACTCTGTCC
901 ACCCACTTTT  TTATTGCACT  GGCTTGAATA  CAGTAGCAGT  GTTGAAAAGA
951 ATCATTTTAT  CCAATAAATA  CTTAAAATGA  AAAAAAAAAA  AAAAAAAAAA
1001 AAAAAAAAAA  AAAAAAAAAA  AAAAAAAAAC  CCAAATTTTT  AGAACCCGAA
1051 ATTCGGAAAC  CCCCAATTAC  ATTAGGGAAC  CGGGAACAAC  CAATTTCTCA
1101 ATTAAAAACC  TTTTGCCTCC  TTTAGGAACA  AACCCGGTTT  CAACCCTGGA
1151 AGTTTACCCC  CGTTTAAAAA  TTTTTAAGGG  CCAAATTAAG  GCCAACTTTT
1201 TTGGCCAAAT  TTCCCCAAGA  AAATTTTTTC  ACGGGGCGAA  AAATTTTTTT
1251 TAAAGGGGTT  TTTCCCAAAA  CCTTTGGGGC  TCCTTCCCCC  CAAAAGGTTT
1301 GGGGCAATTT  CCCCCACCCA  GGGGGGGGGG  GGGTGGTTCC  CCCCTTGCCC
1351 CAAAATTTTT  CAAAAAAAAA  AGGAAAAGGG  GGCAAACCCC  GGGGGG
```

Figure 14. Nucleotide sequence of the clone#4 cDNA. The underlines indicate the putative initiation codon ATG, termination codon TGA and poly A signal. Star: Kozak consensus sequence.

A:

1	MWLQPRPGRQ	GAPLASRHPK	GALDPQSPLQ	RTDLAPVYIS
41	CIYILLHARP	WGENRRGTTP	PHLLRSPPAI	WLAWISFLTR
81	SCPTCPIVSR	IRMEDSEGGP	GSRPLCEEAP	QCPATRCIGH
121	QHSTECSGTT	ETAKPQCCQG	NSPQEEQCWI	REFTPLAPQA
181	NNRRAYGTQE	HKYCSPAALF	QQTCKDPRRQ	SP

192 AA; 21.46 MW

B:

Found Motifs	Position	Description
cAMP_Phospho_Site	55-58	cAMP- and cGMP-dependent protein kinase phosphotylation site
CK2_Phospho_Site	142-145 183-186	Casein kinase II phosphorylation site
PKC_Phospho_Site	132-134 183-185	Protein kinase C phosphorylation site
Myristyl	11-16 98-103 128-133	N-myristolation site

Figure 15. A: Predicted amino acid sequence of ABP cDNA in frame with the Gal4 AD Sequence in expression vector pACT2. AA, amino acid; MW, molecular weight. B: The predicted motifs in ABP. The amino acid positions of motifs are depicted.

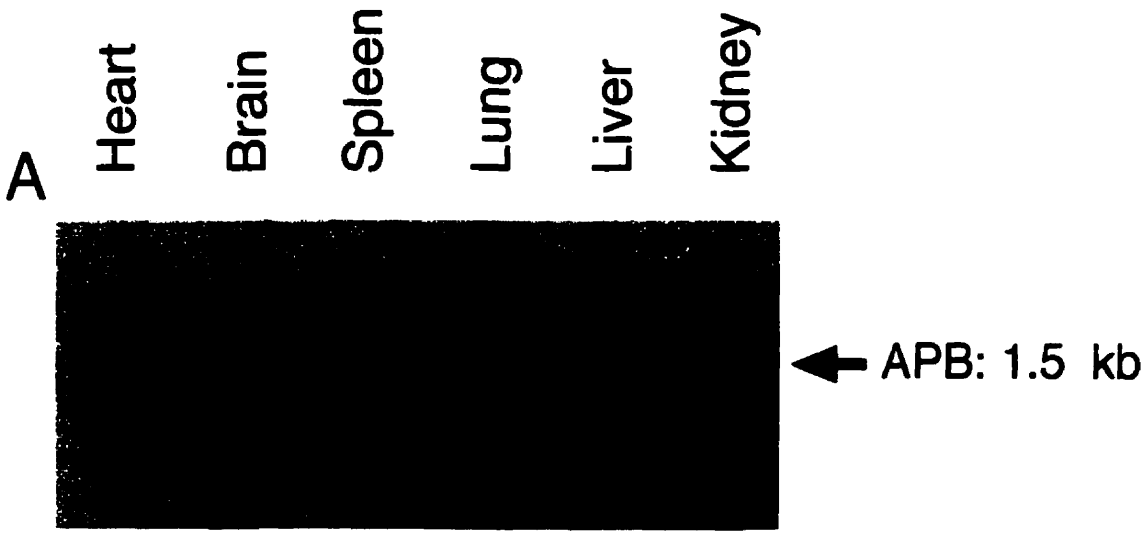


Figure 16. Northern blots analysis of human ABP mRNA. Total RNA were isolated from human tissues (heart, lungs, kidney and liver) by Tri-pure reagrnts, transferred ti Zeta prob nylon membrane, and hybridized with the cloned ABP cDNA . The 1.5 Kb transcript was detected in all tissues examined.

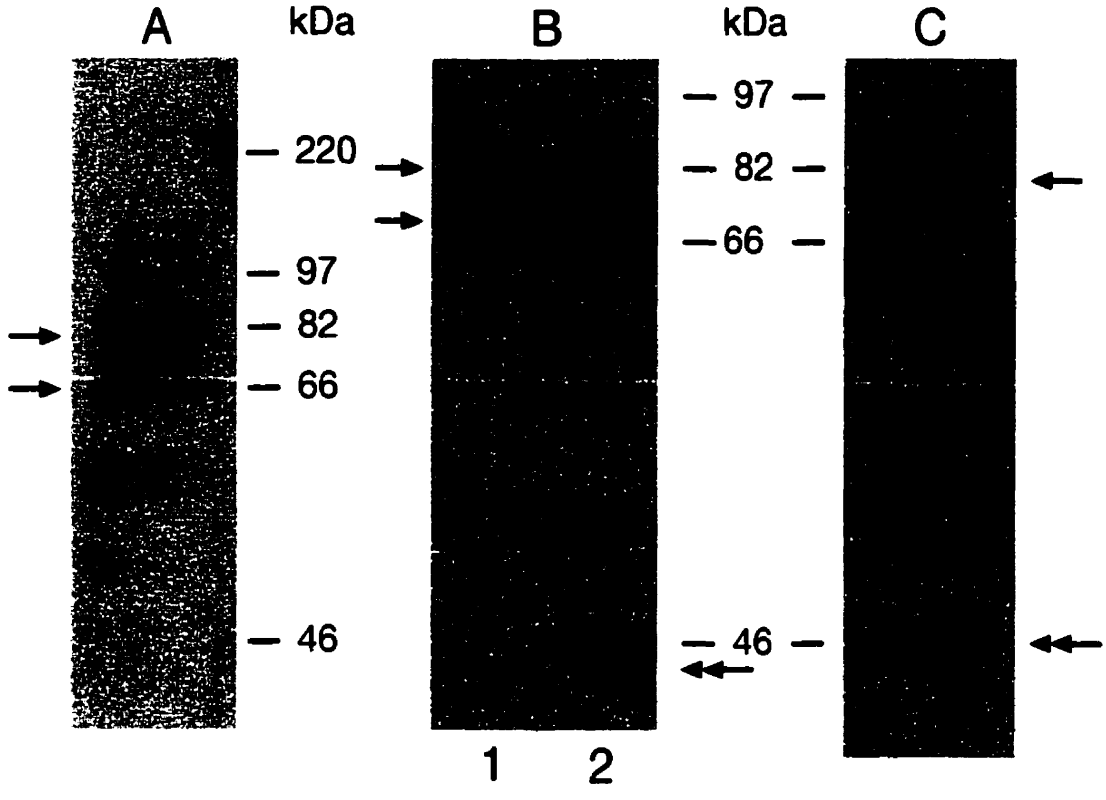


Figure 17. Co-immunoprecipitation of APP with ABP. SK-N-SH cells were stably transfected with pcDNA3.1/ABP cDNA fusion vector. A: Western blot of lysates from untransfected SK-N-SH cells, using monoclonal Anti-APP. B: The lysate from untransfected SK-N-SH cells (Lane1) and transfected (Lane2) were incubated with Anti-APP. Anti-APP immunocomplexes were analyzed by Western blot for the detection of ABP. The blots were then restripped and reprobed with anti-APP. C: Similarly, lysate from transfected cells were incubated with Anti-Gal4 AD and analyzed for APP. The blots were then restripped and reprobed for Gal4 AD. Single arrowhead denotes APP, where as double head arrow denotes AD-tagged ABP.

Discussion

APP has been identified as a causative factor in the pathogenesis of AD (Tanzi *et al,* 1988; Ponte *et al,* 1988; Kitaguchi *et al,* 1988; De-Sauvage *et al,* 1989; Yan *et al,* 1996). Since then, several mutations of APP have been found in familial AD, and it has been confirmed that these mutations contribute to the pathogenesis of familiar AD (Goate *et al,* 1991; Levy *et al,* 1990; Hendricks *et al,* 1992; Mullan *et al,* 1992; Selkoe *et al,* 1994; Haass *et al,* 1997). However, the majority of AD cases are of the sporadic type, in which patients do not carry these mutations. Therefore, knowledge in the physiological functions of APP becomes critical for our understanding of the pathogenesis of both types of AD.

APP was originally described as a cell surface receptor with a single transmembrane domain (Kang *et al,* 1987; Tanzi *et al,* 1987; Goldgaber *et al,* 1987). It is post-transitionally modified and a fraction of the full-length form is then transported to the plasma membrane where they undergo a complex series of events that involve secretion or internalization, degradation, and recycling of various proteolytic fragments (Golde *et al,* 1992; Allsop *et al,* 1994; Koo *et al,* 1994; Cook *et al,* 1997; Kouchi *et al,* 1998). Therefore, APP must interact with a variety of different proteins that determine its fate. These proteins could be involved in mechanisms such as membrane trafficking, signal transduction, or proteolytic processing. Aberrations in any of these processes may be associated with dysfunctions leading to AD.

APP_{COOH}, has been reported to be highly conserved phylogenetically in vertebrates (Iijima *et al*, 1998), it contains signals for APP metabolism (Lai *et al*, 1995; Tomita *et al*, 1998; Kouchi *et al*, 1998; Zheng *et al*, 1998; Borg *et al*, 1998). It is thought to contain an amino acid sequence essential for the transduction of signals from an extracellular ligand that remains to be identified (Neve, 1996). Research on the identification and isolation of factors that interact with APP_{COOH} has been pursued and several factors such as APP_{COOH} binding proteins have been characterized recently. The interaction of the G_0 protein with APP peptide 20 was revealed by co-immunoprecipitation studies (Nishimoto *et al*, 1993) and it has been suggested that the APP peptide 20:G_0 interaction may be involved in apoptosis (Giambarella, 1997). APP-BP1, which was cloned by the yeast two-hybrid system with an APP_{COOH} peptide, is homologous to AXR1, a protein believed to be involved in signal transduction in Arabidopsis cells (Chow *et al*, 1996). Rat Fe65 and its human homologues as APP_{COOH} binding proteins have also been identified (Fiore *et al*, 1995; Guenette *et al*, 1996). For Fe65, two phosphotyrosine binding (PTB) domains, originally identified in the protein Src which was thought to be crucial for the interaction of Src with growth factor receptors in tyrosine kinase signal transduction, appear to mediate the interaction with the last 32 amino acids of APP_{COOH} (Zambrano *et al*, 1997). X11, the other protein with PTB domain, has also been shown to interact with APP_{COOH} (McLoughlin and Miller, 1996; Borg *et al*, 1996a; Borg *et al*, 1998b).

In addition to their potential involvement in signal transduction, APP_{COOH} binding proteins may affect APP metabolism. For example, PTB domains of Fe65 and X11 have been shown to interact with the YENPTY motif in APP_{COOH}. The YENPTY motif has been proven to be an endocytic signal for the internalization of APP. It has been reported that deletion of the YENPTY motif results in increased secretion of the soluble APP fragments (sAPP) while decreasing the production of β-AP (Koo *et al,* 1994; Lai *et al,* 1995). The above results suggest that the consequences of interaction of APP_{COOH} with its different binding proteins are a complex process that may require further studies.

By using the yeast two-hybrid system, we have identified a human brain cDNA encoding a 21.46 KD protein, named ABP, which interacts with APP_{COOH}. The APP_{COOH} : ABP interaction was confirmed by co-immunoprecipitation studies in the neuroblastoma cell line, SK-N-SH, suggesting that ABP does interact with APP_{COOH} in human cells and may contribute to physiological functions of APP. Although ABP is derived from the human brain, Northern blot study showed that the ABP mRNA was expressed in multiple human tissues such as lungs, heart and kidneys. This result indicates that ABP is a ubiquitous protein. Since APP-770 is also widely expressed in mammalian cells, its interaction with ABP may serve a basic role in the normal function of APP.

Analysis of the sequence of the cloned ABP shows that the cDNA encodes a 192 amino acid peptide. We have performed a data base search

for homologues of ABP. There is no evidence indicating any known protein with a significant similarity of sequence to ABP. We conclude that ABP is a novel protein and the exact location of its binding to APP remains to be elucidated. We do not know if the interaction site on APP_{COOH} with ABP contains YENPTY motif or any other phosphorylation sites. Further studies need to be conducted to determine the region on APP_{COOH} that is responsible for its binding to ABP.

Although no known protein is homologous to ABP, there are four motifs identified in ABP. These motifs include PKC, cAMP-dependent protein kinase, CK2 phosphorylation and N-Myristylation sites. PKC is especially abundant in brain tissue, where it is centrally involved in modulating many aspects of synaptic plasticity such as the regulation of neurotransmission, cellular growth differentiation, learning, and memory (Nishizuka, 1989; Nishizuka, 1998). An early PKC deficit in AD has been reported and linked to the pathogenesis of AD (Masliah *et al*, 1991). Of particular interest, the relationship between PKC and APP metabolism has firmly demonstrated that activation of PKC increases the production of non-amyloidogenic soluble APP (sAPP) and decreases the formation of neurotoxic β-AP (Xu *et al*, 1995; Govoni *et al*, 1996; Caputi *et al*, 1997; Marambaud *et al*, 1997; Leblanc *et al*, 1998). However, the mechanisms of PKC-dependent regulation in this APP α-secretase pathway remain unclear. The intracellular targets of PKC have not been defined. Although PKC phosphorylates the APP_{COOH} *in vitro* (Suzuki *et al*, 1992), the effects of PKC on formation and secretion of sAPP and β-AP

do not require phosphorylation of the cytoplasmic tail of APP in intact cells (Hung *et al*, 1994; Jacobsen *et al,* 1994). The discrepancy between the *in vitro* and *in vivo* studies indicates that one or more molecules of the APP trafficking and processing apparatus are PKC substrate phosphoproteins involved in the mechanism by which PKC regulates APP cleavage (Caputi *et al*, 1997). We hypothesize that ABP may be one of these molecules such as an inactive cytoplasmic factor (Tomita *et al*, 1998). If PKC indeed phosphorylates ABP, the phosphorylated ABP may become active and binds to the specific sequence of APP_{COOH}. This process will direct the APP complex into the normal secretory pathway of APP secretion, in which APP is cleaved preferentially at the α- site. In the case of a deficit of PKC, such as in sporadic AD patients, a considerable amount of ABPs are not phosphorylated. Since the unphosphorylated ABPs can not bind to APP_{COOH} , some of APPs may go through the default secretory pathway of APP to produce β-APs, which may contribute to the pathogenesis of AD.

The evidence of alteration in the cAMP pathway in AD has also been reported (Horsburgh and Saitoh, 1994). This pathway is necessary for establishment of long-term but not short-term memory in mammals (Hummler *et al,* 1994). The cAMP-dependent protein kinase in this pathway is PKA. One of the identified PKA substrates is synapsin which is localized in presynaptic terminals and appears to be associated with the cytoplasmic synaptic vesicles anchoring them to the cytoskeleton. The phosphorylation of synapsin by PKA decreases its binding affinity to the vesicles and promotes

its translocation to plasma membrane and the subsequent release of neurotransmitters (Magnoni *et al,* 1991). If this is the case for ABP, its phosphorylation can change its binding affinity and direct down-stream targets to the cytoplasmic tail of APP, to which it binds. This will establish a molecular pathway by which APP works as a cell surface receptor to transduce signals. It enforces us to identify proteins interacting with ABP.

N-myristylated proteins have never been reported in AD. Investigation of the myristylated proteins documented that N-myristylation plays an important role in targeting proteins to the plasma membrane (Wilcox *et al,* 1987; Towler *et al,* 1987). Therefore, it is suggested that ABP could be first N-myristylated and then moved to the plasma membrane, where it interacts with APP, and affects APP metabolism or induces the signal transduction.

CK2, a protein serine/threonine kinase, is ubiquitously distributed in eukaryotic cells, including neurons (Hunter and Karin, 1992) and is an important component of signaling pathways that control the growth and division of cells (Litchfield and Luscher, 1993). CK2 appears to mainly regulate the phosphorylation of nuclear proteins (Litchfield and Luscher, 1993). In AD, CK2 has been shown to be aberrant, there are the decreased amounts and activity of CK2 in the neocortex of the brain (Horsburgh and Saitoh, 1994). ABP seems to be a membrane protein. It appears to be impossible for ABP to work as a regulatory nuclear protein phosphorylated by CK2. However, the phosphorylation of ABP by CK2 may alter the interaction

between APP and ABP, especially in AD patients with a decreased amount or activity of CK2, which could contribute to the abnormal signaling pathway.

In conclusion, we have identified a human brain cDNA, which encodes an APP_{COOH} binding protein, ABP. It is a ubiquitous protein and may be important for APP trafficking and signaling. Obviously, further studies are necessary to elucidate the role of ABP in the normal function of APP.

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Appendix: Solution used in experiments

The following solutions were used in the materials and methods as described above.

Northern blot Solutions:

1xGel-loading buffer	50% glycerol; 1 mM EDTA, pH 8.0; 0.25% bromophenol blue; 0.25% xylene cynanol FF
1xFormaldehyde gel running buffer	0.02 M MOPS 8 mM sodium acetate; 1 mM EDTA
20x SSC stock	175.3 g NaCl; 88.2 g sodium citrate; 1 L water
1xPrehybridization Solutions	50% formamide; 0.12 M Na_2HPO_4; pH 7.2 0.25 M NaCl; 7% (w/v) SDS; 1 mM EDTA

SDS- PAGE Solutions:

8% or 10% resolving gels:	Distilled water 4.6 or 3.3 ml; 30% acrylamide mix 2.7 or 4.0 ml; 1.5 M Tris (pH 8.8) 2.5 ml; 10% SDS 0.1 ml; 10% ammonium persulphate 0.1 ml; TEMED 0.006 or 0.004 ml
5% stacking gel:	Distilled water 2.7 ml; 30% acrylamide mixture 0.67 ml; 1.0 M Tris (pH 6.8) 0.5 ml;

	10% SDS 0.04 ml; 10% ammonium persulphate 0.04 ml; TEMED 0.004 ml
5xgel running buffer (pH 8.3):	0.025MTris base; 0.25 M glycine; 0.1% SDS
4xsample loading buffer:	2M Tris-HCL 1.25 ml, pH 6.8; Glycerol 4.0 ml; 20%(w/v) SDS 4.0 ml; 2-β mercatoethanol 0.5 ml; 0.05%(w/v) bromophenol blue 0.2 ml

Western blotting Solutions:

1x Transfer buffer	25 mM Tri; 192 mM glycine; 20%(w/v) methanol
TBS (pH 7.6)	0.02 M Tris base; 0.5 M NaCl; 0.02% Na Azide
TBS-T	TBS; $5x10^{-4}$%(v/v) Tween 20

β-galactosidase filter assay solutions:

Z buffer	$Na_2HPO_4\_7H_2O$, 16.1g/L

	$Na_2HPO_4\_H_2O$, 5.5g/L KCL, 0.75g/L $MgSO_4\_7H_2O$, 0.246g/L
Z buffer/X-gal solution	Z buffer, 100 ml β-mercaptoethanol, 0.27 ml X-gal stock solution (20mg/ml), 1.67 ml