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A

ABNORMAL HYPERPHOSPHORYLATION OF BETA-TUBULIN AND BETA-CATENIN IN ALZHEIMER DISEASE BRAIN

by

SHRIJAY VIJAYAN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment
of the requirements for the degree of Doctor of Philosophy,
The City University of New York

2000

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

ABNORMAL HYPERPHOSPHORYLATION OF BETA-TUBULIN AND BETA-CATENIN IN ALZHEIMER DISEASE BRAIN

by

SHRIJAY VIJAYAN

Advisor: Professor Khalid Iqbal

Microtubule associated protein tau, the major protein subunit of Alzheimer paired helical filaments is abnormally hyperphosphorylated and an imbalance in the protein kinase - phosphatase system has been implicated in the abnormal phosphorylation. Furthermore, the activities of phosphoseryl / phosphothreonyl protein phosphatase - 1 and - 2A, which are expressed in neurons and regulate the phosphorylation of neuronal proteins phosphorylated at serines and threonines, are decreased in Alzheimer disease (AD) brain. We undertook a study to investigate whether proteins other than tau are also hyperphosphorylated in the AD brain. Frontal gray matter from AD and age-matched control cases were homogenized and centrifuged at 100,000 x g for 30 min. While in the 100,000 x g supernatant there were no significant differences in phosphate levels between the two groups, the pellet showed an increase of ~11 pmoles phosphate per microgram protein in AD over the control cases. Hyperphosphorylation of proteins was examined by employing antibodies to phosphoserine and

phosphothreonine on Western blots. Two non-tau phosphoproteins with molecular masses of ~54 kD and ~94 kD were found to be hyperphosphorylated in AD. The ~54 kD non-tau protein was purified by preparative SDS-PAGE and identified as β-tubulin, by immunolabeling with specific antibody to β-tubulin, by mass spectrometry and by N-terminal amino acid sequencing. The ~94 kD protein was identified as β-catenin, by immunolabeling with antibodies to β-catenin on Western blots. Hyperphosphorylation of β-catenin was confirmed by immunoprecipitating β-catenin followed by Western blotting with P-thr antibodies. We also found that the steady state levels of particulate pool, but not the cytosolic pool, of β-catenin was ~39 % higher in AD compared with age-matched controls. We investigated the role of APP in the regulation of β-catenin levels in cultured cos-7 cells. Cos-7 cells were transfected with APP full length (APP FL), ΔcAPP (lacking the C-terminal 44 amino acids), APPswe (M670N,K671L) or as control with vector alone. The APP FL was found to downregulate particulate pool of β-catenin. This ability was lost when APP was mutated as in APPswe or when 44 amino acids from its cytoplasmic tail were deleted. These studies suggest that normal APP may function by downregulating β-catenin and when its normal functions are altered as in APPswe it is unable to downregulate β-catenin levels, resulting in an increase in the particulate pool of β-catenin. Furthermore, the restoration of normal levels of β-catenin in the ΔcAPP transfected cells suggest that APP downregulates β-catenin through its cytoplasmic tail.

Our findings that two non-tau proteins - β-catenin and probably β-tubulin are hyperphosphorylated support the hypothesis that there is an imbalance in the phosphorylation - dephosphorylation system in the AD brain, resulting in the hyperphosphorylation of several neuronal phospho-proteins, which in turn may result in the alteration of their biological activity, as has been demonstrated for tau.

Dedicated

in loving memory of

my father ***Mr. V. Vijayan***

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ABBREVIATIONS

AD	Alzheimer disease
APO	apolipoprotein
APP	amyloid precursor protein
CDK	cyclin dependent kinase
CRMP	collapsin response mediator protein
EDTA	Ethylene diamine tetraacetic acid
FAD	familial Alzheimer disease
GSK	Glycogen synthase kinase
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
HMW	high molecular weight
HU	human
MT	microtubule
MAP	microtubule associated protein
NFT	neurofibrillary tangles
PDPK	proline directed protein kinases
PHF	paired helical filament
PP	protein phosphatase
PS	presenilin
PSP	phosphoseryl/phosphothreonyl protein phosphatases
PTP	phospho tyrosyl protein phosphatase
TCF	T cell factor
TG	transgenic
WT	wild type

INTRODUCTION:

Alzheimer's disease is the most common cause of dementia in mid-to-late life. Clinically, the most common and salient feature is loss of memory. Histopathologically, it is defined by the presence of two characteristic lesions, namely the intracellular neurofibrillary tangles and the extracellular deposits of β-amyloid which makes the core of senile plaques. Neurofibrillary tangles correlates with the degree of dementia, whereas, some of the normal aged persons have as many β-amyloid plaques as seen in AD brains. Therefore, understanding the molecular mechanisms of neurofibrillary degeneration is crucial in understanding the progression of the disease. Microtubule associated protein tau in an abnormally hyperphosphorylated form is the major component of the neurofibrillary tangles. Phosphorylation of any protein is a reflection of the activities of kinases and phosphatases that act upon them. Thus, it is conceivable that hyperphosphorylation of tau is due to either the increase in the activities of kinases or a decrease in the activities of phosphatases or a combination of both. Studies from our laboratory and others suggest alterations in the activities of these enzymes, implying a general imbalance in the protein phosphorylation / dephosphorylation system is the basis for hyperphosphorylation of tau. In this study we have sought to test the hypothesis that there is a general imbalance in the protein phosphorylation / dephosphorylation system and that neuronal phosphoproteins other than tau are also hyperphosphorylated in AD brain.

SPECIFIC AIMS:

The goal of this thesis is to study if phosphoprotein(s) other than tau, are also hyperphosphorylated in AD. AD is a dementing disorder with polyetiology. Studies carried out in our laboratory and elsewhere have shown that microtubule associated protein tau is abnormally hyperphosphorylated in AD brain (Grundke-Iqbal et al., 1986a,1986b, Iqbal et al., 1986, 1989, Lee et al., 1991). The state of phosphorylation of any protein is a reflection of the activities of kinases and phosphatases that act upon them. The activities of cdk5 might be upregulated in the brains of AD patients (Patrick et al., 1999). The exact role of any of the other kinases in hyperphosphorylation of tau is not known. Studies carried out in our laboratory suggest that a decrease of phosphoseryl / phosphothreonyl phosphatase activity might be a cause of abnormal hyperphosphorylation of tau in AD (Gong et al., 1993, 1995). These studies suggest a general imbalance in the protein phosphorylation / dephosphorylation system in AD brain implying abnormal hyperphosphorylation of other neuronal phosphoprotein(s).

We hypothesize that there is a general imbalance in the protein phosphorylation / dephosphorylation system in AD brain. To test this hypothesis, we proposed two specific aims:

1. To detect abnormally hyperphosphorylated protein(s) other than tau in the brains of Alzheimer disease (AD) patients.
2. To isolate and identify the abnormally hyperphosphorylated protein(s) from specific aim#1.

BACKGROUND AND SIGNIFICANCE:

Alzheimer disease (AD) is a neurodegenerative disease that affects predominantly the middle-aged and elderly humans. It is the most common cause of dementia in mid-to-late life (Evans et al., 1989). Other causes of dementia include cerebrovascular disease, Parkinsonism, Lewy body disease, Pick's disease, Frontotemporal dementia, Wernicke-Korsakoff syndrome, drug intoxication, infections, such as AIDS and syphilis, trauma, particularly subdural hematoma, brain tumors, normal pressure hydrocephalus, vitamin deficiencies (e.g. B_{12}), hypothyroidism, and a variety of other metabolic disorders. Familial British Dementia (FBD) and Familial Danish Dementia (FDD) are also other causes of dementia and are similar to AD, neuropathologically (Ghiso et al.,2000 ; Vidal, R et al., 2000).

Currently, there are approximately 4 million Americans believed to suffer from AD. The prevalence of AD is rapidly approaching epidemic proportions. A major reason for this is that the prevalence of this disease increases with age , from middle age onwards, and improvements in modern medicine has markedly increased the human lifespan. The problem becomes even more acute in the light of recent estimates that the number of people living beyond the age of 65 is expected to continue to increase. By the year 2030, it is estimated that between 17 and 20% or approximately 50 million of the population of the United States will be over age 65. The impact of these statistics on the family and the health care industry in terms of time, effort and cost are staggering. A report issued by the Michigan Task force on Alzheimer disease and Related

Conditions (1987) effectively underscores this last point. "Each person with a dementing disease requires an average of seven years of care, either at home or in a residential care facility. Care provided at home is estimated to cost about $12,000 annually, for a total of $84,000 per person. This is a conservative figure, however, because many people with dementia spend their last few years in a nursing home at an average cost of $22,000 per year and some spend from 10 to 15 years in a nursing home, for a total cost of $220,000 to $330,000. The human cost cannot be calculated so easily, because it includes exhausted caregivers and shattered families as well as depleted financial assets. All in all, the disease represents a great human and economic toll.

Clinical symptoms:

The insidious onset of AD is characterized by a subtle decline of memory functions in a state of clear consciousness. In many elderly, memory loss represents the earliest sign of AD (McKhann et al., 1984; Evans et al., 1989; Price et al., 1991; Arrigada et al., 1992). In addition to memory loss, patients in the early stages of AD often show impairments in at least one other area of cognition i.e., praxis, language, calculation, perception, and judgement. With advanced disease, the deficits become more profound, whereas patients may exhibit gross behavioral disturbances such as aggression, agitation, social inappropriateness or even frank psychosis (with delusions and hallucinations). In late stages, virtually all patients are profoundly demented, incontinent, and bedridden.

The steady aggravation of symptoms reflects the gradual expansion of AD-related brain destruction, which begins in specific limbic areas of the cortex and then spreads in a predictable pattern, across the hippocampus to the neocortex and a number of subcortical nuclei.

Diagnosis:

To make a diagnosis of AD and to exclude other causes of dementia, clinicians rely on histories from patients and informants; physical, neurological, and psychiatric examinations; neuropsychological testing; and a variety of diagnostic tests, including neuroimaging studies, examination of levels of specific proteins in the serum or cerebrospinal fluid, such as the Aβ peptide and tau, and apoE genotyping. Computerised tomography or magnetic resonance imaging of the brain, performed on many patients with this syndrome, can identify other causes of dementia (brain tumors, normal pressure hydrocephalus, etc.) and disclose patterns consistent with AD (i.e., diffuse cortical atrophy). Positron emission tomography and single photon emission computerised tomography, which are not generally used as diagnostic tools, usually show decreased regional blood flow in the parietal and temporal lobes with involvement of other cortical areas at later stages. The clinical profile and results of diagnostic studies allow clinicians to make a diagnosis of possible or probable AD (Mckhann et al., 1984). However, definitive diagnosis of AD requires demonstration of two characteristic histopathologic lesions in the post-mortem brain viz. the extracellular senile plaques and the intracellular neurofibrillary changes as tangles and neuropil threads.

Neuropathology:

Correlative neuropathological - neurochemical studies have shown that symptoms and signs of AD are associated with abnormalities in neuronal populations in the amygdala, hippocampus, neocortex, anterior thalamus, basal forebrain cholinergic system, and monoaminergic brainstem systems (i.e., locus coeruleus, raphe complex) (Whitehouse et al., 1982; De Souza et al., 1986). In the cortex, degenerative changes in AD involve both the large glutamatergic pyramidal neurons of layers III and V and various populations of interneurons, some of which use somatostatin or corticotropin-releasing factor as a transmitter (De Souza et al., 1986). In the medial temporal lobe, abnormalities occur in neurons, particularly of entorhinal cortex and hippocampal areas CA1 and CA2 (Braak and Braak., 1991 ; West et al., 1994). Stereological studies of hippocampus in AD have shown that the most distinctive abnormality is an age-independent loss of neurons in the CA1 region (West et al., 1994). Degenerative changes are also observed in the amygdala, basal forebrain cholinergic system (Whitehouse et al., 1982), anterior nucleus of the thalamus, and locus coerulus and raphe complex.

The two hallmark pathological lesions of AD brain are senile plaques and neurofibrillary tangles (fig. 1 & 2). The senile plaques are composed of beta-pleated sheets of a 39-43 amino acid peptide that is derived from a larger precursor protein called APP (Glenner et al., 1984) whereas, the chief proteinaceous component of the neurofibrillary tangles is the microtubule associated protein tau in an abnormally hyperphosphorylated state (Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b;

Iqbal et al., 1989). Besides the formation of plaques and tangles, the loss of synapses is an important factor of Alzheimer's pathology. This has been illustrated in ultrastructural studies of the frontal cortex from biopsies as well as autopsies (De Kosky et al.,1990) and by immunocytochemical studies (Hamos et al., 1989 ; Lippa et al., 1992 ; Masliah et al., 1990 Masliah et al., 1990; Terry et al., 1991).

Etiology :

The etiology of the disease is not very well understood. The two main risk factors are (1) age and (2) genes.

Age- The most common cause of dementia occuring in mid-to-late life, AD affects 7% - 10% of individuals > 65 years of age and perhaps 40% of persons > 80 years of age (Evans et al., 1989). Thus, with the exception of some early onset cases, the majority of which are familial, AD is a disease of late life. The risk of developing AD doubles approximately every 5 years between the ages of 65 and 85 years.

Genes- Approximately 10% of cases of AD are familial and show autosomal dominant inheritance, and subsets of these cases are linked to mutations of specific genes including amyloid precursor protein (APP), presenilin 1 (PS-1) and presenilin-2 (PS-2). Only 1-3 % of the early onset and autosomal dominantly inherited AD is related to mutations in APP. A large majority of the early onset familial cases are linked to mutations in PS1 and PS2 genes. Another locus linked to FAD has been found on chromosome 12; however, the gene has not yet been unambiguously identified. The

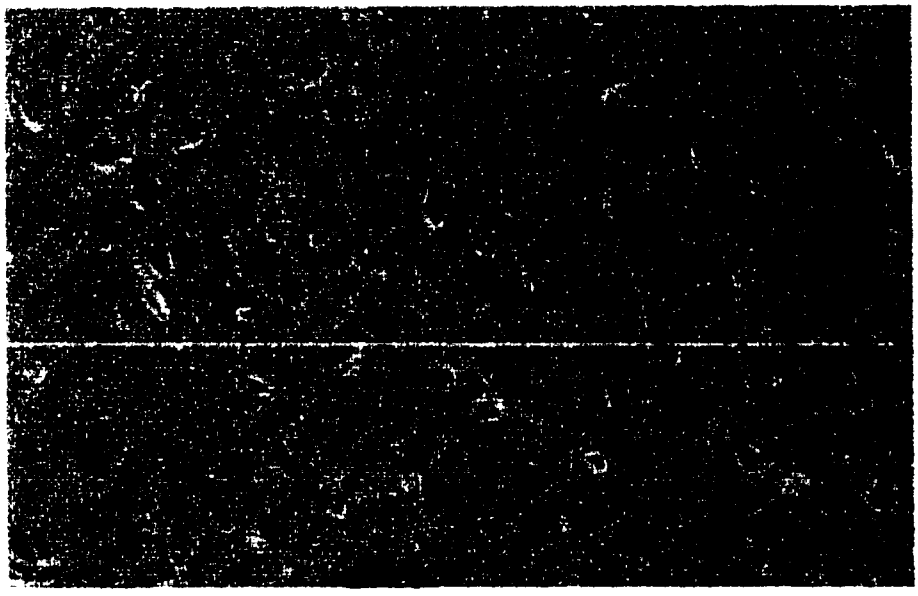


Fig. 1. Histological section of hippocampus from an AD patient, stained by Bielschowsky's silver technique, showing neurons with neurofibrillary tangles (NFT), neuropil threads (NT) and neuritic (senile) plaques (NP) (reproduced from Iqbal, K and Grundke-Iqbal, I (1999) Neurofibrillary degeneration In Mony J. de Leon, eds. *An Atlas of Alzheimer's Disease.* Parthenon publishing group ;75 - 88)

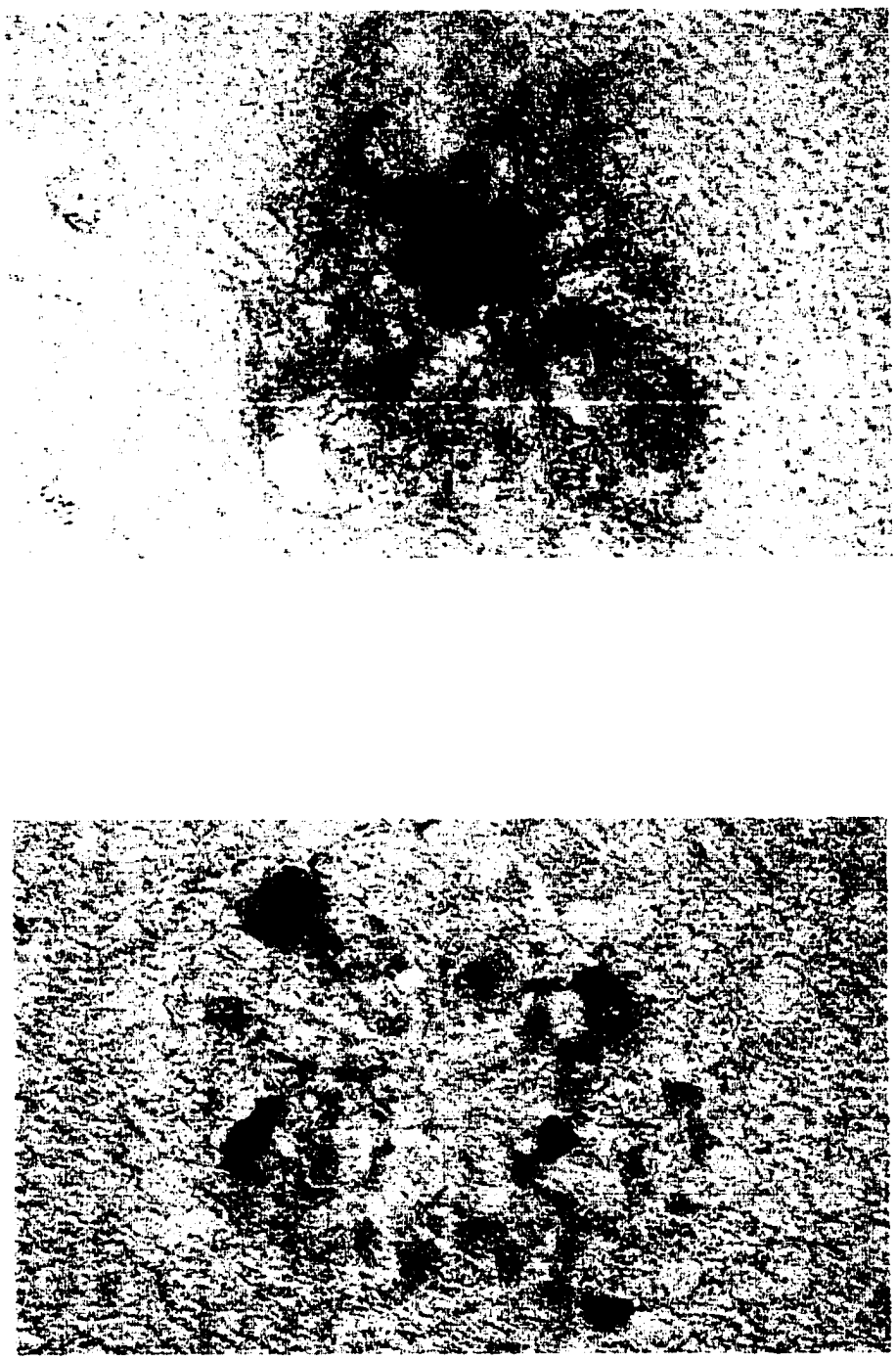


Fig. 2 .Neuritic (senile) plaque , immunostained with antibody to B-amyloid (top) and with antibody to hyperphosphorylated tau (bottom), showing the amyloid core and the degenerating neurites at the corona of the plaque. (reproduced from Iqbal, K and Grundke-Iqbal, I (1999) Neurofibrillary degeneration In Mony J. de Leon, eds. *An Atlas of Alzheimer's Disease.* Parthenon publishing group ;75 - 88)

risk of late-onset disease is associated with the presence of the apolipoprotein E4 allele (ApoE4).

APP, a type I integral membrane glycoprotein is encoded by a gene on chromosome 21. APP is expressed in brain cells, particularly neurons, and is rapidly transported anterogradely in axons (Koo et al., 1990; Sisodia et al., 1993). The functions of APP in neural tissues are not well understood. APP contains the Aβ region comprised of 28 amino acids of the ectodomain and 11-14 amino acids of the adjacent transmembrane domain of APP (Kang et al., 1987 ; Robakis et al., 1987 ; Selkoe et al., 1987 ; Probst et al., 1991). In a small fraction of cases, autosomal dominant mutations have been documented in the APP gene (Goate et al., 1991; Hendricks et al., 1992; Mullan et al., 1992). In some families, the valine residues at position 717 (four amino acids downstream of the C-terminus of $A\beta_{42}$ is substituted with either isoleucine, or glycine, or phenylalanine (Goate et al., 1991). Cells that express APP harboring 717 mutations secrete a higher fraction of longer Aβ peptides (i.e., extending to Aβ residue 42) relative to cells that express wild-type (wt) APP (Suzuki et al., 1994). In two large, related, early onset AD families from Sweden, Lys-Met codons immediately N-terminal to Asp 1 of Aβ are substituted with Asn-Leu (Mullan et al., 1992). Cells that express APP harboring the "Swedish" substitutions secrete higher levels of $A\beta_{40}$ as compared to cells expressing wt constructs (Citron et al., 1992; Cai et al., 1993).

PS-1 and PS-2, encoded by genes on chromosomes 14 and 1, respectively, are highly homologous proteins (Sherrington et al., 1995) predicted to contain eight transmembrane helices (Doan et al., 1996). The N- and C-terminal regions and a large loop domain are cytoplasmic; the loop is believed to interact with cytosolic constituents.

In cultured cells and in brain tissue (human, monkey, and mouse), PS-1 is endoproteolytically processed to form an N-terminal, ~28 kD fragment and a C-terminal 18 kD fragment which are thought to be functional units (Thinakaran et al., 1996). Although the biological functions of PS are not yet well understood, the finding of significant homology between PS and sel-12, a gene product that plays a role in the determination of cell fates during development in Caenorhabditis elegans, suggest that PS may be important in developmental processes(Baumeister et al., 1997)

The majority (~70%) of cases of early onset autosomal dominant FAD are linked to mutations in the PS-1 gene (St. George Hyslop et al., 1992; Sherrington et al., 1995). Approximately 50% of mutations occur within or immediately adjacent to the predicted loop domain (Sherrington et al., 1995). Mutations in the PS-2 gene cause autosomal dominant AD in Volga-German kindreds and in an Italian pedigree (Levy-Lahad et al., 1995a, b; Rogaev et al. 1995).

The mechanisms by which mutations in PS-1/-2 predispose individuals to AD are not clear, but recent studies indicate that mutaions in PS1 influence levels of $A\beta_{42}$. Plasma and conditioned media from fibroblasts obtained from carriers of PS-1 and PS-2 mutations show elevated levels of $A\beta_{42}$ compared to samples from unaffected family members. In the medium of cell lines expressing variable levels of either the A246E, M146L, or ΔE9 PS-1 variants, the ratio of $A\beta_{42}$: $A\beta_{40}$ is elevated compared to ratios in media of cells that express wt PS-1 (Borchelt et al., 1996; Borchelt et al., 1997). Similarly, the $A\beta_{42}$: $A\beta_{40}$ ratio is elevated in the brains of young transgenic (Tg) mice coexpressing a chimeric APP and an FAD-linked PS-1 variant compared with the brains of Tg mice expressing APP alone or Tg mice that coexpress wt human PS-1 and APP

(Borchelt et al., 1996). Thus, mutant PS-1 may cause AD by increasing the extracellular concentration of $A\beta_{42}$. In contrast, the consequence of PS1 null state, which is associated with developmental defects and late embryonic lethality (Wong et al., 1997) is a reduction of levels of Aβ (De Strooper et al., 1998; Naruse et al., 1998). PS1 has also been suggested to have a role in the stability of β-catenin, a member of the armadillo family of proteins. PS-1 can associate with members of the catenin family of signalling proteins (Yu G et al., 1998 ; Zhou et al., 1997). A study has shown that PS-1 forms a complex with β-catenin *in vivo* that increases β-catenin stability (Zhang et al., 1998) and pathogenic mutations in the presenilin-1 gene reduce the ability of PS-1 to stabilize β-catenin in the brains of transgenic mice. Moreover, β-catenin levels are reduced in the brains of Alzheimer's disease patients with PS-1 mutation. In a somewhat contradictory report, Kang et al (1999) reported that pathogenic mutations of PS-1 increase the stability of β-catenin. These differences have been attributed to different experimental approaches between the studies.

Located on chromosome 19, ApoE encodes three alleles of a ~34 kD glycoprotein: ApoE3 has a cysteine at position 112 and an arginine at position 158; ApoE4 has arginine at both positions; and ApoE2 has cysteine at both positions. The ApoE3 allele is most common in the general population (0.78 frequency); the allelic frequency of ApoE4 is 0.14. However, in clinic-based studies, patients with late-onset disease (>65 years of age) have an ApoE4 allelic frequency of 0.50 (Mayeux et al., 1993; Saunders et al., 1993). This impact of ApoE allele type is considerably less in community-based populations. In the brain, ApoE has been shown to be synthesized and secreted by glial cells, predominantly astrocytes, but not by neurons. However,

ApoE may be internalized by neurons in vivo. Several cell surface receptors for apoE are known to be expressed on one or many of the different cell types that constitute the brain parenchyma. These receptors are members of a single family and include the low-density lipoprotein (LDL) receptor, the very low-density lipoprotein (VLDL) receptor, the apo E2 receptor (apo-ER2), the LDL receptor-related protein (LRP) and the megalin / gp330 receptor. (Poirier et al., 1999). The mechanisms whereby the ApoE allele type influences age of onset is not well understood.

Other factors - Based on epidemiological studies, other potential risk and protective factors have been identified. Case control and, more recently, prospective studies have found increased risk in subjects with poor education, head trauma, and perhaps myocardial infarction, thus providing other clues to the pathogenesis and progression of disease. The identification of potential protective factors such as described above have encouraged new therapeutic approaches, including the value of hormone replacement, anti-inflammatory compounds, and anti-oxidant therapies.

Pathogenesis:

Many AD researchers believe that Aβ is central to the pathogenesis of AD, either directly or indirectly through a number of downstream events and is cause of the disease. This notion is part of the *amyloid cascade hypothesis* (Hardy J., 1996). Some of the evidence in support to this hypothesis are as follows: (a) In Down's Syndrome, amyloid deposition precedes neurofibirillary degeneration (Mann et al., 1989). (b) Studies *in vitro* using Aβ synthetic peptides have shown that toxicity is dependent on the presence of a fibrillar, predominantly β-sheet conformation (Kosik et al., 1992 ;

Lorenzo et al., 1994). (c) Transgenic mice expressing high levels of mutant human β-APP which cosegregate with the disease in humans show the development of diffuse and amyloid plaques (Games et al., 1995 ; Hsiao et al., 1996). Alternative hypothesis for the pathogenesis of AD suggest that the deposition of Aβ in the form of amyloid is only a marker of the disease process that is not directly linked to the causation of dementia. Evidence against the amyloid hypothesis are as follows: (a) In two mouse models, APP_{717} mouse (Games, et al., 1995) and the $APP_{670,671}$ mouse (Hsiao et al., 1996), despite extensive amyloid deposition, no significant neuronal loss and cognitive deficit have been detected (Irizarry et al., 1997a ; Irizarry et al., 1997b). One of the strongest point against amyloid cascade hypothesis is that some of the normal aged humans have as much amyloid in the form of plaques as in AD. These studies indicate that fibrillar deposits *per se* are not necessarily neurotoxic in vivo. Other factors are necessary for the development of toxicity *in vivo*.

To date, the exact relationship between the neurofibrillary degeneration and β-amyloidosis, the two hallmark lesions of AD, is not understood. The bulk of the data suggest that these two lesions can be formed independent of each other and that neither might be the direct cause of the formation of the other in AD. The frequency of NFTs directly correlates with the degree of dementia, whereas non-demented brains have been found to contain a high frequency of extracellular amyloid plaques, but few intracellular NFTs (Tomlinson et al., 1970; Alafuzoff et al., 1987;Arigada et al., 1992; Dickson et al., 1991). β-amyloidosis alone, in the absence of neurofibrillary degeneration does not produce the disease clinically. Thus, understanding the molecular mechanism of

neurofibrillary degeneration is critical to devising a rational therapeutic treatment of AD.

Neurofibrillary degeneration

Neurons with neurofibrillary tangles lack microtubules, and microtubule assembly from AD brain cytosol is not observed (Iqbal, et al., 1986). The neuronal cytoskeleton in AD is progressively disrupted and replaced by the appearance of bundles of paired helical filaments (PHF) as the neurofibrillary tangles. In addition to the neuronal perikaryon, the PHF also accumulates in the neuropil as neuropil threads and as dystrophic neurites surrounding wisps or a core of β-amyloid in the neuritic plaques. PHFs are comprised mainly of the microtubule associated protein (MAP) tau in an abnormally hyperphosphorylated state (Grundke-Iqbal et al.,1986a ; Grundke-Iqbal et al., 1986b ; Iqbal et al., 1989).

Tau promotes the assembly of tubulin monomers (α and β) into microtubules and maintains the structure of microtubules. Microtubules, in turn, are required for axonal transport. These functions of tau are regulated by its degree of phosphorylation. The normal brain tau, which is optimally active, has 2-3 moles of phosphate per mole of the protein. Tau in PHF and in AD brain, which is abnormally hyperphosphorylated, contains 5-9 moles of phosphate per mole of the protein (Kopke et al., 1993). Unlike normal tau, the AD abnormally hyperphosphorylated tau(AD P-tau) does not promote the *in vitro* assembly of microtubules, bind to microtubules or stabilize their structure (Alonso et al.,1994, 1996; Iqbal et al., 1994). The AD P-tau competes with tubulin in binding to normal tau and inhibits the assembly of microtubules. Also, unlike normal

tau, the abnormally hyperphosphorylated tau in AD brain is glycosylated and deglycosylation of AD neurofibrillary tangles by endoglycosidase F/N glycosidase F converts them into tangles of thin straight filaments (Wang et al., 1996) similar to those formed by the association of AD P-tau and normal brain tau (Alonso et al., 1996).

In addition to tau, the neuron contains high molecular weight-microtubule associated proteins (HMW-MAPs) MAP1 and MAP2, which also promote microtubule assembly and maintain the structure of microtubules. Like tau, MAP1 and MAP2 associate to AD P-tau and the sequestration of the HMW-MAPs from microtubules by AD P-tau results in the disassembly of microtubules(Alonso et al., 1997). Both, the disassembly of microtubules and the sequestration of tau, MAP1 and MAP2 by AD P-tau are inhibited by its dephosphorylation. Moreover, the affinity of binding between the AD P-tau and normal tau is higher than that between AD P-tau and the HMW-MAPs. Furthermore, unlike the association between the AD P-tau and normal tau, the binding of AD P-tau to MAP1 or MAP2 does not result in the formation of tangles or individual long filaments. This may be the basis of the degeneration of many neurites without any accumulation of PHF in AD brain. HMW-MAPs have not been observed in isolated PHFs.

Role of Protein kinases in the hyperphosphorylation of tau

Employing phosphorylation dependent antibodies and mass spectrometry, twenty-one phosphorylation sites in the AD P-tau have been identified (Morishima-Kawashima et al., 1995 ; Iqbal et al., 1995). Ten of the 21 sites are canonical sites for proline directed protein kinases (PDPKs) and the rest are the non-PDPK sites (fig.3). The state of

phosphorylation of a protein is the function of the activities of protein kinases, as well as the protein phosphatases that regulate its phosphorylation. The hyperphosphorylation of tau in AD might be the result of either higher activities of protein kinases or lower activities of protein phosphatases, or both. Tau can be phosphorylated by several PDPKs and non-PDPKs *in vitro* (Ishiguro et al., 1992; Baudier, et al., 1987 ; Roder et al., 1991 ; Drewes, et al., 1992 ; Ledesma et al., 1992 ; Litersky et al., 1992 ; Singh et al., 1994). However, in AD, the exact role of any of these kinases in the abnormal hyperphosphorylation of tau is not yet known. A recent report (Patrick et al., 2000) suggested an increase in cdk5 activity in AD brain mediated by the conversion of a rather unstable regulatory subunit p35 to a relatively more stable p25, which accumulates and activates cdk5 in AD brain. It may be possible that there are other kinases as well, activities of which are upregulated in AD brain, although that has yet to be demonstrated.

Role of Protein Phosphatases in the hyperphosphorylation of tau

A large number of phosphoprotein phosphatases have been described in mammalian tissues(Cohen, P., 1989). These enzymes can be broadly divided into two types, i.e. Phosphoseryl/phosphothreonyl-protein phosphatases (PSPs) and phosphotyrosyl protein phosphatases (PTPs). The PSPs have been further classified into four subtypes i.e. protein phosphatase (PP)-1, PP-2A, PP-2B and PP-2C. These phosphatase activities differ in substrate specificity, dependence on divalent cations, and sensitivities to specific inhibitors (Cohen,., 1989 ; Ingebritsen et al., 1983)

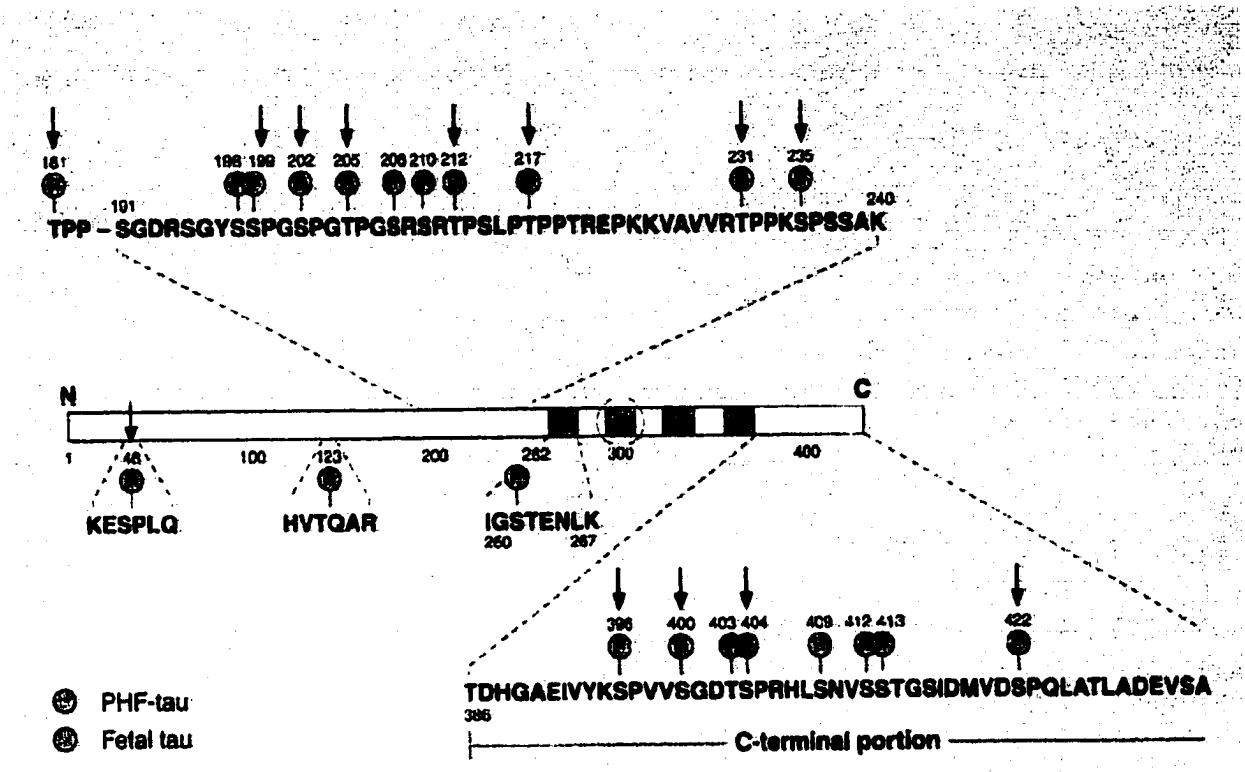


Fig.3. Phosphorylation sites of PHF-tau and fetal tau. Ten of the 21 sites in PHF-tau are canonical sites for the proline-directed protein kinases (PDPKs) ; the remaining 11 sites are non- PDPK sites. Microtubule binding repeats are indicated by the squares.

(**reproduced from** Iqbal, K and Grundke-Iqbal, I (1999) Neurofibrillary degeneration In Mony J. de Leon, eds. *An Atlas of Alzheimer's Disease.* Parthenon publishing group ;75 - 88)

To date, only phosphorylation of serines and threonines have been observed in normal tau and AD abnormally hyperphosphorylated tau (AD P-tau). Thus, only PSPs are expected to dephosphorylate tau. Immunocytochemical studies have revealed that these protein phosphatases are present both in granular and pyramidal neurons, including the tangle-bearing neurons (Pei et al., 1994). Employing ^{32}P-labeled (with protein kinase A)

phosphorylase kinase as a substrate and specific inhibitors, it has been shown (1) that the activities of PP-1, PP-2A, PP-2B and PP-2C can be determined in autopsied (2-7 hrs.) and frozen human brains, and (2) that the activities of PP-1 and PP-2A are decreased in AD neocortex (Gong, et al., 1993). Furthermore, the studies on dephosphorylation of the AD abnormally hyperphosphorylated tau (AD P-tau) have revealed that (1) PP-2A and PP-2B and, to a lesser extent PP-1, are involved in the dephosphorylation of tau and (2) that the phosphatase activity towards dephosphorylation of ser198 / ser199 / ser202, major abnormal phosphorylation sites in the abnormal tau is decreased by about 30% in the brain of patients with AD (Gong et al., 1995). These findings suggest that a decrease of tau phosphatase activity might be a major cause of the abnormal hyperphosphoryltion of tau in AD. The abnormal hyperphosphorylation of tau and changes in enzyme activities that regulate the intracellular protein phosphorylation / dephosphorylation are consistent with the hypothesis that there is a general imbalance in the protein phosphorylation-dephosphorylation system in AD brain. If the hypothesis were to be true, then it is conceivable that there are other non-tau phosphoproteins in AD brain that could also be abnormally hyperphoshporylated. ***To test our hypothesis***, we defined the goal of this thesis to identify and characterize non-tau phosphoproteins that are abnormally hyperphosphorylated in AD brain.

At the time, this work was started, there were only a few reports on abnormal hyperphosphorylation of non-tau proteins. Based on immuno-cytochemical studies neurofilaments in the affected neurons of AD brain were reported to be abnormally hyperphosphorylated (Sternberger et al., 1985 ; Morrison et al., 1987 ; Lee, et al., 1988 ; Monte, et al., 1994 ; Vickers et al., 1994 and Shetty et al., 1995). However, the

neurofilament antibodies employed in these studies cross-reacted with hyperphosphorylated tau (Kriezak-Reding et al., 1987 ; Nukina et al., 1987).

Results:

In order to test our hypothesis that there is an imbalance in the protein phosphorylation-dephosphorylation system in AD brain and phosphoproteins other than tau could also be abnormally hyperphosphorylated, we

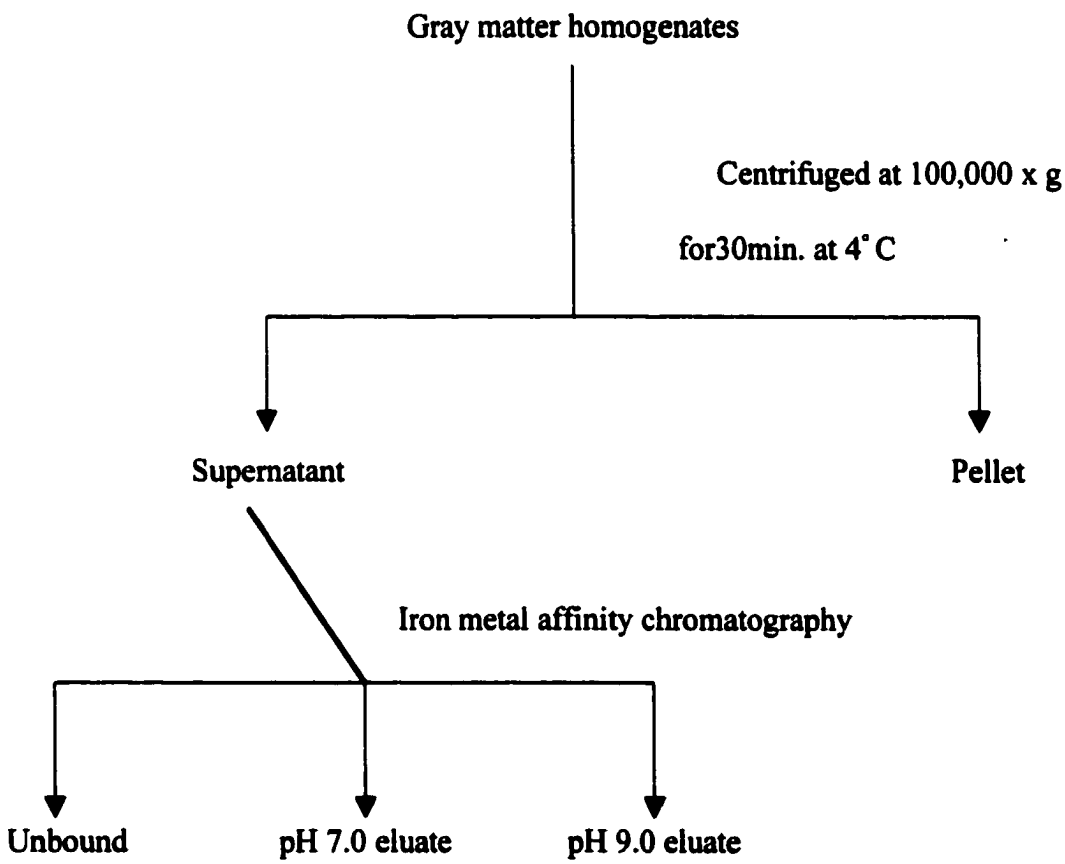
(1) assayed phosphate bound to proteins in AD v/s age-matched controls and

(2) studied the phosphorylation of polypeptides from AD and control brains by immuno-labeling with antibodies to phosphoserine and phosphothreonine on Western blots.

Fractionation of proteins by Iron Metal Affinity Chromatography

The cerebral gray matters from five AD and five age-matched controls were dissected on ice and homogenized in 100 mM MES, pH 5.7, 25 mM NaF, 1 mM Na_3VO_4 and 1 mM DTT, using 20 strokes at 2500 r.p.m. on a Potter-Elvehjem homogenizer. The resultant homogenate was centrifuged at 100,000 x g for 30 min. at 4°C. The pellets were stored at -80°C. The supernatants were further fractionated by phosphoprotein specific chromatography, Immobilized Metal Affinity Chromatography (IMAC) as described in the Materials and Methods section. Upon equilibration of the column with MES (pH 5.7), 25 mM NaF, 1 mM Na_3VO_4 and 1 mM DTT, 1 mg protein was loaded

onto the column. Bound proteins were eluted, after collecting the flowthrough, by step elution with 0.1 M MOPS (pH 7.0), 25 mM NaF, 1 mM Na_3VO_4 and 1 mM DTT, followed by 0.1 M Tris (pH 9.0), 25 mM NaF, 1 mM Na_3VO_4 and 1 mM DTT. Typically, three fractions were obtained viz. unbound, pH 7.0 eluate and pH 9.0 eluate.



(1) Estimate phosphate bound to proteins

(2) Western blot analyses with antibodies to P-Ser and P-Thr residues

Phosphoprotein phosphate levels

The 100, 000 x g pellet, supernatant and the IMAC fractions - unbound, pH 7.0 and pH 9.0 of 5 AD and 5 age-matched control cases were assayed for protein-bound phosphate as described in the Materials and Methods.

The data revealed no statistically significant differences between AD and control cases in the supernatant, unbound, pH 7.0 and pH 9.0 fractions. However, the pellet from AD revealed an increase by ~11 pmoles phosphate per microgram protein compared with age-matched controls (Table 1, Fig.4 and 5)

Table 1. picomoles of phosphate per microgram of protein in the supernatant, the three IMAC fractions and the 100,000 x g pellet of AD and control gray matter homogenates.

	AD	Control
Supernatant	10.01 + 0.84	10.35 + 1.92
Unbound	9.87 + 5.01	10.29 + 4.4
pH 7.0 eluate	6.33 + 1.4	6.70 + 1.4
pH 9.0 eluate	19.83 + 6.8	22.51 + 5.84
Pellet	75.53 + 4.7	63.90 + 5.41

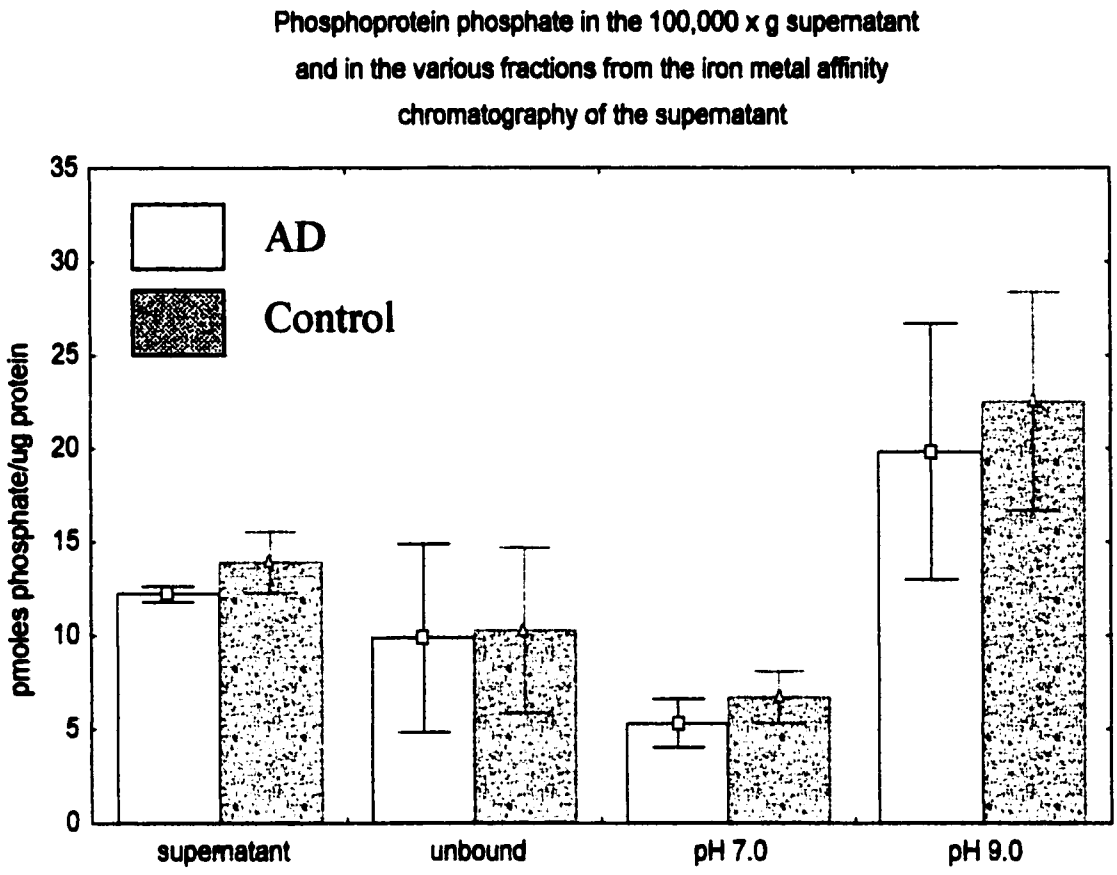


Fig. 4. Quantitation of phosphoprotein phosphate in the 100,000 x g supernatant and iron metal affinity chromatography fractions of AD and control brains. Frontal gray matter was homogenised in 0.1 M MES buffer, pH 5.7, 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4 and 1 mM PMSF and centrifuged at 100,000 x g for 30 min. at 4°C. The 100,000 x g supernatant was subjected to iron metal affinity chromatography. The unbound fraction and the eluates (pH 7.0 and pH 9.0) were collected. The data revealed no significant difference in the phosphoprotein phosphate content between the AD and control cases in the 100,000 x g supernatant and the iron metal affinity chromatography fractions (Values are expressed as mean ± S.D).

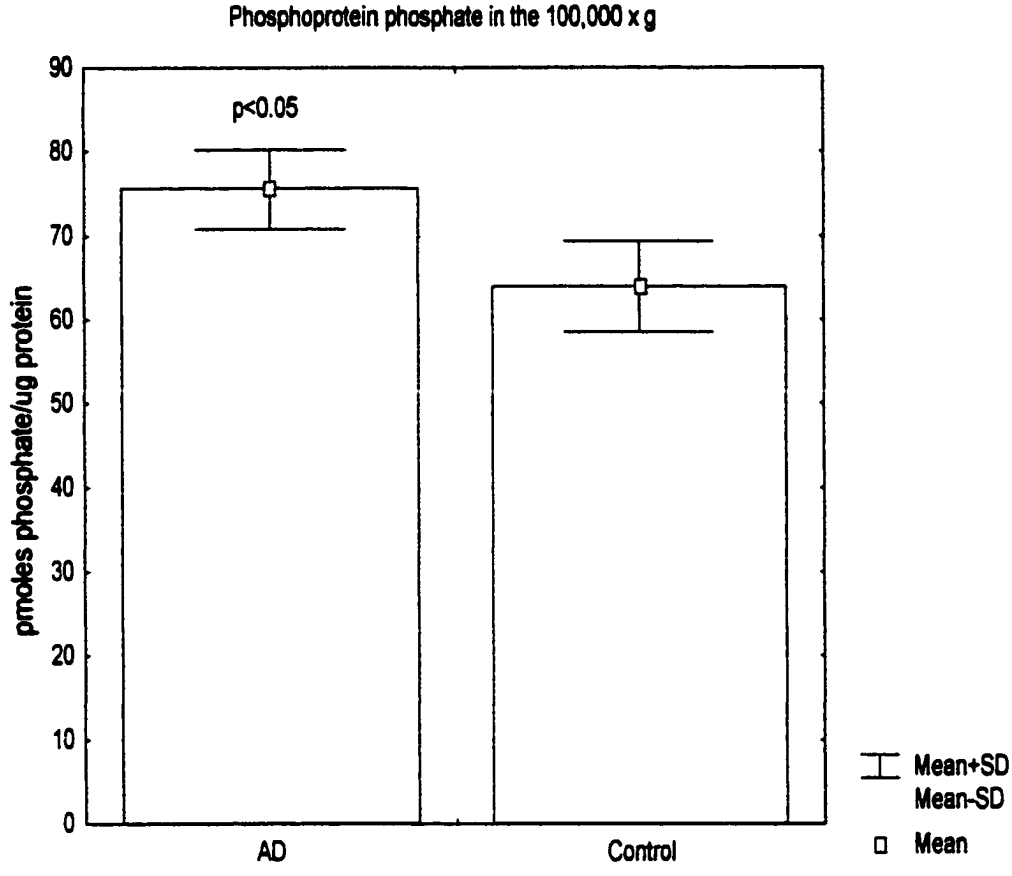


Fig. 5. Quantitation of phosphoprotein phosphate in the 100,000 x g pellet of AD and control brains. Frontal gray matter was homogenised in 0.1 M MES buffer, pH 5.7, 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4 and 1 mM PMSF and centrifuged at 100,000 x g for 30 min. at 4°C. The 100,000 x g pellet showed an increase of ~11 pmoles phosphate per microgram protein in AD over age-matched controls. This data suggests that there are protein(s) other than tau that are also abnormally hyperphosphorylated in AD brain (Values are expressed as mean ± S.D)

Hyperphosphorylation of non-tau phosphoproteins based on phosphate analyses:

A 11pmoles / μg increase in phosphate levels in AD observed is highly significant because of the following reason. AD P-tau has 5-9 moles phosphate per mole of tau. One mole of tau is approximately 50,000 gm. Therefore, taking 9 moles of phosphate per mole of AD P-tau, there would be 9 pmoles of phosphate per 50 ng tau. In AD brain, tau levels are approximately 5 -13 ng per microgram protein (~1.6 ng / μg protein x 3 - 8 fold increase as compared with normal aged brain). Therefore, AD P-tau contributes to approximately 0.9 - 2.3 pmoles phosphate / 5-13 nanogram tau / microgram protein. In other words, abnormally hyperphosphorylated tau contributes to less than three picomole phosphate per microgram protein in AD brain. The 100, 000 x g pellet, which is ~80% of all tissue proteins has almost all of the AD P-tau. By these calculations, if the protein bound phosphate is higher than 3 pmoles phosphate per microgram protein in AD brain, it can be said that the additional pmoles phosphate per microgram protein (~8 pmoles phosphate per microgram protein) must be contributed by other abnormally hyperphosphorylated proteins.

Phosphoserine hyperphosphorylated proteins

Having found an increase in phosphate bound to proteins in the 100,000 x g pellet of AD brain, we investigated the protein(s) that are hyperphosphorylated. We performed Western blots using antibodies to phosphorylated amino acids, first phosphoserine and then phosphothreonine antibodies. The 100,000 x g pellet protein from AD and age-matched controls were electrophoresed on a 5-15% gradient SDS-PAGE. After

transferring onto an Immobilon-P (PVDF) membrane and blocking with 5% BSA-TBST (bovine serum albumin-tris buffer saline Tween-20), the membrane was incubated with polyclonal anti-phosphoserine antibodies. The blots were then developed with anti-rabbit secondary antibody linked to horse-radish peroxidase and ECL reagents.The immunoreactive bands were quantitated by densitometric scanning. At least four proteins were found to be hyperphosphorylated at serine in AD compared with age-matched controls (fig. 6, 6A & 6B). Three of the four proteins co-migrated with tau as judged by parallel Western blotting with anti-tau antibody (figure not shown). However, a ~54 kD protein did not co-migrate with tau and was further investigated.

P-Ser immunoreactivity in the 100,000 x g pellet

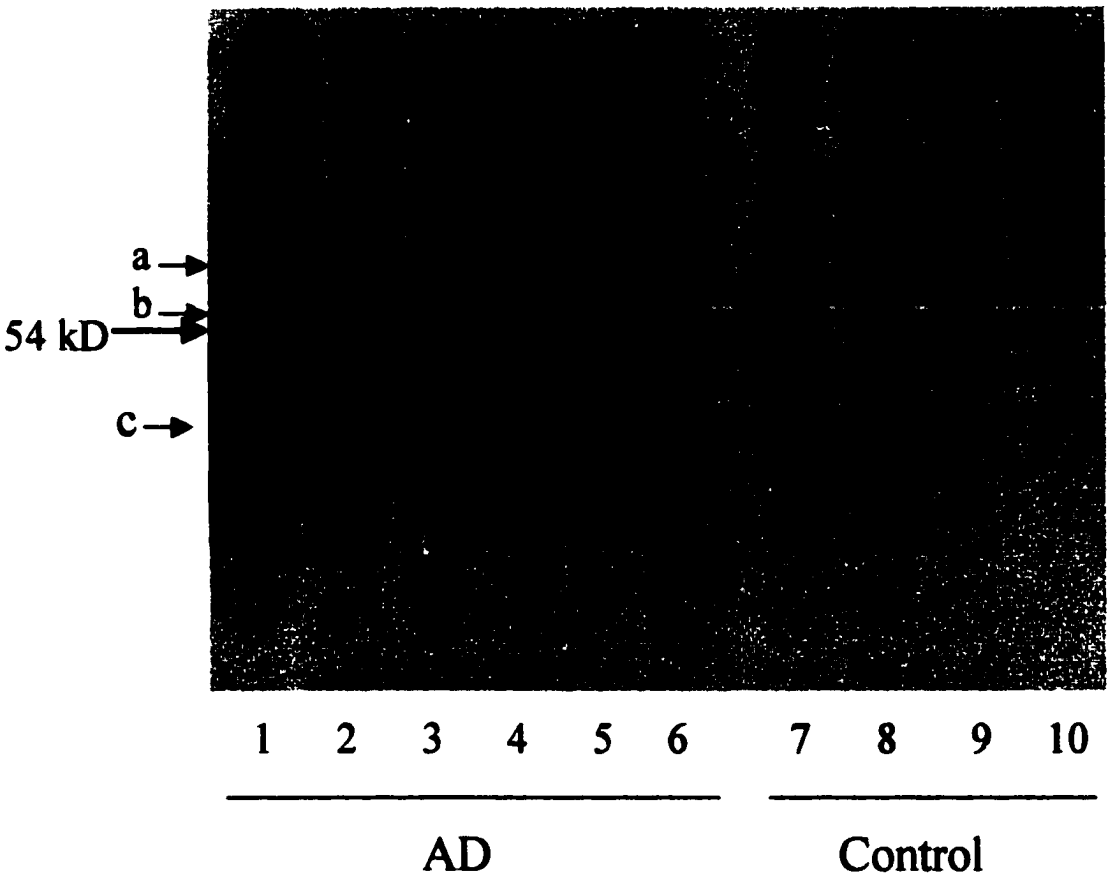


Fig.6. Western blot of the 100,000 x g pellet of homogenates of frontal gray matter from AD and age-matched control cases developed with antibody to phosphoserine. The 100,000 x g pellet of brain homogenates from AD and age-matched control cases was electrophoresed (30 ug/ lane) on a 5 - 15% SDS-PAGE and the protein transferred to a PVDF membrane. After blocking with 5 % BSA-TBST, the phosphorylated serine residues of proteins were immuno-detected using polyclonal P-ser antibodies (1:750) and HRP-conjugated secondary antibody (1:25000). The blot was developed by ECL reagents. Lanes 1 to 6 - AD; Lanes 7 to 10 - age-matched controls. The intensity of immunoreactivity was quantitated by densitometry. Arrows : a - 68 kD; b - 60 kD; c - 40 kD. P-ser immunoreactivity at ~54 kD was increased by 36% in AD compared with age-matched controls ($p < 0.05$).

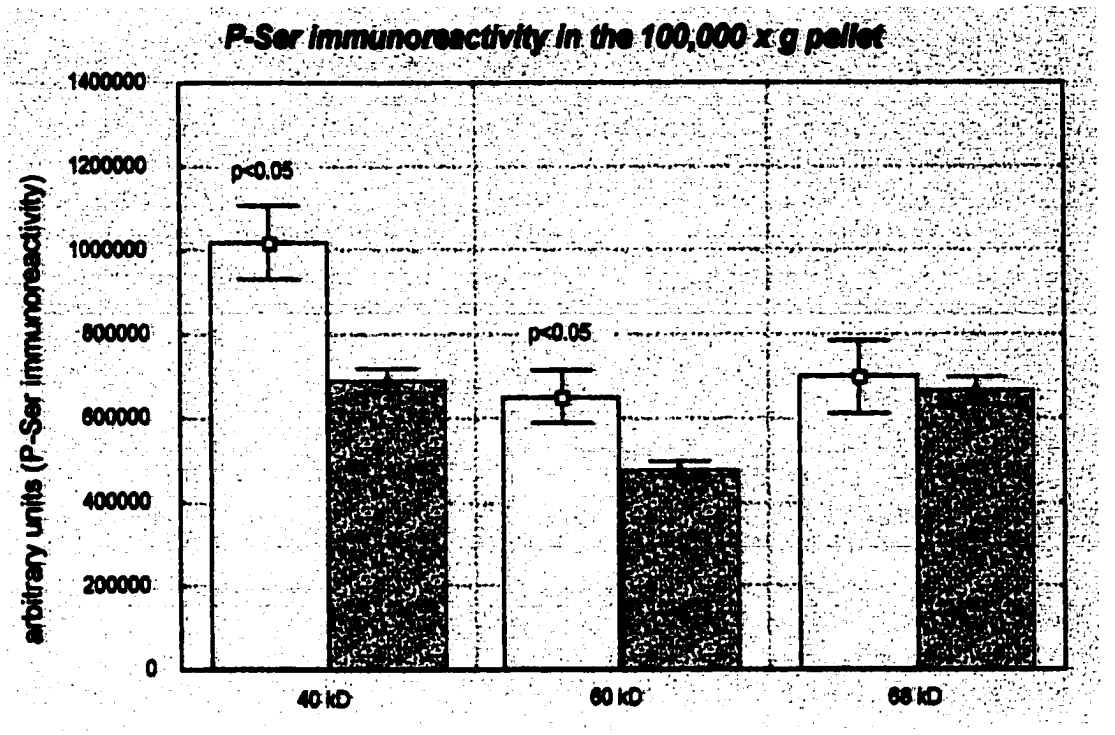


Fig. 6A. Quantitation of the P-Ser immunoreactivity in the 100,000 x g pellet of AD and age-matched contol cases. P-ser immunoreactivity was found to be significantly higher in ~40 kD and ~60 kD proteins ($p < 0.05$) in AD compared with age-matched controls. A ~68 kD protein was also hyperphosphorylated although the differences between AD and age-matched control cases were not statistically significant. ***Open rectangle*** - AD ; ***Closed rectangle*** - control (n= 7 for AD and n = 5 for age-matched control cases ; values are expressed as Mean ± S.E.M).

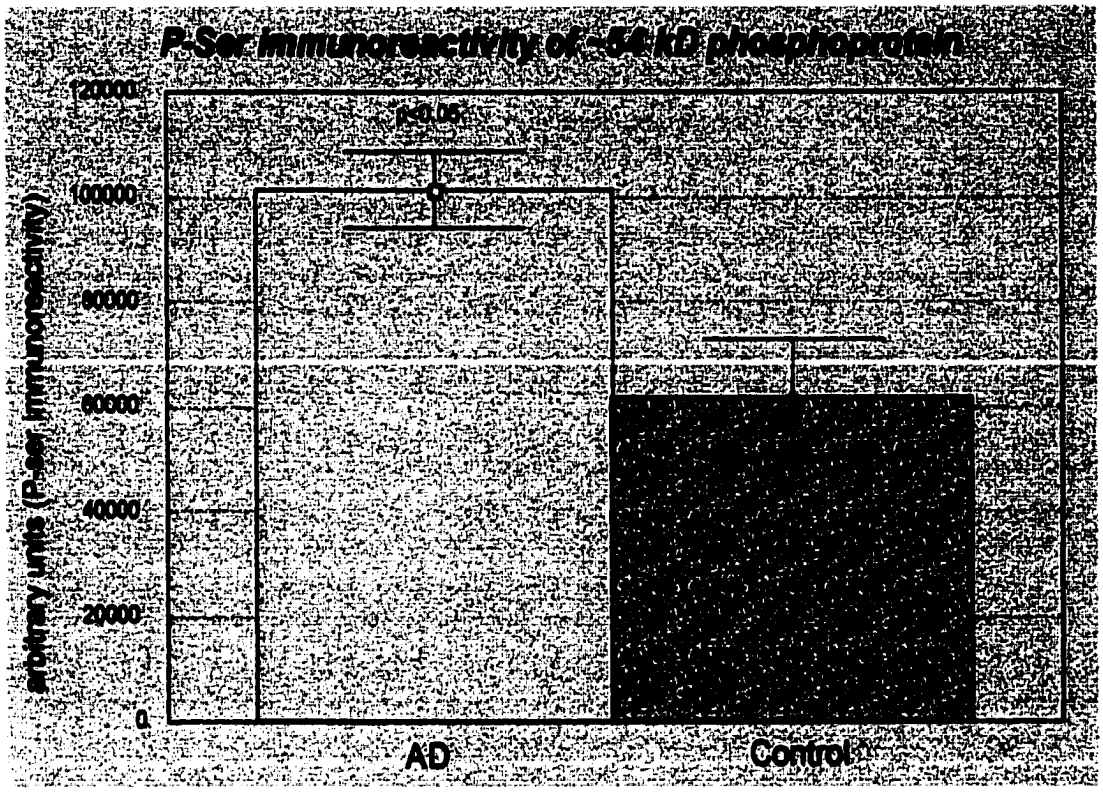


Fig.6B. Quantitation of the P-Ser immunoreactivity of a ~54 kD protein in the 100,000 x g pellet of AD and age-matched control cases. P-Ser immunoreactivity of ~54 kD protein was 36% more in AD than age-matched control cases ($p<0.05$). The differences were statistically significant (n = 7 for AD & n = 5 for controls. Values are expressed as Mean ± S.E.M)

Solubilisation of the particulate proteins:

Since the ~54 kD protein of interest was in the 100,000 x g particulate fraction, it was necessary to solubilise the protein in order to be able to purify using conventional chromatographic techniques. So, we decided to determine the solubility of the 100,000 x g pellet using a panel of detergents/denaturants as listed below. The percentage solubility of proteins is listed in Table. 2

Table.2. Solubility of 100,000 x g proteins in various detergents incubated for 3 hours at room temperature with intermittent mixing.

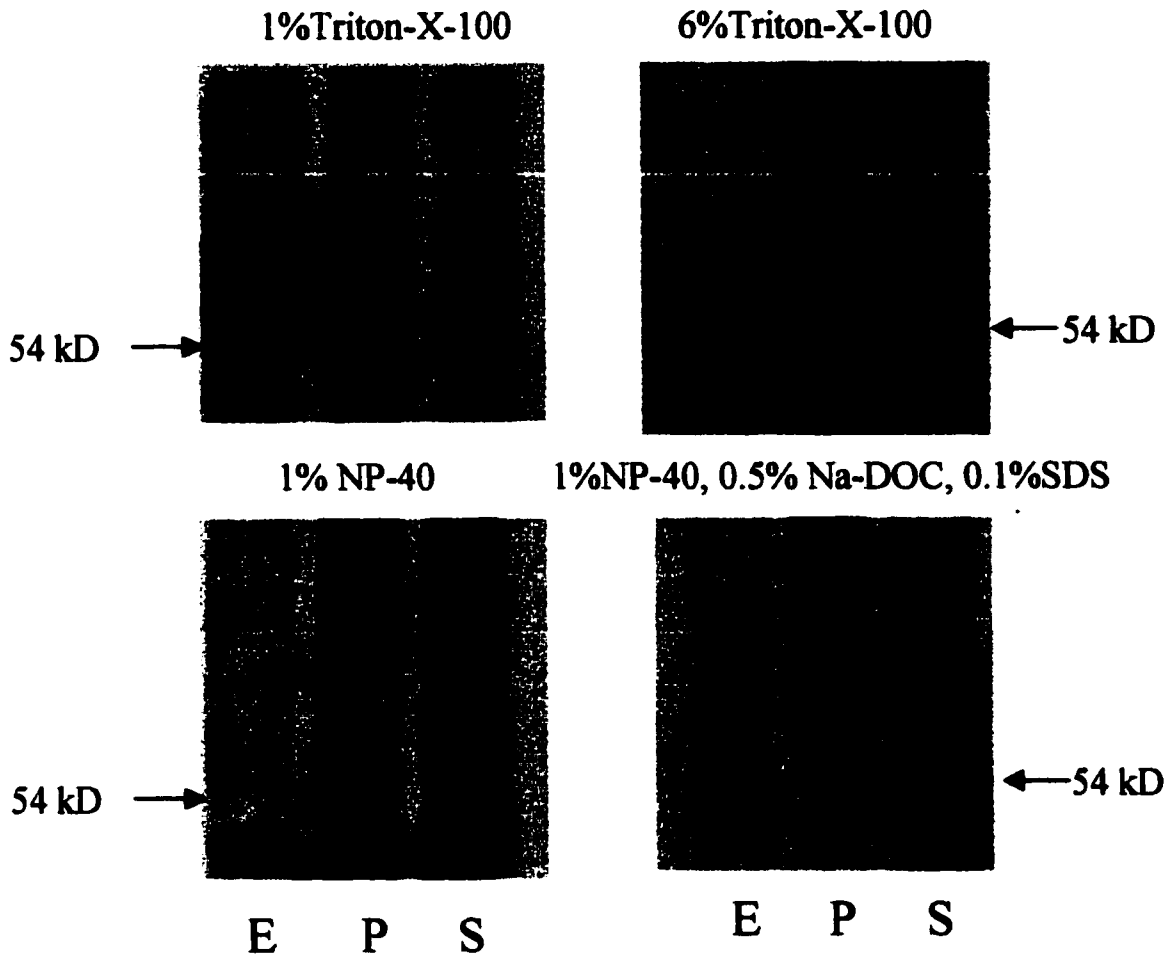
Detergents	% solubility
(1) 1% Triton-X-100	44
(2) 6% Triton-X-100	21
(3) 1% NP-40	44
(4) 1% NP-40 + 0.5% sodium deoxycholate + 0.1% SDS	60
(5) 8 M urea	25
(6) 6 M GnHCl	64
(7) B-PER reagent	09

Note : B-PER reagent (PIERCE, IL, U.S.A.) is a commercially available detergent used to solubilise bacterial cell wall and plasma membrane)

B-PER reagent (PIERCE) was the least effective in solubilising the 100,000 x g pellet proteins. Whereas, 6 M GnHCl and mixture of detergents (1%NP-40 + 0.5% sodium deoxycholate + 0.1% SDS) were the most effective in solubilising proteins in the 100,000 x g pellet (Table.2). Our studies revealed that the ~54 kD protein was soluble only in 6 M GnHCl (fig. 7 & 8).

Fig. 7. Solubility of ~54 kD protein in a variety of detergents. ~ 6 milligram of 100,000 x g pellet protein was incubated individually with 3 ml of (a) 1 % Triton-X-100, (b) 6 % Triton-X-100, (c) 1 % NP-40 and (d) 1 % NP-40, 0.5% sodium deoxycholate and 0.1% SDS. For 3 hrs. at room temperature with intermittent mixing before centrifugation at 100,000 x g for 30 min. at 4°C. The supernatant (S) was considered the detergent soluble pool of proteins and the pellet (P) , the insoluble pool of proteins. The protein in the detergent before centrifugation was called extract (E). All the different pools of protein were then analysed for the presence of ~54 kD protein by Western analyses with P-Ser antibodies (1:750). The ~54 kD protein remained insoluble in all the cases.

Solubilization of 100,000 x g pellet with various detergents



Western blot - P-ser

Figure. 7

Solubilization of 100,000 x g pellet with 6 M GnHCl

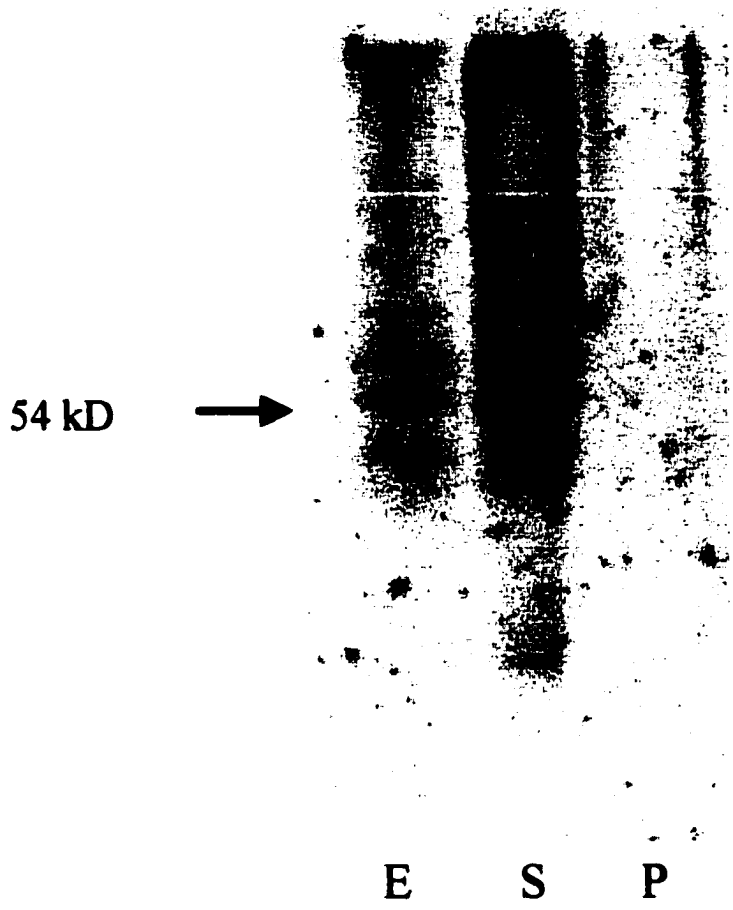


Fig.8. Solubility of ~54 kD protein in 6 M GnHCl. . ~ 6 milligram of 100,000 x g pellet protein was incubated with 3 ml of 6 M GnHCl for 3 hrs. at room temperature with intermittent mixing before centrifugation at 100,000 x g for 30 min. at 4°C. The supernatant (S) was considered the guanidine soluble pool of proteins and the pellet (P), the insoluble pool of proteins. The protein in guanidine before centrifugation was called extract (E). The ~54 kD protein was completely soluble in 6 M GnHCl as judged by Western blotting with P-Ser (1:750) antibodies.

Purification of ~54 kD protein by preparative SDS-PAGE:

We decided to take advantage of the fact that the ~54 kD protein was insoluble in the mixture of detergents and soluble in 6 M GnHCl. The 100,000 x g pellet was first extracted with mixture of detergents (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) by incubating for 3 hrs. at RT with intermittent mixing. After centrifugation at 100,000 x g for 30 min. at 4°C, the mixture of detergents insoluble fraction was incubated in 6 M GnHCl for 3 hrs. at RT with intermittent mixing, followed by centrifugation at 100,000 x g for 30 min. at 4°C. The 6 M GnHCl soluble fraction was dialysed against 2 M Urea containing 50 mM Tris, pH 7.6, 25 mM NaF, 1 mM Na3VO4, 1 mM DTT. After quantitating protein using modified Lowry method, ~1 mg protein was loaded on a 10% preparative SDS-PAGE (Fig.9 & Fig. 10) and 288 fractions were collected at an elution rate of 60 ml/hr. Fractions containing the ~54 kD protein were detected by monitoring all fractions by both Coomassie stain as well as Western blot analyses with phosphoserine (p-ser) antibodies.

The fractions from preparative SDS-PAGE containing the ~54 kD protein were located by slab gel SDS-PAGE and Coomassie Blue staining of each fraction, followed by Western blots of the fractions in the 54 kD range developed with phosphoserine antibody (Figs. 10 & 11). Preparative electrophoresis enabled us to purify the ~54 kD protein as a single protein band (Fig. 11).

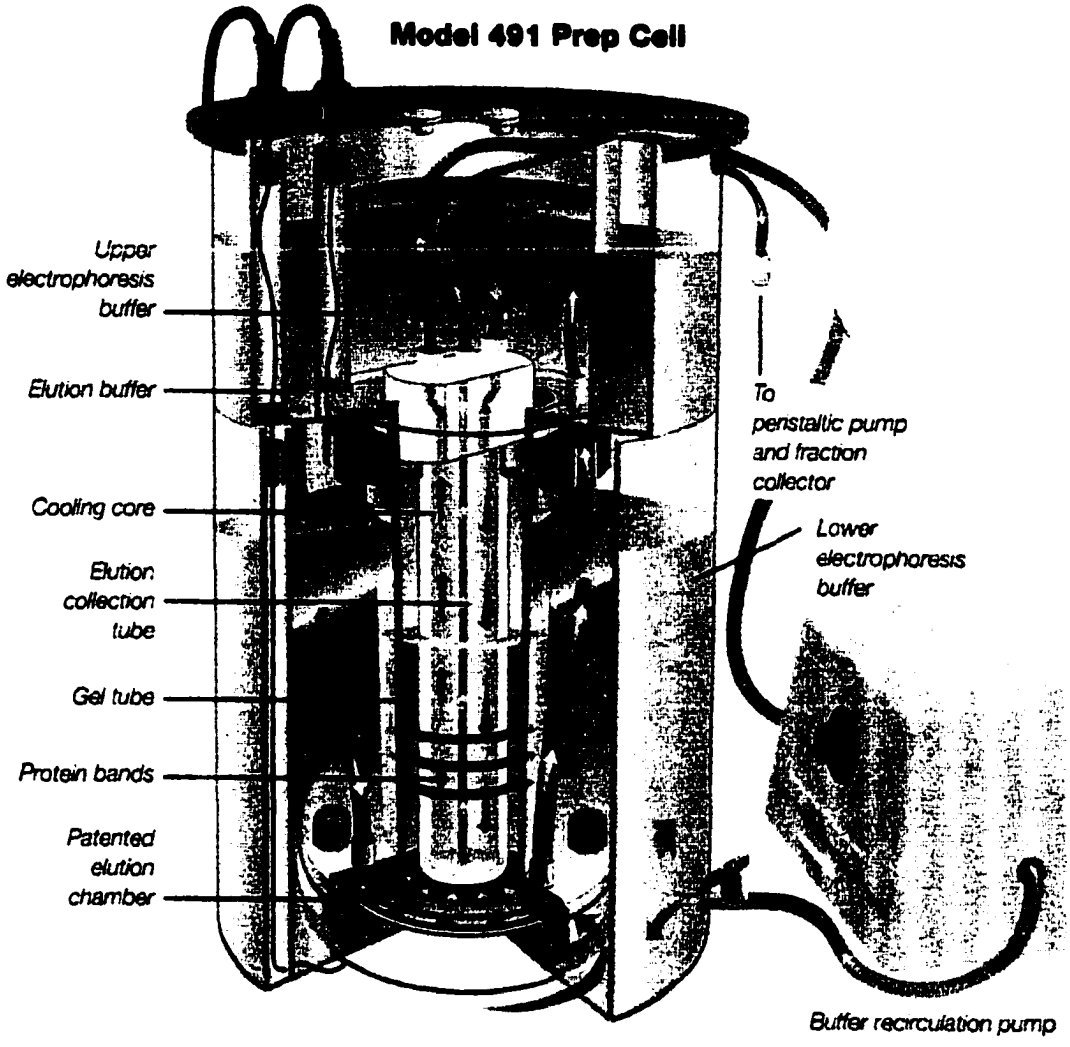


Fig. 9. Cartoon of the Preparative electrophoresis apparatus (reproduced from BIO-RAD catalogue 2000/2001 pp. 164).

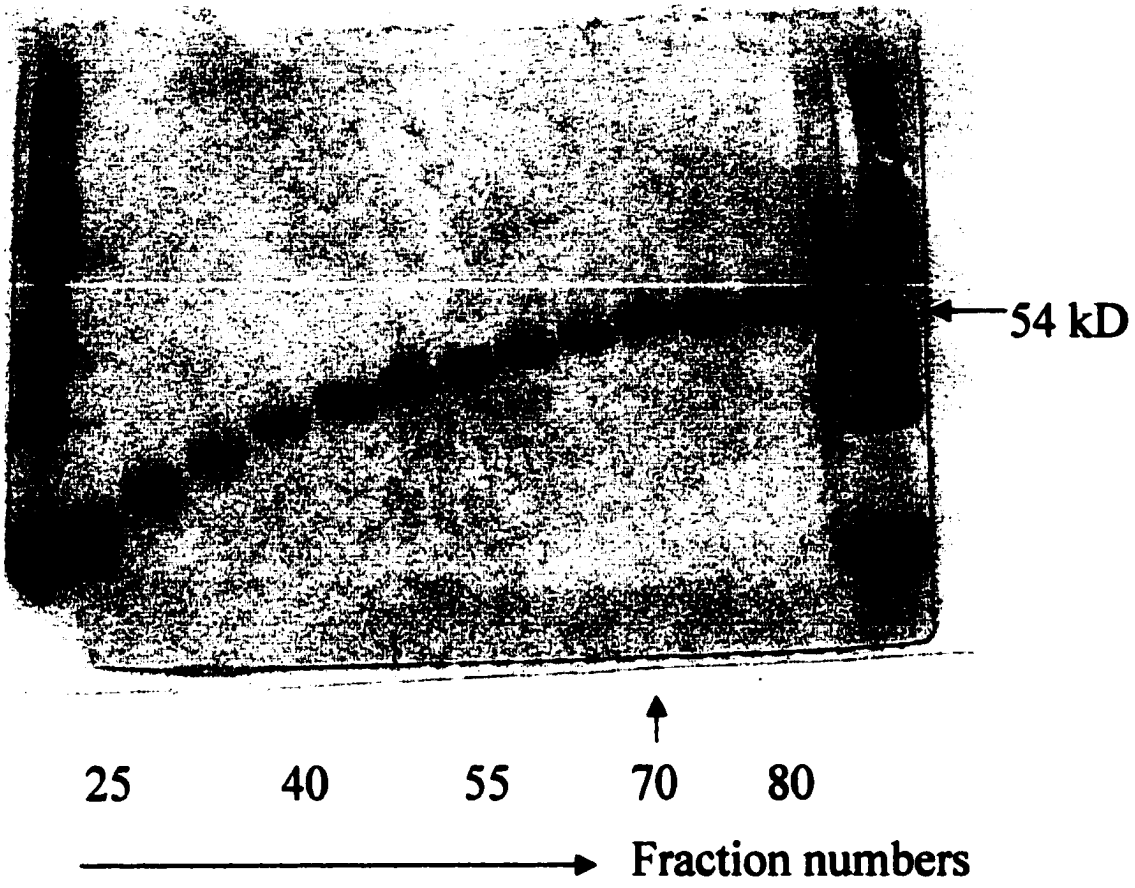


Fig.10. Representative profile of the protein fractions obtained using preparative electrophoresis. The fractions were resolved on a 10% SDS-PAGE followed by staining with Coomassie Blue stain. Fraction#70 contained the ~54 kD protein.

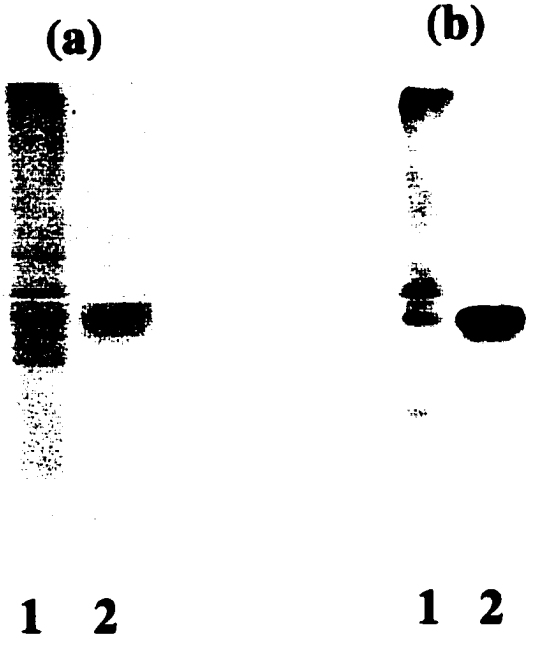


Fig.11. Purification of the ~54 kD protein by 10% Preparative SDS-PAGE. The 100,000 x g pellet from an AD brain was subjected to detergent (1% NP-40, 0.5 % sodium deoxycholate and 0.1 % SDS) extraction. The ~54 kD protein remained exclusively insoluble. The detergent insoluble pellet proteins were subjected to 6 M guanidine hydrochloride (GnHCl) solubilisation, wherein the ~54 kD protein was almost exclusively solubilised. After dialysing overnight against 50 mM Tris, 2 M Urea, 1 mM PMSF, 1 mM Na_3VO_4, and 25 mM NaF, 1 mg. of dialysed protein was subjected to preparative electrophoresis. Protein fractions were collected and ~54 kD protein fraction was identified by both Coomassie staining of the fraction on a 10 % SDS-PAGE and Western blots with P-Ser antibodies. **(a)** Coomassie staining of proteins on a 10 % SDS-PAGE. Lane 1 - GnHCl soluble protein before subjecting to preparative electrophoresis, Lane 2 - a purified single band of ~54 kD protein. **(b)** Western blot with P-Ser. Lane 1 - GnHCl soluble protein before subjecting to preparative electrophoresis, Lane 2 - a purified single band of ~54 kD protein.

Mass Spectrometry of the purified protein:

The Coomassie Blue-stained protein was excised from the 10% SDS-PAGE, washed 25 times with distilled water in an Eppendorf tube and dried using a vacuum concentrator before sending it to Beckman Facility at Stanford University for Mass spectrometric (MS-MALDI) analyses. After digesting the ~54 kD purified protein with a protease - LysC, the resulting peptide mixture was bombarded with laser on a matrix, in a Mass spectrometry chamber. Twenty three mass/charge peptides were obtained (fig.12). When these peptides were matched with the data bank using a program - ***Prospector***, there were 15 matches at the stringency shown in Table.3. All of them matched with β-tubulin. Since the sample was from human brain, the relevant match was that with accession number ***X79535***. There are at least 15 different isotypes of β-tubulin. This particular one has not been typed and is only referred to as β-tubulin. The mass spectrometric analysis was repeated with three different AD and age-matched controls, all leading to the identification of the ~54 kD protein as β-tubulin.

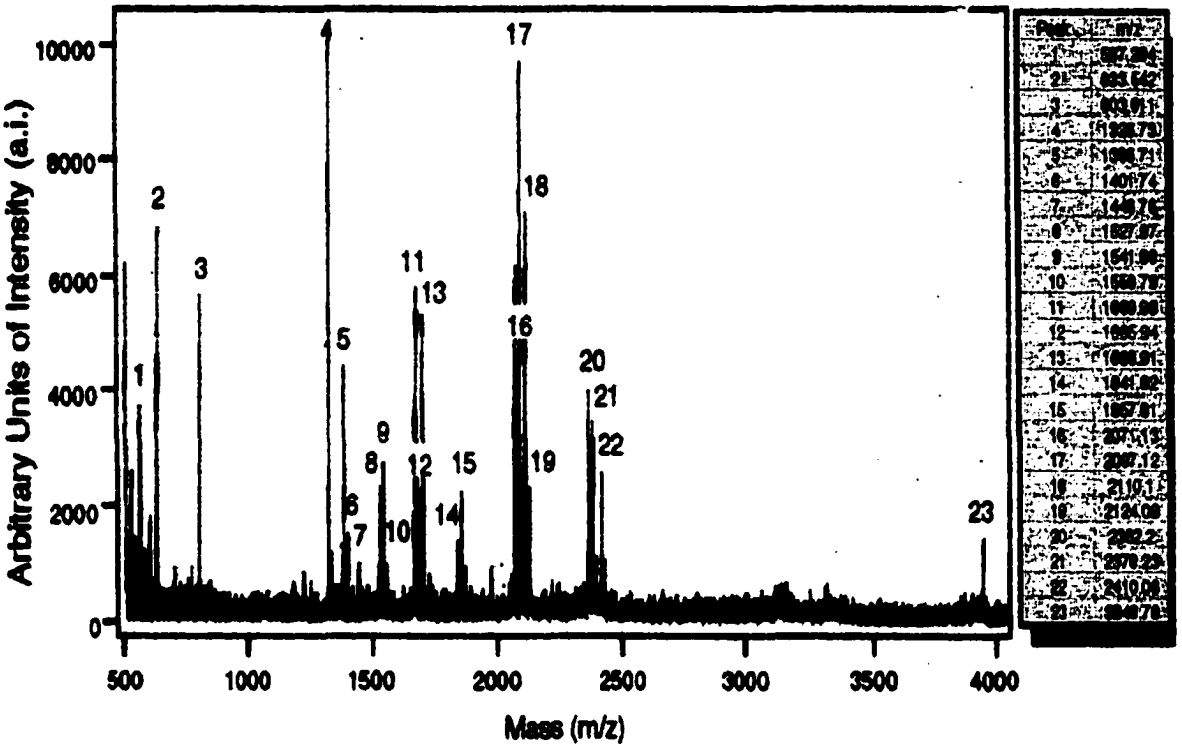


Fig.12. MS-MALDI Spectrum of Lys-C peptides obtained from ~54 kD protein. The 100,000 x g pellets of brain homogenates were subjected to detergent (1% NP-40, 0.5% sodium deoxycholate and 0.1 % SDS) extraction. The ~54 kD protein remained almost exclusively insoluble. The detergent insoluble pellets were then solubilised in 6 M Guanidine Hydrochloride (GnHCl) and subjected to preparative SDS-PAGE. Protein fractions were collected and the ~54 kD protein fraction was identified by both Coomassie Blue staining of the fractions on SDS-PAGE and Western blotting with P-Ser antibodies. The Coomassie Blue-stained ~54 kD protein band was excised from the gel and analyzed by MS-MALDI for identification.

Abscissa - mass/charge ratio (m/z) of the peptide fragments. ***Ordinate*** - arbitrary units of intensity (a.i). The table lists the mass signals (1 to 23) attributed to LysC fragments of ~54 kD protein with measured m/z values.

MS-FIT SEARCH RESULTS

Sample ID: Iqbal AD
Database Searched: NCBInr.12.15.98
Molecular Weight search(1000-100000 Da) selects 325855 entries.
MS-Fit Search selects 15 entries.
Considered Modifications:[Oxidation of M]

Min. # Peptides to Match	Peptide Mass Tolerance (+/-)	Peptide masses are	Digest Used	Max# missed Cleavages	Cysteines modified by	Input# peptide masses
10	200 ppm	monoisotopic	Lys-C	1	carbamido-methylation	23

Result Summary

Table.3

Rank	*MS-Digest Index#*	*# (%)Masses Matched*	*NCBInr.12.15.98 Accession#*	*Species*	*Protein MW(Da)*	*Protein Name*
1	39374	11/23 (47%)	91858	Unreadable	49586.1	tubulin beta-4 chain-mouse
1	252256	11/23 (47%)	2443346	Halocynthia Roretzi	50036.5	beta-tubulin
2	39365	10/23 (43%)	92930	Rattus Norvegicus	49937.4	tubulin T beta 15
2	39368	10/23 (43%)	86468	Unreadable	49802.3	tubulin beta-3 chain-chicken
2	39373	10/23 (43%)	135459	Mus Musculus	49831.3	tubulin Mbeta3
2	53973	10/23 (43%)	109432	Cricetulus Griseus	49748.2	beta-tubulin
2	77109	10/23 (43%)	631483	***Homo Sapiens***	49907.3	***(X79535) beta tubulin***
2	89856	10/23 (43%)	135464	Gallus Gallus	49861.3	c-beta-3 beta-tubulin
2	90840	10/23 (43%)	417855	Gallus Gallus	49953.4	tubulin beta
2	94332	10/23 (43%)	135446	Gallus Gallus	49909.3	beta-1 tubulin
2	94335	10/23 (43%)	401166	Xenopus Laevis	4981 .3	beta tubulin
2	94337	10/23 (43%)	135490	Pig	49861.2	Tubulin beta chain
2	252255	10/23 (43%)	2443344	Halocynthia Roretzi	49812.2	beta-tubulin
2	323544	10/23 (43%)	3745822	Unreadable	47894.4	1tub/B chain B, Tubulin Alpha-Beta di
2	337764	10/23 (43%)	3907633	Gadus Morhua	49749.2	beta-1 tubulin

Table.3. MS-FIT search results of the ~54 kD protein analyzed by Mass spectrometry. The search results indicate the presence of β-tubulin and no other protein.

Confirmation of ~54 kD protein as β-tubulin by Western Blot analysis:

Having identified the ~54 kD protein as β-tubulin with mass spectrometry, we decided to immunodetect the ~54 kD protein by Western blots with commercially available monoclonal antibody to β-tubulin. The preparative SDS-PAGE purified ~54 kD protein was immuno-labeled with antibody to β-tubulin, further confirming ~54 kD protein as β-tubulin (Fig. 13)

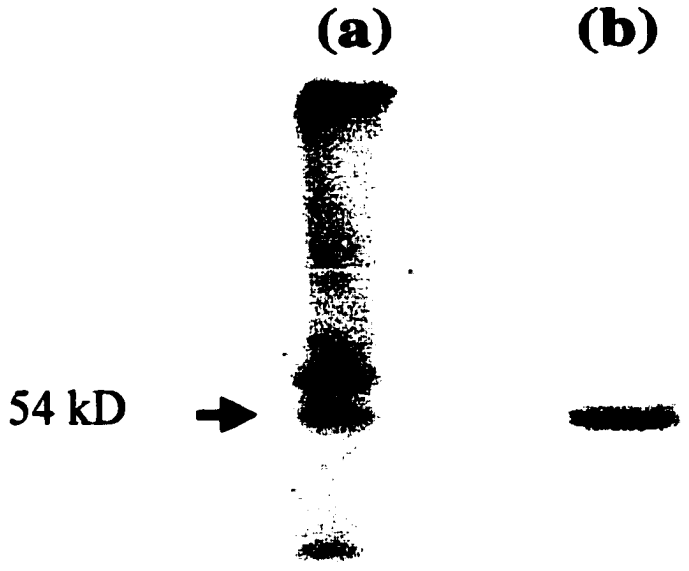


Fig. 13. Western blots of the preparative electrophoresis purified ~54 kD protein developed with an antibody to β-tubulin. Proteins were electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% BSA-TBST (Bovine Serum Albumin - Tris Buffer Saline Tween-20), β-tubulin and proteins phophorylated at serine residues were immunodetected using monoclonal antibodies to β-tubulin (1:750) and polyclonal antibodies to P-Ser (1:750), followed by corresponding HRP-conjugated secondary antibody (1;25000). The blots were developed by ECL reagents. (a) GnHCl-soluble proteins 30 ug/lane immunodetected with anti-P-Ser antibodies and (b) Preparative electrophoresis purified ~54 kD protein immunodetected with anti- β-tubulin antibody. The ~54 kD protein is β-tubulin immunopositive.

N-terminal Amino acid sequencing of the purified ~54 kD protein:

The amino terminal amino acid sequencing was carried out with two-fold purpose: (1) to confirm the identity and (2) to determine the purity of the protein. The coomassie-stained purified ~54 kD protein was excised from the gel, washed 25 times with distilled water in a 1.5 ml Eppendorf tube and dried using a vacuum concentrator before N-terminal amino acid sequencing at Beckman Center, Stanford University. The analyses was carried out with a cut off sensitivity fixed at 0.5 pmoles for each residue. The results are shown in Fig.14. The first ten N-terminal residues obtained were as follows: MREIVHIQAG which matched with the N-terminal sequence of β-tubulin (Accession # X79535) confirming that not only the protein being analysed was β-tubulin, but it was also practically pure.

(A)

Alzheimers disease				Control		
Residues	Amino Acids	Yield (pmoles)		Residues	Amino Acids	Yield (pmoles)
1	M	3		1	M	3
2	R	6		2	R	5
3	E	3		3	E	3
4	I	3		4	I	3
5	V	3		5	V	3
6	H	2		6	H	1
7	I	2		7	I	2
8	Q	1		8	Q	2
9	A	2		9	A	2
10	G	1		10	G	1

(B)

>gi|631483|pir||S45140 tubulin beta chain - human
MREIVHIQAGQCGNQIGAKFWEVISDEHGIDPTGSYHGDSDLQLERINVYYNEAAGNKYVPRAILVD
LEPGTMDSVRSGPFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKESESCDCLQGFQ
LTHSLGGGTGSGMGTLLISKIREEYPDRIMNTFSVMPSPKVSDTVVEPYNATLSVHQLVENTDETYS
IDNEALYDICFRTLKLTTPTYGDLNHLVSATMSGVTTCLRFPGQLNADLRKLAVNMVPFPRLHFFMP
GFAPLTSRGSQQYRALTVPELTQQMFDSKNMMAACDPRHGRYLTVAAIFRGRMSMKEVDEQMLNVQN
KNSSYFVEWIPNNVKTAVCDIPPRGLKMSATFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEG
MDEMEFTEAESNMNDLVSEYQQYQDATADEQGEFEEEEGEDEA

Fig. 14. N-terminal amino acid sequence analysis of the ~54 kD protein purified by 10% preparative SDS-PAGE. (A) Preparative electrophoresis purified ~54 kD protein was electrophoresed on a 10% SDS-PAGE. The Coomassie-stained protein band was excised from the gel and subjected to amino acid sequence analysis. The N-terminal sequence MREIVHIQAG obtained by this analysis matched with that of β-tubulin confirming the identity as well as purity of the ~54 kD protein. **(B)** The sequence of β-tubulin that identified with ~54 kD purified protein by MS-MALDI

Hyperphosphorylation of β-catenin

Phosphothreonine hyperphosphorylated proteins:

When we analysed the 100,000 x g pellet of AD (n = 7) and control (n = 5) gray matter homogenates by Western blots using polyclonal anti-phosphothreonine antibodies, we observed hyperphosphorylation of several proteins (Fig.15 & 15A). Three of these proteins also labeled with antibody to tau. Whereas, a fourth protein with a molecular mass of ~94 kD, which was also hyperphosphorylated in AD did not label with tau antibody.

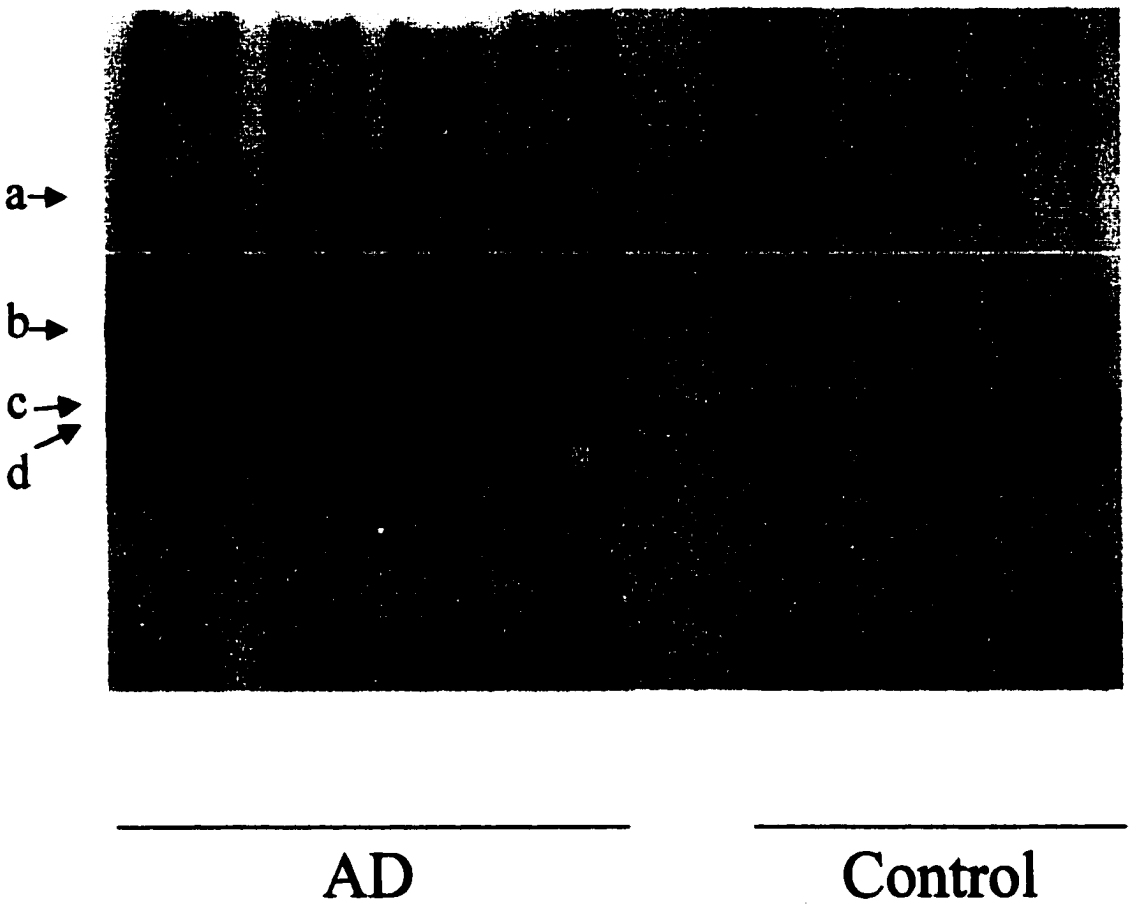


Fig. 15 Western blot of the 100,000 x g pellet of homogenates of frontal gray matter from AD and age-matched control cases developed with antibody to phosphothreonine. The 100,000 x g pellet of brain homogenates from AD and age-matched control cases was electrophoresed (30 ug/ lane) on a 5 - 15% SDS-PAGE and the protein transferred to a PVDF membrane. After blocking with 5 % BSA-TBST (bovine serum albumin-tris buffer saline tween-20), the phosphorylated threonine residues of proteins were immuno-detected using polyclonal phospho-threonine antibodies (1:750) and HRP-conjugated secondary antibody (1:25000). The blot was developed by ECL reagents. Lanes 1 to 4 - AD; Lanes 5 to 7 - age-matched controls. The intensity of immunoreactivity was quantitated by densitometry. ***Arrows*** : a - 94 kD ; b - 55 kD ; c - 43 kD ; d - 40 kD. P-Thr immunoreactivity on ~40 kD, ~43 kD and ~55 kD in AD was signficantly higher when compared with age-matched controls ($p < 0.05$). { Statistical significance was obtained by using a total of 7 AD and 5 age-matched control cases }.

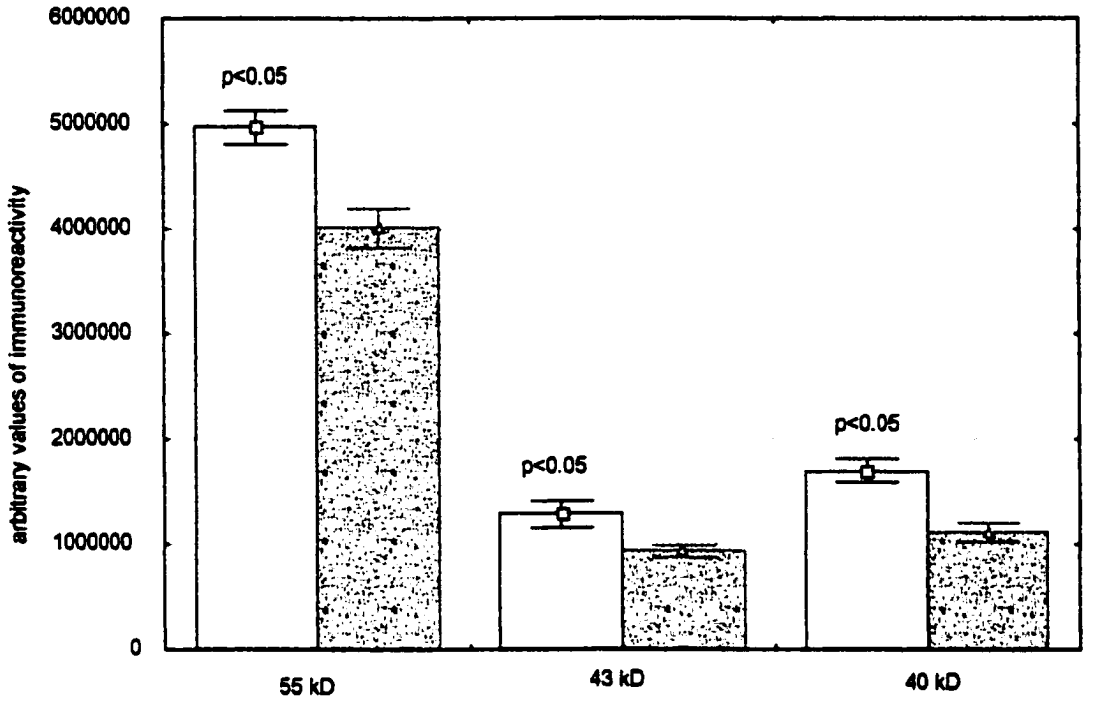
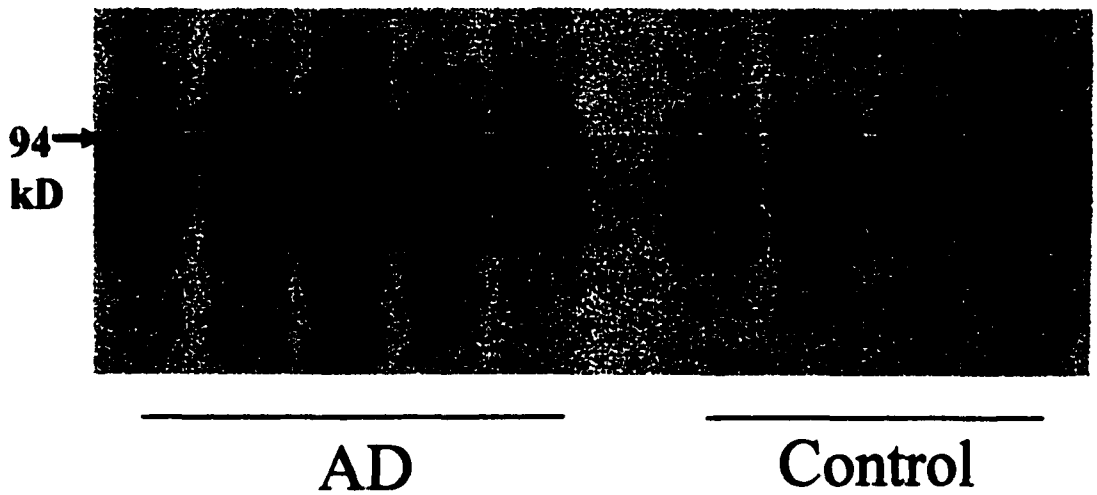


Fig. 15-A. Quantitation of the P-Thr immunoreactivity in the 100,000 x g pellet of AD and age-matched contol cases. P-Thr immunoreactivity was found to be significantly higher in ~40 kD, ~43 kD and ~55 kD proteins ($p < 0.05$) in AD compared with age-matched controls. ***Open rectangle*** - AD ; ***closed rectangle*** - control. Statistical significance was obtained by using a total of 7 AD and 5 age-matched control cases (Values are expressed as Mean ± S.E.M).

The hyperphosphorylation was much more obvious when ^{125}I- secondary antibody was used as the secondary antibody instead of HRP-conjugated secondary antibody (Fig.16). Since, β-catenin migrates as a ~94 kD protein, we investigated whether the ~94 kD phosphothreonine protein was β-catenin. Upon Western blots with monoclonal anti-β-catenin, we found that the ~94 kD phosphothreonine protein was indeed labeled with anti-β-catenin antibody. In order to answer the question if β-catenin was hyperphosphorylated at threonine in AD brain, we purified the β-catenin from the 100,000 x g pellet of gray matter homogenates.

Fig. 16. Western blot of the 100,000 x g pellet of homogenates of frontal gray matter from AD and age-matched control cases developed with antibody to phosphothreonine and developed with ^{125}I- secondary antibody. The 100,000 x g pellet of brain homogenates from AD and age-matched control cases was electrophoresed (30 ug/ lane) on a 5 - 15% SDS-PAGE and the protein transferred to a PVDF membrane. After blocking with 5 % BSA-TBST (bovine serum albumin-tris buffer saline tween-20), the phosphorylated threonine residues of proteins were immuno-detected using polyclonal phospho-threonine antibodies (1:750) and ^{125}I - conjugated secondary antibody (1:5000). The blot was developed by exposing to a phosphorimager screen. Lanes 1 to 5 - AD; Lanes 6 to 9 - age-matched controls. The intensity of immunoreactivity was quantitated by densitometry. P-Thr immunoreactivity on **~94 kD** protein in AD was signficantly higher when compared with age-matched controls ($p < 0.02$). { Statistical significance was obtained by using a total of 7 AD and 5 age-matched control cases }.

P-thr immunoreactivity on a ~94 kD protein of 100,000 xg pellet of brain homogenates



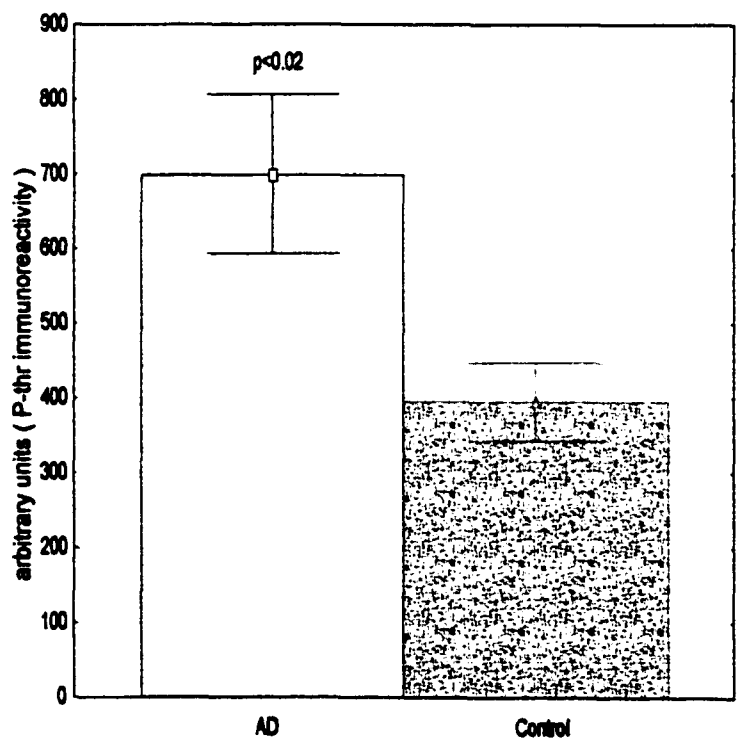


Fig. 16

Purification of β-catenin by Preparative electrophoresis:

The 100,000 x g pellets of gray matter homogenates from AD and age-matched controls were solubilised in 50 mM Tris, pH 7.6 containing 6 M GnHCl, 25 mM NaF, 1mM Na3VO4, 1mM DTT and 1 mM PMSF by incubating for 3 hrs. at room temperature with intermittent mixing. After 3 hrs. of incubation, the denaturant treated samples were centrifuged at 100,000 x g for 30 min. at 4 degrees celsius. The supernatant was considered 6 M GnHCl (guanidine)- soluble fraction, which was then dialysed against 50 mM Tris, pH 7.6 containing 2 M urea, 25 mM NaF, 1mM Na3VO4, 1mM DTT and 1 mM PMSF with two 30 min. changes followed by overnight dialysis. After dialysis, the protein was assayed by modified Lowry method. Then 1 mg protein was heated in 1 X Laemmli sample buffer before loading on a 10% preparative SDS-PAGE. The fractions were collected at a rate of 60 ml/hr. The β-catenin fraction was followed by first monitoring with coomassie stained 10% analytical SDS-PAGE, followed by Western blots developed with monoclonal anti-β-catenin antibodies. After zeroing in on the β-catenin fractions, parallel Western blots were performed of β-catenin containing fractions with (a) monoclonal anti-β-catenin antibody and (b) polyclonal anti-phosphothreonine antibodies (Fig.17).

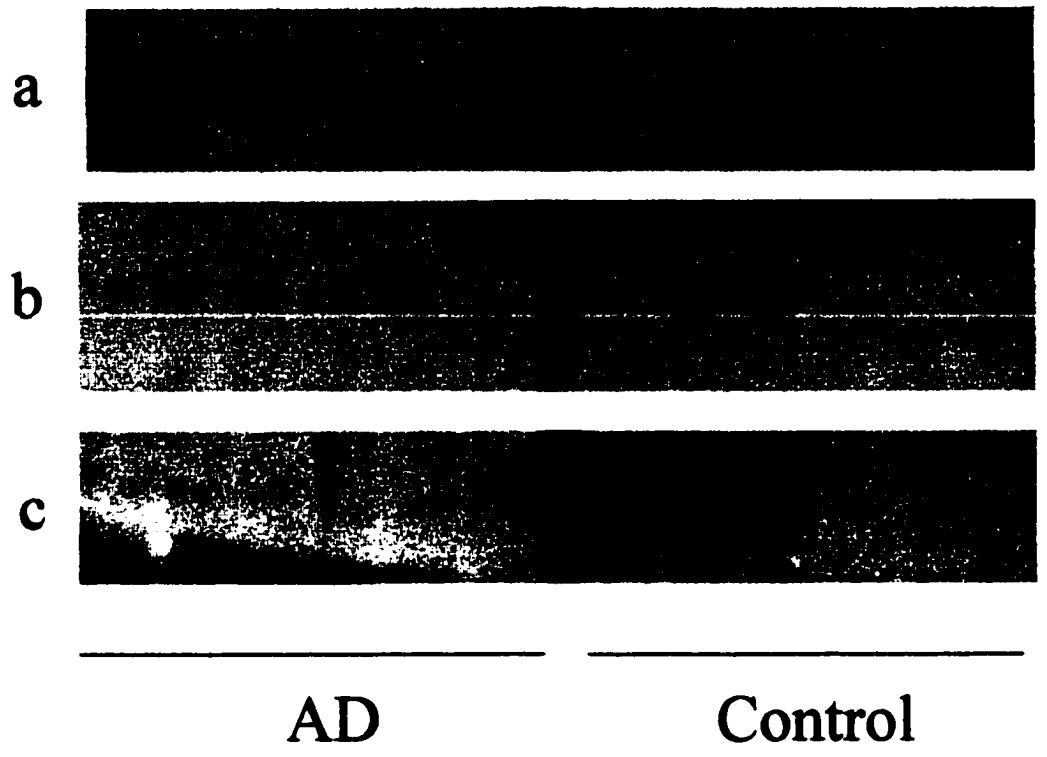


Fig. 17. Hyperphosphorylation of β-catenin in preparative SDS-PAGE fractions from an AD and age-matched control. Western blots of SDS-PAGE purified β-catenin-containing fractions developed with (a) mouse monoclonal β-catenin antibody (1: 1000), (b) rabbit polyclonal phosphothreonine antibody (1:750) and (c) rabbit polyclonal phosphoserine antibody (1:750). The level of phosphorylation at threonine residues was 200% more in AD compared with age-matched controls.

The Western blots in Fig.17., clearly demonstrates that β-catenin is hyperphosphorylated at threonine residues. The level of phosphorylation in AD is ~200% more than in age-matched controls as determined by phosphorimaging analyses of the Western blots. However, the purity of β-catenin cannot be guaranteed based on Western blots, alone. Therefore, in order to confirm that the hyperphosphorylation seen in the β-catenin fraction was contributed by β-catenin alone and not by any contaminating source, we isolated β-catenin by immunoprecipitation.

Immunoprecipitation of β-catenin

After solubilising the 100,000 x g pellet of gray matter homogenates from AD (n = 8) and age-matched controls (n = 8) in 50 mM Tris pH 7.6 containing 6 M GnHCl, 25 mM NaF, 1mM Na3VO4, 1mM DTT and 1 mM PMSF, the final concentration of guanidine was adjusted to 3.2 M with 1% digitonin buffer (50 mM Tris pH 7.6, 25 mM NaF, 1mM Na3VO4, 1mM DTT and 1 mM PMSF and 1% digitonin) before preclearing with Protein-G agarose for 3 hrs. The protein sample was then incubated overnight with mouse monoclonal anti-β-catenin antibody, followed by incubation with Protein-G agarose for another 3 hrs before precipitating the immune complex. The immune complex was washed thrice with 1% digitonin buffer before heating to 70° C for 5 min. in Laemmli buffer. After separation in SDS-polyacrylamide gels, proteins were transferred onto an Immobilon-P membrane. Immunoprecipitated proteins were detected by the incubation of mouse monoclonal anti-β-catenin antibody followed by HRP-conjugated secondary antibody and enhanced chemiluminescence. As seen in Fig.18., immune-

purified β-catenin from AD brains was observed to be hyperphosphorylated at threonine residues. Upon quantitation by densitometric scanning and comparing the ratio of P-thr / β-catenin immunoreactivity between AD and age-matched controls, we found that β-catenin in AD was ~200% more phosphorylated at threonine, similar to the data obtained from preparative electrophoresis purified β-catenin. These studies demonstrated the hyperphosphorylation of β-catenin on threonine residues in the brains of AD patients, as determined by comparison of the ratios of P-thr / β-catenin (obtained from Western blotting data with β-catenin and P-thr antibodies) in preparative electrophoresis-purified β-catenin as well as immune-purified β-catenin from AD and age-matched control cases.

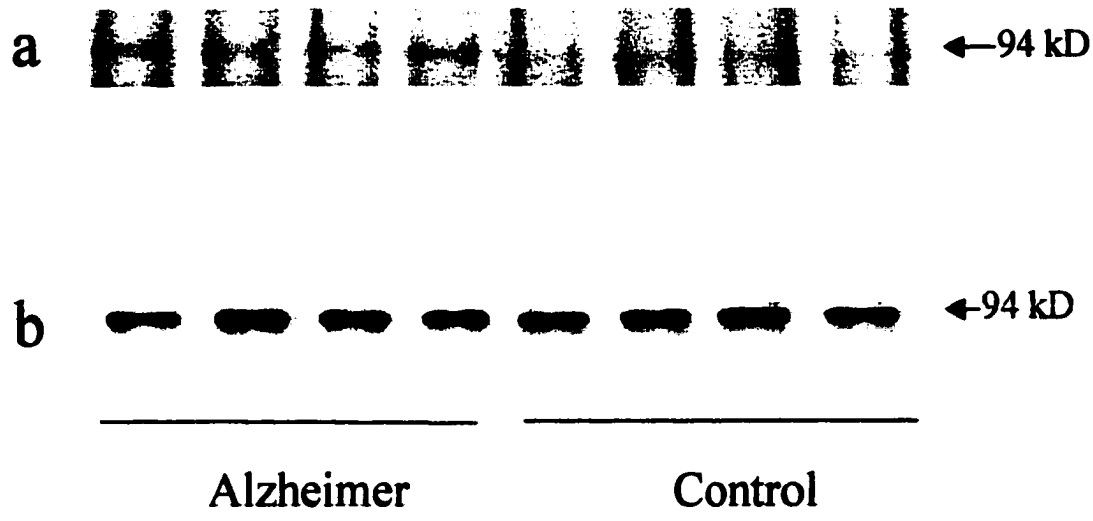


Fig.18. Hyperphosphorylation of β-catenin confirmed by immunoprecipitation. Western blots of β-catenin immunoprecipitates of 6 M GnHCl extract of AD and control brains developed with antibodies to (a) phospho-threonine and (b) β-catenin. The phosphothreonine / β-catenin ratio as determined by densitometric analyses revealed a 200 % increase in phosphorylation of βcatenin in AD as compared with age-matched control cases. ($p<0.05$). ($n = 8$ for AD ; $n=8$ for age-matched controls).

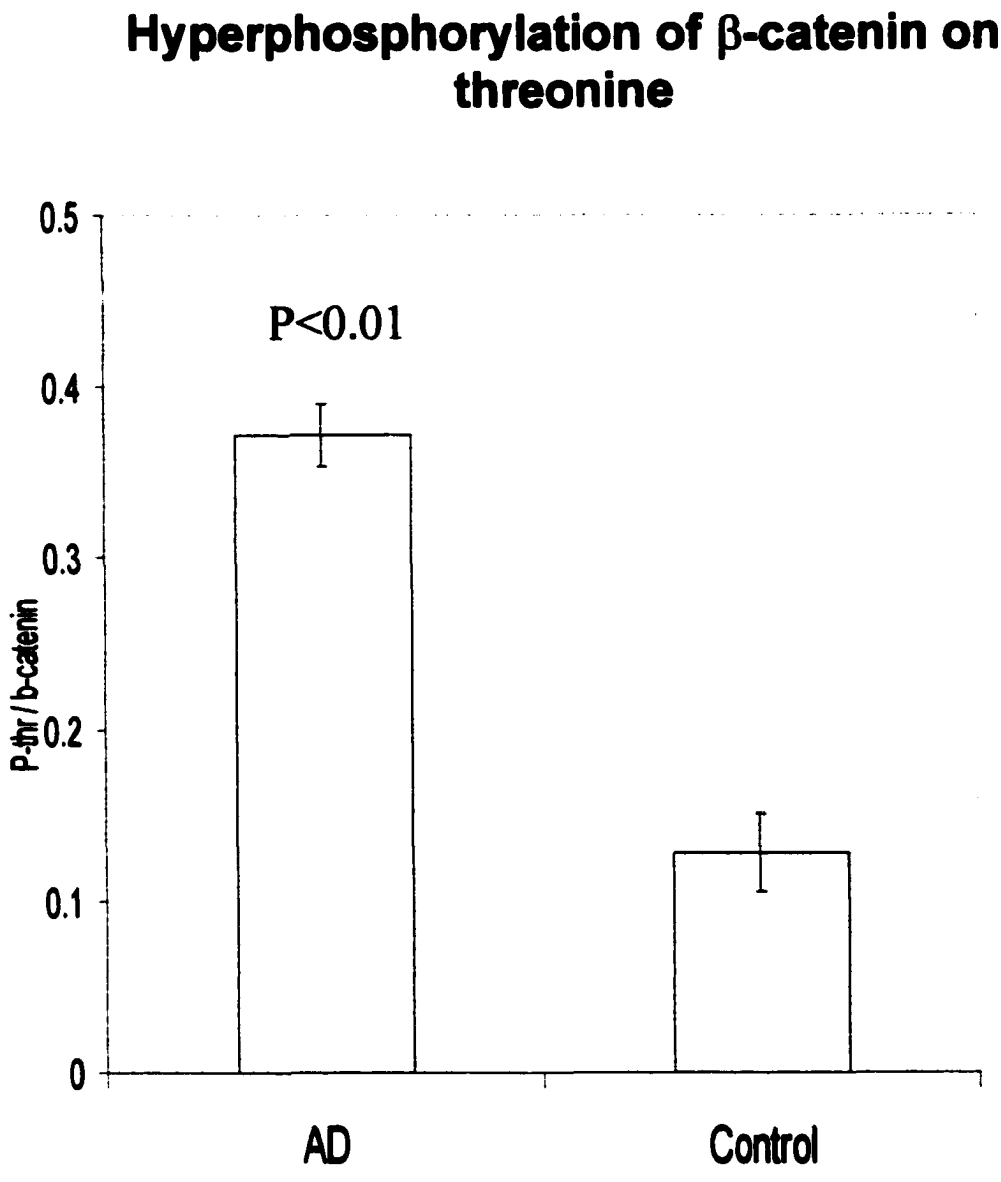


Fig. 18-A. Quantitation of hyperphosphorylation at threonine in β-catenin immunoprecipitates of AD and age-matched contol cases. Hyperphosphorylation was quantitated by calculating the ratio of P-Thr / β-catenin. There was a ~200 % increase in phosphorylation at threonine residues on β-catenin in AD compared with age-matched control cases. (n = 8 for AD as well as age-matched controls ; values are expressed as Mean ± S.E.M).

Phosphorylation of β-catenin at Thr^{41}:

Having established the hyperphosphorylation of β-catenin in AD brains, we attempted to investigate the phosphorylation sites of this protein. We decided to take advantage of the availability of a polyclonal antibody to Thr^{41} of β-catenin. This site is under the regulation of GSK-3 (Glycogen synthase kinase-3), which is one of the candidate kinases involved in the hyperphosphorylation of tau in AD (Pei et al., 1997, 1999 ; Wang et al., 1998. Hong et al., 1997 Utton et al., 1997; Singh et al., 1994 ; Hanger et al., 1992)

Our data (Figs. 19 & 19A) revealed that there were no significant differences between the AD and age-matched control cases in the phosphorylation state of β-catenin at Thr^{41}, suggesting that the hyperphosphorylation on threonine residues, as indicated by Western blotting with P-thr antibody, is at some other site (s) on β-catenin. This may also raise the possibility that GSK-3 is not involved in the hyperphosphorylation of β-catenin since Thr^{41} is a preferred site for GSK-3 activity.

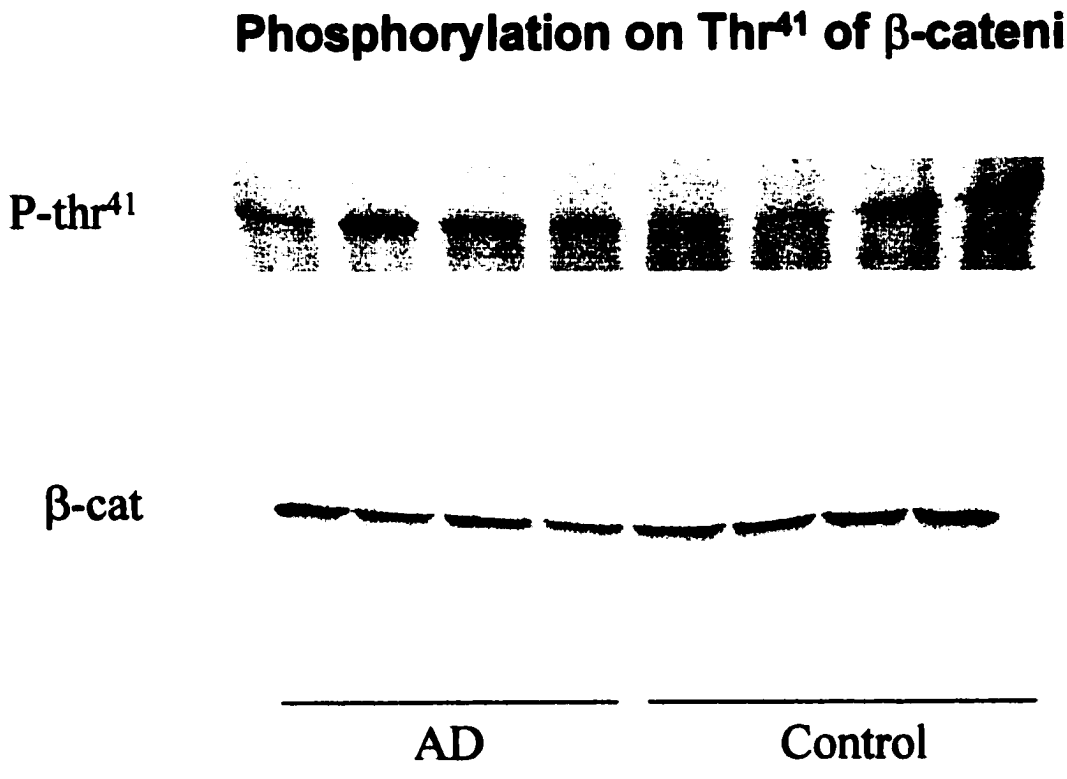


Fig. 19. Western blot of the 100,000 x g pellet of homogenates of frontal gray matter from AD and age-matched control cases developed with antibody to P-Thr^{41}. The 100,000 x g pellet of brain homogenates from AD and age-matched control cases was electrophoresed (30 ug/ lane) on a 10% SDS-PAGE and the protein transferred to a PVDF membrane. ***Top*** - P-Thr^{41} residues of β-catenin were immuno-detected using polyclonal P-Thr^{41} antibodies (1:1000) and ***Bottom*** - β-catenin was detected using monoclonal anti-β-catenin antibody (1: 1000) followed by HRP-conjugated secondary antibody (1:25000). The blot was developed by ECL reagents. Positions of AD and age-matched controls as marked.

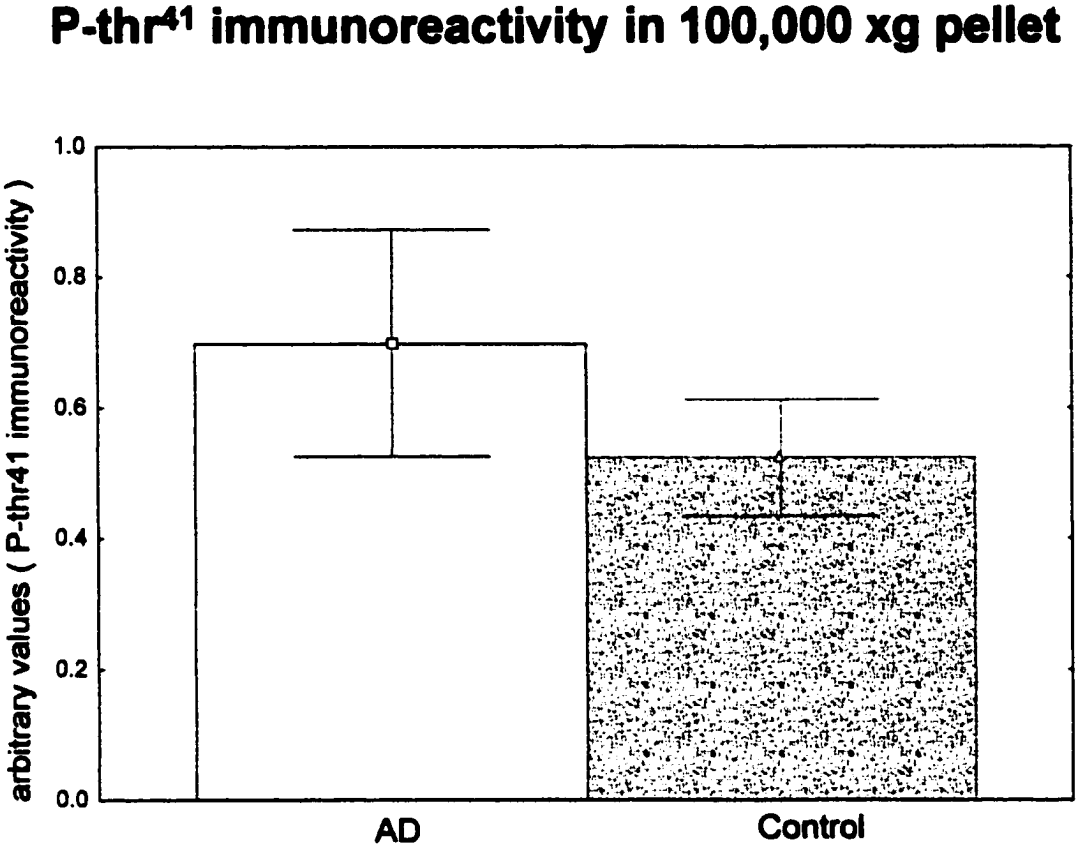


Fig. 19-A. Quantitation of the P-Thr^{41} immunoreactivity in the 100,000 x g pellet of AD and age-matched contol cases. P-Thr^{41} immunoreactivity was quantitated by calculating the ratio of P-Thr^{41} / β-catenin. There was no significant difference in phosphorylation of β-catenin at Thr^{41} between AD and age-matched control cases. (n = 8 for AD as well as age-matched controls ; values are expressed as Mean ± S.E.M).

β-catenin levels are elevated in AD brain 100,000 x g pellet

Western blots developed with antibody to β-catenin, revealed a significant increase in the levels of β-catenin in the 100,000 x g pellet of AD brains compared with age-matched controls (Fig. 20 and 20A). AD brain pellet had 39% more β-catenin compared with age-matched control cases. We investigated whether the increase reflected a general increase in β-catenin or that a selective pool of β-catenin was affected in AD. We analyzed the cytosolic pool (100,000 x g supernatant) of β-catenin for changes between AD and age-matched controls.

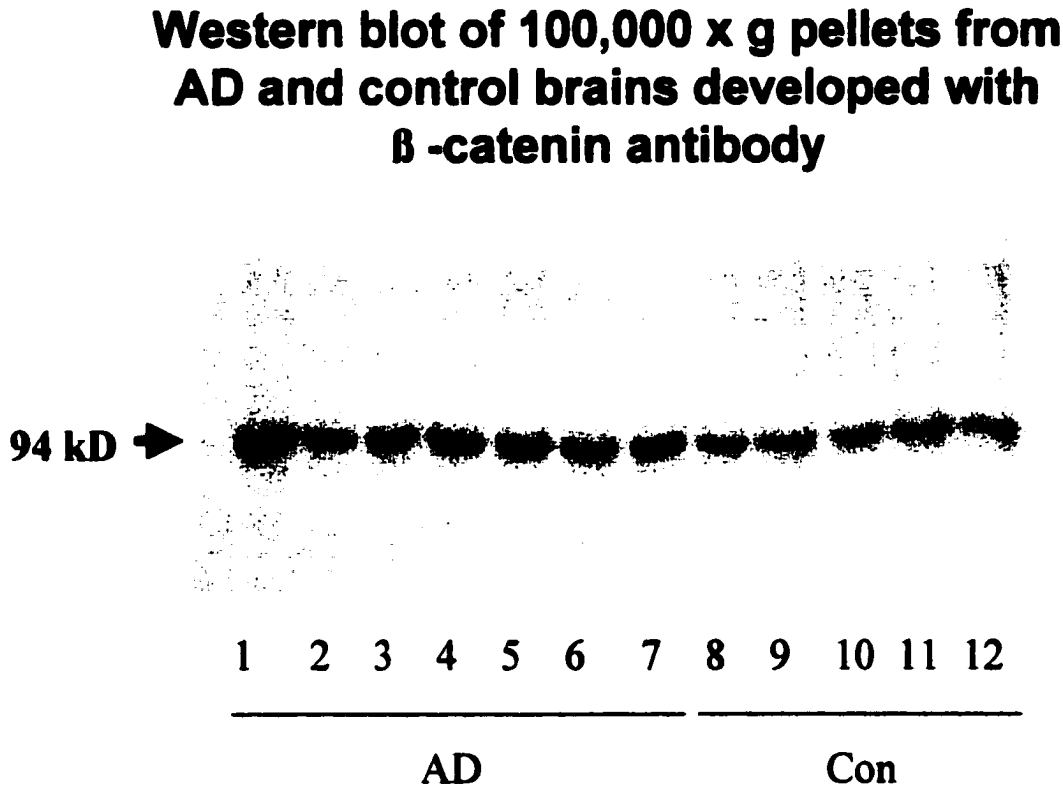


Fig.20. Western blot of the 100,000 x g pellet of homogenates of frontal gray matter from AD and age-matched control cases developed with antibody to β-catenin. The 100,000 x g pellet of brain homogenates from AD and age-matched control cases was electrophoresed (30 ug/ lane) on a 10% SDS-PAGE and the protein transferred to a PVDF membrane. After blocking with 5 % BSA-TBST (bovine serum albumin-tris buffer saline tween-20), β-catenin levels were measured using monoclonal anti-β-catenin antibody (1: 1000) followed by HRP-conjugated secondary antibody (1:25000). The blot was developed by ECL reagents. Lanes 1 to 7 - AD; Lanes 8 to 12 - age-matched controls. The intensity of immunoreactivity was quantitated by densitometry. β-catenin levels were found to be ~39% more in AD compared with age-matched controls.

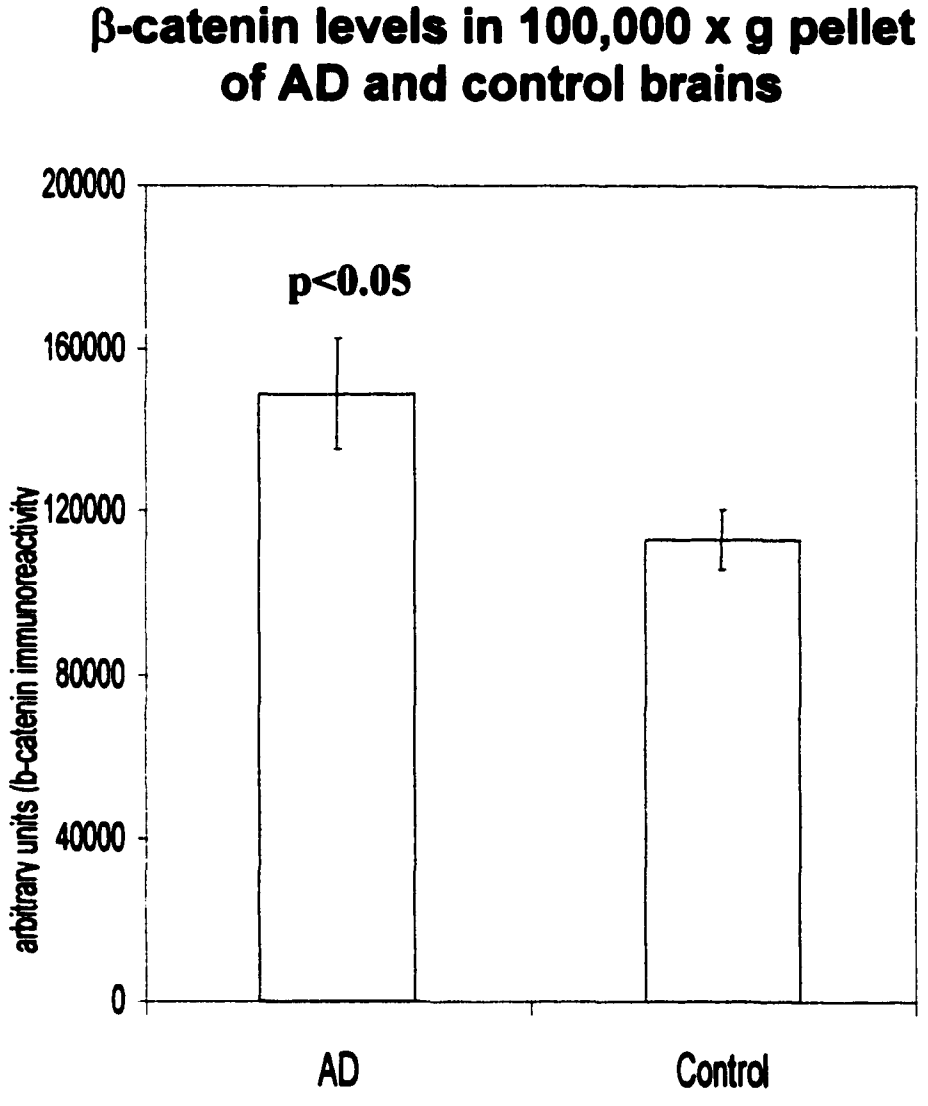


Fig.20-A. Quantitation of β-catenin levels in 100,000 x g pellets of homogenates of frontal gray matters from AD and age-matched controls. β-catenin immunoreactivity in seven AD and five age-matched controls were quantitated by densitometry using FUJI BAS-1500 phosphorimager. β-catenin levels were ~39% more in AD compared with age-matched controls ($p < 0.05$; values are expressed as Mean ± S.E.M).

β-catenin levels in the 100,000 x g supernatant

The 100,000 x g supernatants of AD and age-matched controls were electrophoresed on a 10% SDS-PAGE followed by Western blots developed with monoclonal anti-β-catenin antibodies. The blots were developed using HRP-labeled anti-mouse antibodies and ECL reagents. The β-catenin immunoreactive protein bands were then quantitated using FUJI BAS-1500 phosphorimager. The data revealed no statistically significant differences in the levels of β-catenin in the cytosolic pool between the AD and age-matched control cases. (Fig.21 and Fig.21-A)

Western blot of 100,000 x g supernatant from AD and control brains developed with β-catenin antibody

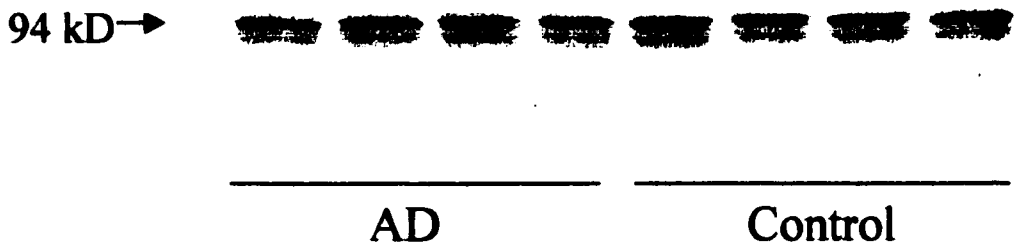


Fig.21. Western blot of the 100,000 x g supernatant of homogenates of frontal gray matter from AD and age-matched control cases developed with antibody to β-catenin. The 100,000 x g supernatant of brain homogenates from AD and age-matched control cases was electrophoresed (30 ug/ lane) on a 10% SDS-PAGE and the protein transferred to a PVDF membrane. After blocking with 5 % BSA-TBST (bovine serum albumin-tris buffer saline- tween-20), blots were incubated with monoclonal anti-β-catenin antibody (1: 1000) followed by HRP-conjugated secondary antibody (1:25000). The blot were developed by ECL reagents. The intensity of immunoreactivity was quantitated by densitometry. The levels of β-catenin were unchanged in AD versus age-matched controls.

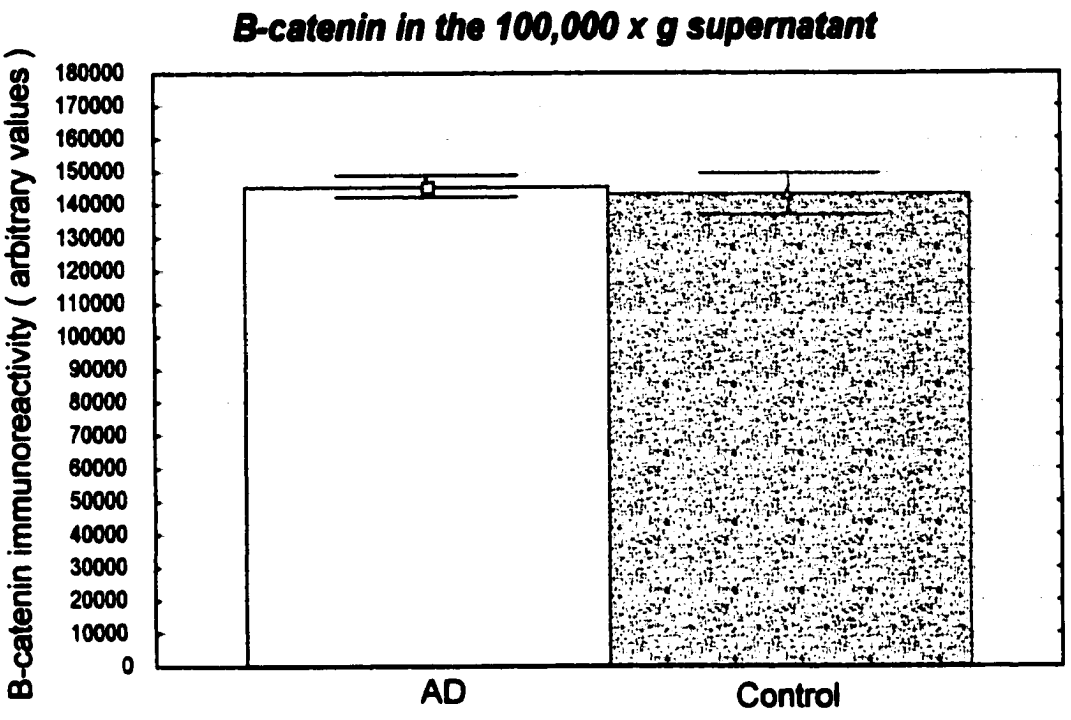


Fig. 21-A. Quantitation of β-catenin levels in 100,000 x g supernatants of homogenates of frontal gray matters from AD and age-matched controls. β-catenin immunoreactivity in seven AD and five age-matched controls were quantitated by densitometry using FUJI BAS-1500 phosphorimager. β-catenin levels remained unchanged in AD versus age-matched controls. Values are expressed as Mean ± S.E.M).

β-catenin mRNA analysis:

Since we observed that the levels of β-catenin were elevated in AD brain, we attempted to study the levels of mRNA for this protein in the brains of AD and control patients. An increase in the level of a particular protein can be due to two principal reasons - either the synthesis has increased or the breakdown has decreased. In order to investigate if there has been an increase in the gene expression, we decided to measure the levels of β-catenin mRNA by Northern blotting.

RNA was extracted with TRI reagent (Molecular Research Center, Inc. Cincinnati, OH, U.S.A.), from 7 AD and 5 age-matched controls, according to the manufacturers instructions. RNA pellets were dissolved in small volumes of nuclease-free water. RNA was electrophoresed on 1 % agarose / 0.66 formaldehyde gels in MOPS buffer. Following electrophoresis, RNA was transferred to Immobilon Ny+ membranes using downward capillary transfer. After crosslinking, the membranes were incubated in prehybridization solution (5 X saline-sodium phosphate-EDTA buffer, 5 X Denhardt's, 1% sodium dodecyl·sulfate, 10% dextran sulfate, 50 % formamide, 100 ug / ml calf thymus DNA) for 1 - 4 h at 42°C. The membranes were hybridized with [^{32}P]-β-catenin cDNA probe or [^{32}P]- G3PDH cDNA probe, overnight at 42°C with fresh prehybridization solution that contained ~10^9 dpm of probe per membrane.

We were unable to obtain any signal with [^{32}P]-β-catenin probe in the lanes that had RNA isolated from AD or age-matched controls (Fig. 22 b). This may be attributed to the poor integrity of RNA isolated from the post-mortem brains of AD and age-matched controls. (Fig. 22 a)

RNA analyses of AD and control brains

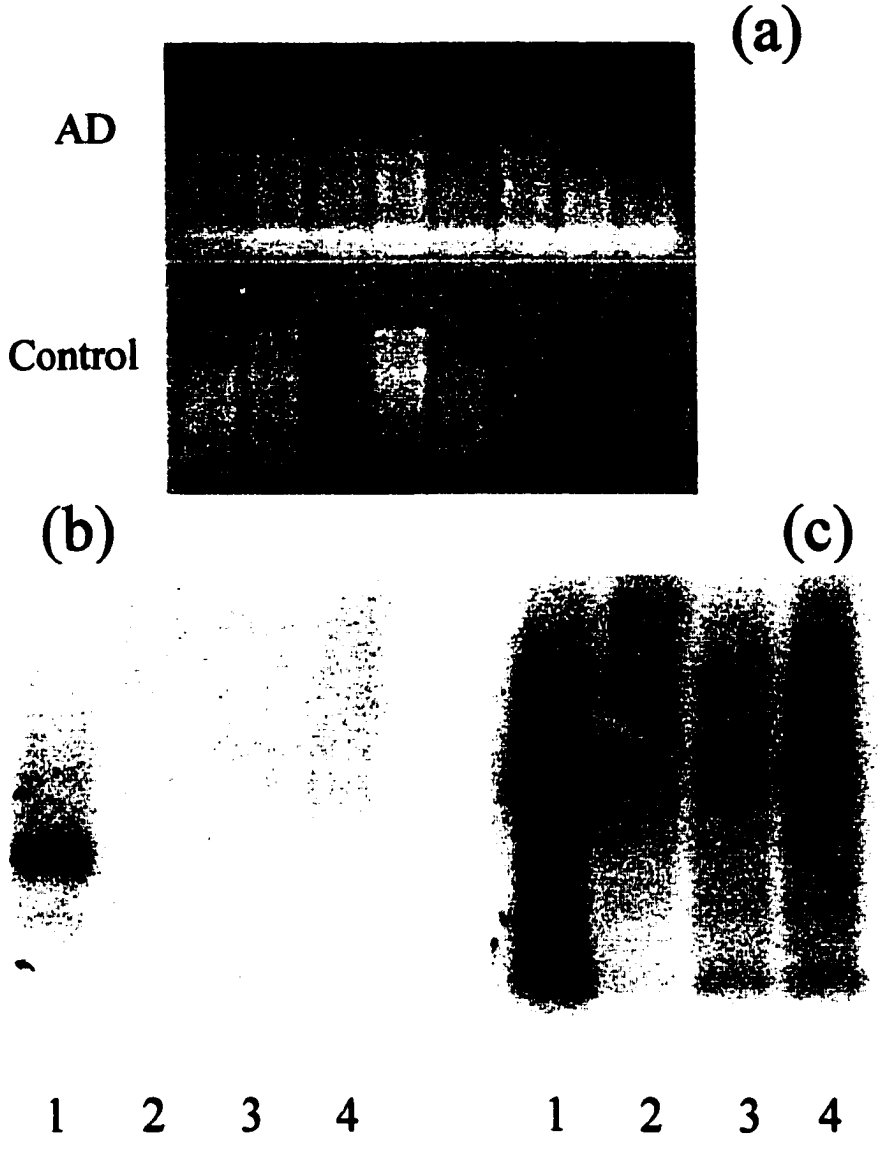


Fig.22.Quantitation of -β-catenin message from AD and age-matched control brains. RNA was isolated using the TRI reagent from 7 AD and 5 age-matched control cases.(**a**) RNA (10 ug / lane) was separated on 1% agarose / 0.66 M formaldehyde gels in MOPS buffer. Following electrophoresis, RNA was transferred to Immobilon Ny+ membranes using downward capillary transfer. Membranes were cross-linked and incubated in prehybridization solution for 1 - 4 h at 42°C. The membranes were then hybridized with either (**b**) [^{32}P]-β-catenin cDNA probe or (**c**) [^{32}P]- G3PDH cDNA probe, overnight at 42°C with fresh prehybridization solution that contained ~10^9 dpm of probe per membrane. Lane 1 - Rat brain RNA ; Lanes 2 & 3 - AD brain RNA ; Lane 4 - Control brain RNA.

Effects of APP on β-catenin levels in cos-7 cell lysates:

Certain mutations in APP are known to associate with the disease in some familial cases of AD (Goate et al., 1991). Although we had studied only sporadic cases of AD in which we found elevated levels of β-catenin, we investigated the role of APP in the regulation of β-catenin because both cases with APP mutations as well as sporadic cases have the same histopathology. For these studies, we employed cos-7 cells transfected with various APP constructs as a model system.

After transfecting the cells with plasmids containing vector alone, full length APP and cytoplasmic deletion $\Delta APP_{708-751}$), the cells were cultured for 48 hrs. in Dulbecco's Eagle medium before harvesting. The cells were lysed in 1 X Laemmli's buffer by heating in a boiling water bath for 5 min. Lysates of Cos-7 cells transfected with different constructs were analysed on a Western blot for β-catenin levels.

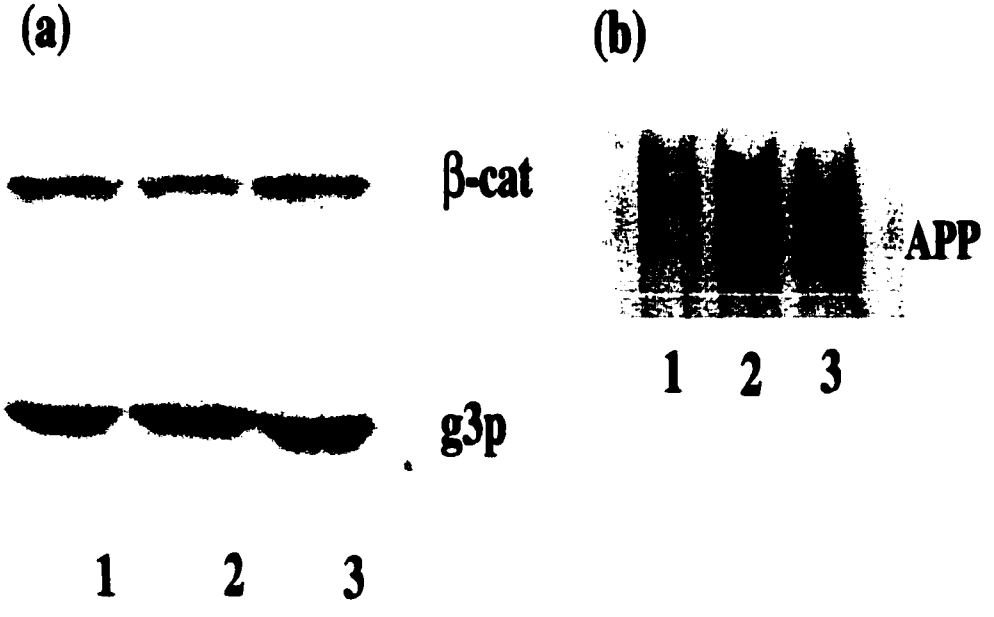


Fig. 23. Western blot of lysates of APP transfected cos-7 cells. Lysates were electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane followed by incubation with (a) mouse monoclonal β-catenin antibody or (b) rabbit polyclonal APP antibody. Blots were developed with HRP-conjugated anti-mouse (for β-catenin antibody) or anti-rabbit (for APP antibody) and ECL reagents. Band intensities were calculated by densitometric scanning using a phosphorimager. Lane 1 - Vector control, Lane 2 - APP full length (APP 751), Lane 3 - ΔcAPP. The β-catenin values were normalised against that of G3PDH. Levels of expression of APP were comparable in the two APP construct transfected cells. Three determinations were made to insure reproducibility

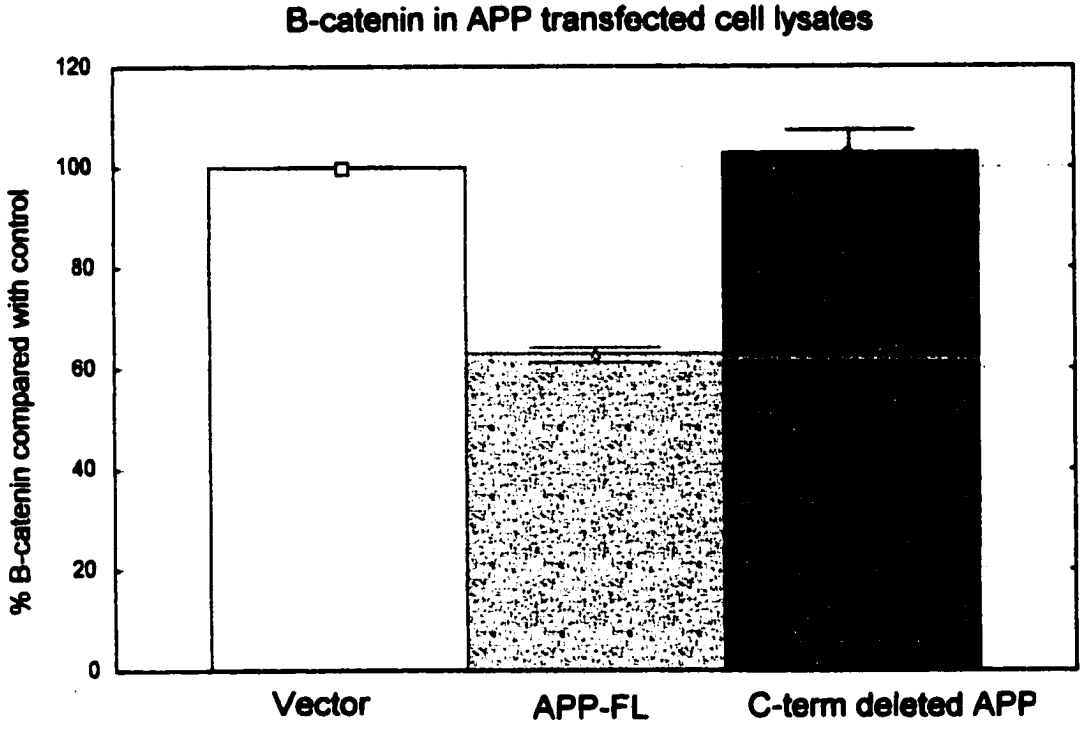


Fig. 23-A. Quantitation of β-catenin in the lysates of APP transfected cos-7 cells. Band intensities were quantitated by densitometric scanning using a phosphorimager. The β-catenin values were normalised against that of G3PDH. β-catenin levels decreased ~40% in full length APP (APP-FL) transfected cells. Truncation of 44 amino acids from the cytoplasmic tail (ΔcAPP) abolished this effect of APP-FL indicating the importance of the cytoplasmic tail in downregulating β-catenin levels. (values are expressed as Mean ± S.E.M).

The levels of APP expression were comparable in the cells transfected with full length APP and $\Delta APP_{708-751}$ as determined by Western blots developed with a polyclonal anti-APP antibody (fig. 23 b). The quantitations were done by normalising against a housekeeping gene product - glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The levels of β-catenin were significantly decreased in the cells transfected with full length APP as compared with those transfected with vector control. Interestingly, the levels of β-catenin in $\Delta APP_{708-751}$ transfected cells were similar to vector controls. (figure 23 a). These results revealed that β-catenin levels are downregulated by full length APP in a physiological way. Whether this is a direct or indirect effect is yet to be determined. Nevertheless, when the C-terminal 44 amino acids of APP which is in the cytoplasmic domain are deleted as in the case of the $\Delta APP_{708-751}$ transfected cells, the ability of APP to downregulate β-catenin is lost. These observations suggest that the downregulating effects of APP are mediated through its cytoplasmic tail. The C-terminal of APP has been implicated to play a role in its metabolism (; Borg et al., 1998 ; Guenette et al., 1999) Mutations of APP also affects its metabolism. We investigated if the Swedish mutation (APPswe) has an effect on APP's ability to downregulate β-catenin.

Effects of ^{Swe}mutant APP on β-catenin levels in cos-7 cell lysates.

To examine if the ability of APP to downregulate β-catenin levels is lost with the Swedish mutation, we transfected cos-7 cells with plasmid containing APP_{Swe} (670,671). After 48 hrs. of transfection, the cells were lysed in 1 X Laemmli's buffer, followed

by heating in a boiling water bath for 5 min before loading on a 10 % SDS-PAGE for Western blot analysis.

Examination of Western blots revealed no statistically significant difference compared with the vector control, suggesting that ^{Swe}mutation of APP (670, 671) influences the function of APP such that it can no longer downregulate β-catenin levels as does the full length APP (Fig. 24)

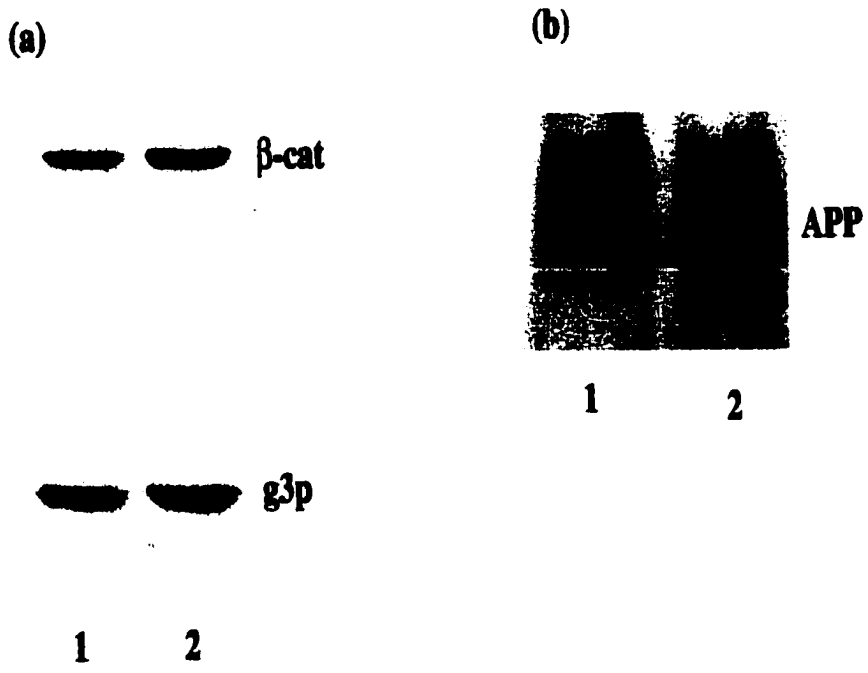


Fig. 24. Western blots of lysates of APPswe transfected cos-7 cells. Lysates were electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane followed by incubation with (a) mouse monoclonal β-catenin antibody or (b) rabbit polyclonal APP antibody. Blots were developed with HRP-conjugated anti-mouse (for β-catenin antibody) or anti-rabbit (for APP antibody) and ECL reagents. Band intensities were calculated by densitometric scanning using a phosphorimager. Lane 1 - Vector control, Lane 2 - APPswe. The β-catenin values were normalised against that of G3PDH. APP levels in the APPswe transfected cells were significantly higher than that in the vector control transfected cells. Three determinations were made to insure reproducibility

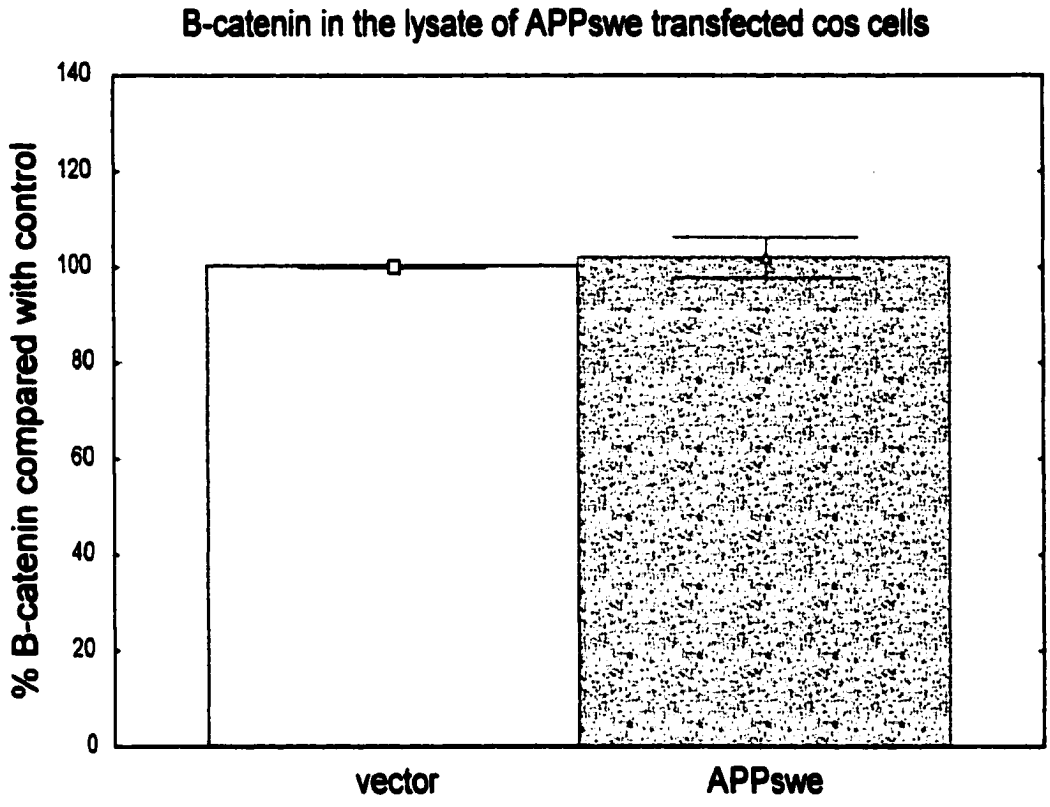


Fig. 24-A. Quantitation of β-catenin in the lysates of APPswe transfected cos-7 cells. Band intensities were quantitated by densitometric scanning using a phosphorimager. The β-catenin values were normalised against that of G3PDH. β-catenin levels were unchanged upon transfection of cos-7 cells with APPswe, indicating that mutation of APP-FL at positions 670, 671 was enough to abolish it's ability to downregulate β-catenin. (values are expressed as Mean ± S.E.M).

Since, β-catenin is found both in the particulate and soluble pools in the cell, we asked the question - Which pool of β-catenin is regulated by APP? In order to answer this question, we fractionated Cos-7 cells into cytosolic and particulate fractions.

Effects of APP on β-catenin levels in the cytosolic and particulate fractions:

After transfecting the cells with plasmids containing vector alone, full length APP and cytoplasmic deletion ($\Delta APP_{708-751}$), the cells were cultured for 48 hrs. in Dulbecco's Eagle medium before harvesting. The cells were lysed in 50 mM Tris buffer, pH 7.6 containing 25 mM NaF, 1mM Na_3VO_4, 1 mM DTT and 1 mM PMSF by probe sonication on ice with 15 sec. pulse five times with 15 sec. intervals. The resultant homogenates were centrifuged at 100,000 x g for 30 min. at 4°C. The supernatant was considered the cytosolic fraction and the pellet, the particulate fraction. The cytosolic and particulate fractions of Cos-7 cells transfected with different constructs were analysed by Western blots developed with anti-β-catenin antibodies. The levels of APP expressed were comparable in the cells transfected with full length APP and $\Delta APP_{708-751}$ as determined by Western blots with polyclonal anti-APP antibody. The quantitations of β-catenin levels were done by normalising against a house-keeping gene product - glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Although the levels of β-catenin were slightly decreased in the cytosolic pool from full length APP transfected cells compared with those transfected with vector control, this decrease was statistically insignificant whereas a significant decrease was observed in the particulate pool. Interestingly, the $\Delta APP_{708-751}$ did not show any difference in the β-catenin levels in either the cytosolic or particulate pool compared with vector controls (Fig. 25)

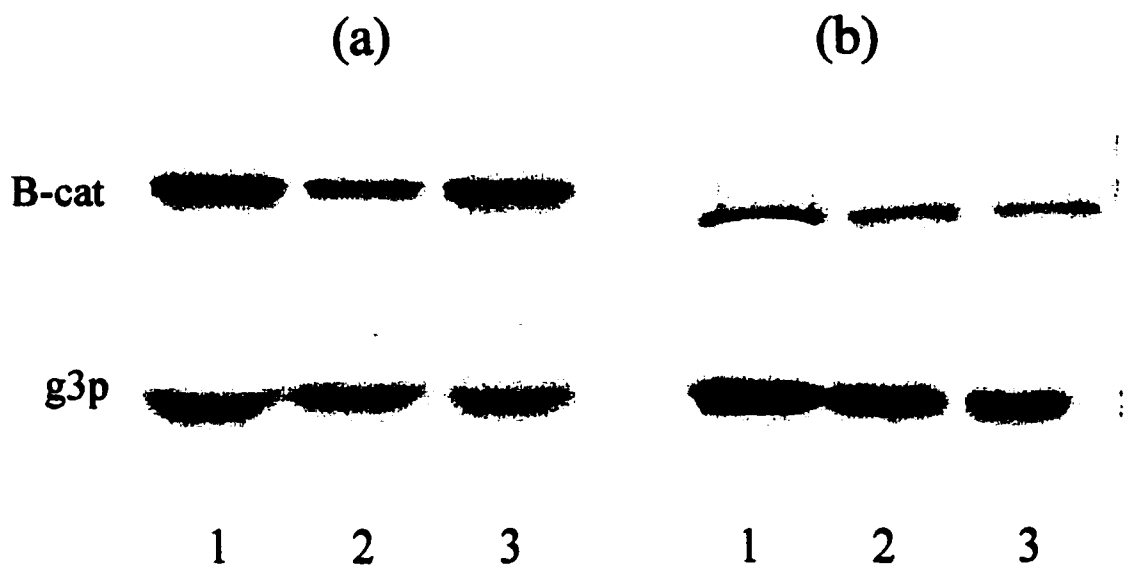


Fig. 25. Western blots of particulate and cytosolic fractions of APP transfected cos-7 cells. (a) The 100,000 x g pellet (particulate pool of β-catenin) and (b) supernatant (cytosolic pool of β-catenin) were electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane followed by incubation with mouse monoclonal β-catenin antibody. Blots were developed with HRP-conjugated anti-mouse secondary antibody and ECL reagents. Band intensities were calculated by densitometric scanning using a phosphorimager. Lane 1 - Vector control, Lane 2 - APP full length (APP 751), Lane 3 - ΔcAPP. The β-catenin values were normalised against that of G3PDH. Levels of expression of APP were comparable in the two APP construct transfected cells. Three determinations were made to insure reproducibility

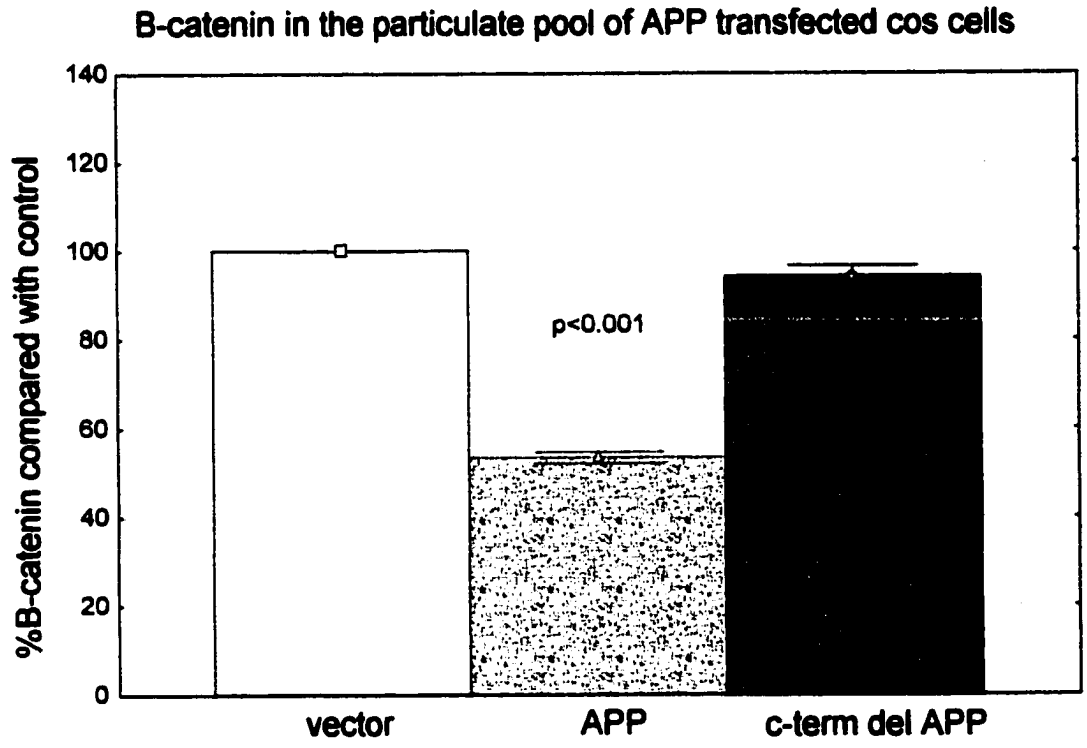


Fig. 25-A. Quantitation of β-catenin in the particulate fraction of APP transfected cos-7 cells. Band intensities were quantitated by densitometric scanning using a phosphorimager. The β-catenin values were normalised against that of G3PDH. β-catenin levels decreased ~40% in the particulate fraction of APP-FL transfected cells ($p<0.05$). Truncation of 44 amino acids from the cytoplasmic tail abolished this effect of APP-FL indicating the importance of the cytoplasmic tail in downregulating β-catenin levels. (values are expressed as Mean ± S.E.M).

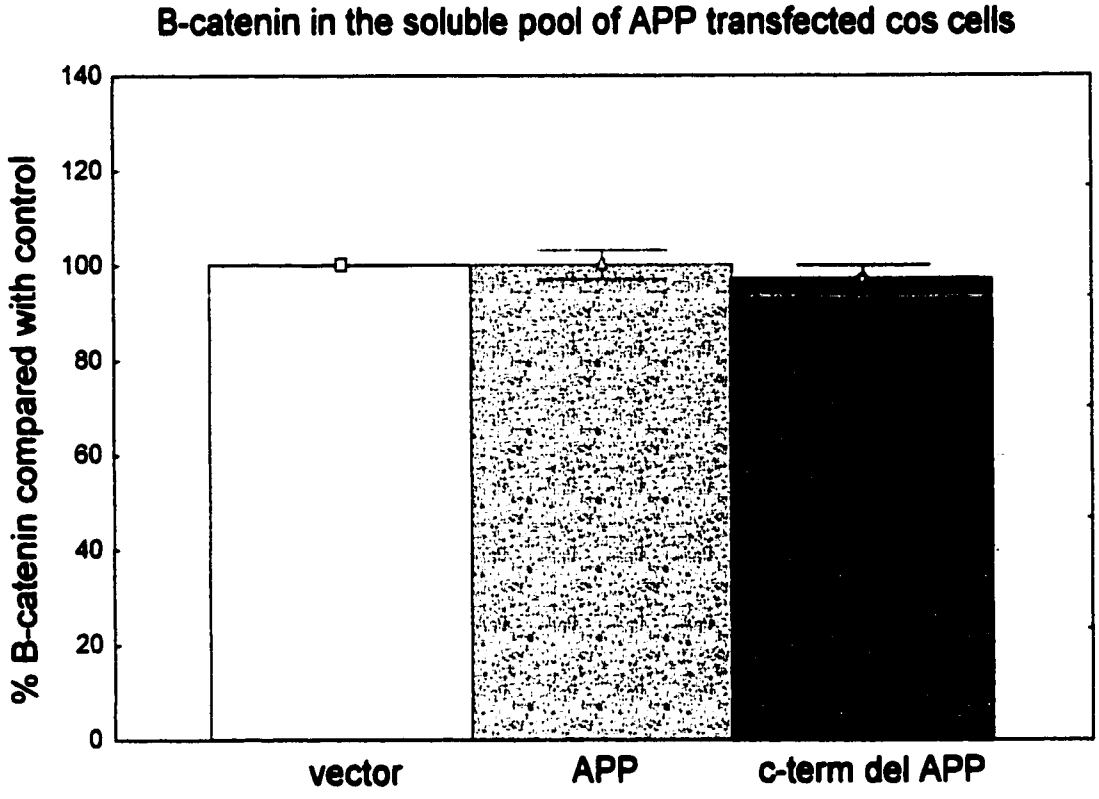


Fig. 25-B. Quantitation of β-catenin in the cytosolic fraction of APP transfected cos-7 cells. Band intensities were quantitated by densitometric scanning using a phosphorimager. The β-catenin values were normalised against that of G3PDH. β-catenin levels in the cytosolic fractions were not statistically different among the groups (Values are expressed as Mean ± S.E.M).

Effects of ^{Swe}mutant APP on β-catenin levels in cos-7 cell cytosolic and particulate fractions:

To determine whether the ability of APP to downregulate β-catenin levels is lost with the introduction of Swedish mutation in the APP gene, we transfected cos-7 cells with plasmid containing ^{Swe}mutant APP (670,671). After 48 hrs. of transfection, the cells were lysed and the cytosolic, membrane and particulate fractions were obtained as described above. Examination on Western blots revealed no significant difference between the ^{Swe}mutant APP and vector control, suggesting that ^{Swe}mutant APP is unable to downregulate β-catenin levels as the full length APP (Fig. 26 & 26A)

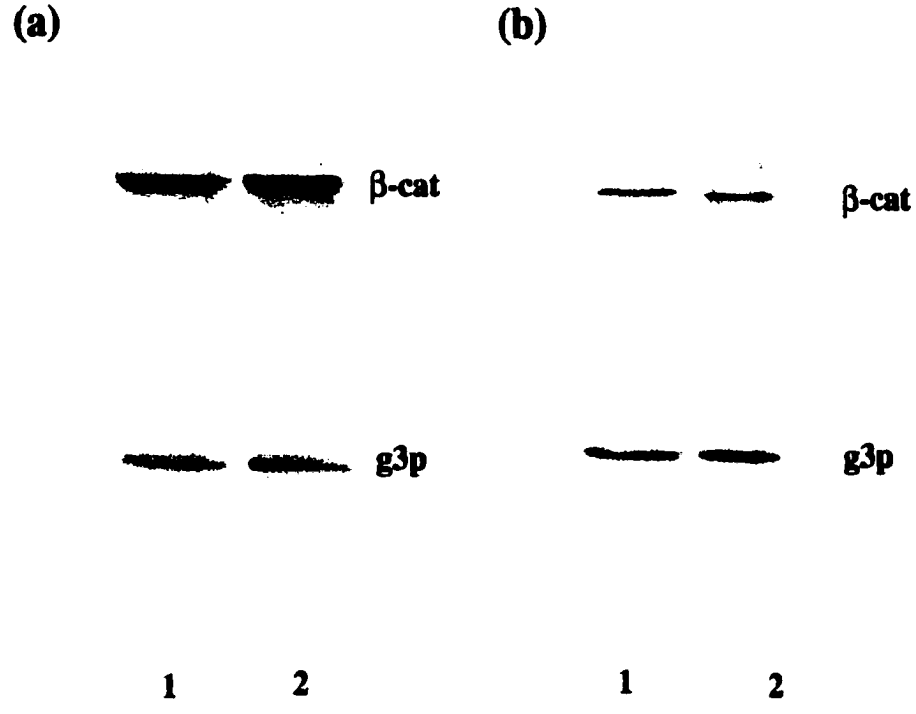


Fig. 26. Western blot of particulate and cytosolic fractions of APP swe transfected cos-7 cells. (a) The 100,000 x g pellet - particulate pool of β-catenin and (b) supernatant - cytosolic pool of β-catenin were electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane followed by incubation with mouse monoclonal β-catenin antibody. Blots were developed with HRP-conjugated anti-mouse secondary antibody and ECL reagents. Band intensities were calculated by densitometric scanning using a phosphorimager. Lane 1 - Vector control, Lane 2 - APPswe. The β-catenin values were normalised against that of G3PDH. APP levels in the APPswe transfected cells were significantly higher than those in the vector control transfected cells. Three determinations were made to insure reproducibility.

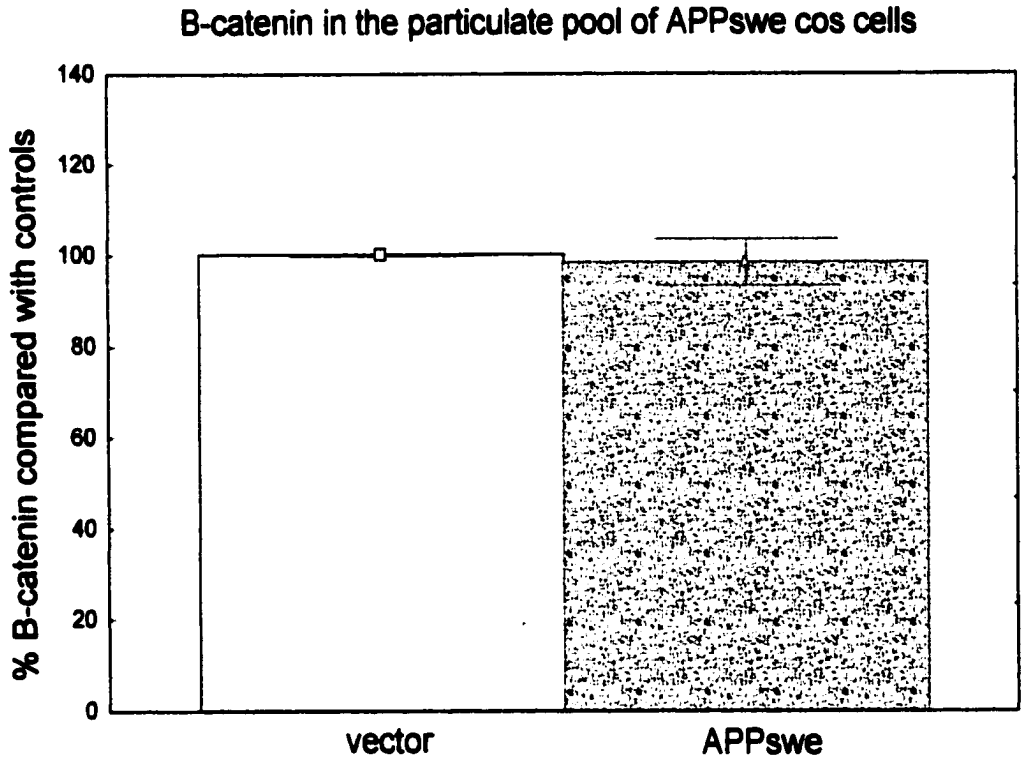


Fig. 26-A. Quantitation of β-catenin in the particulate fraction of APPswe transfected cos-7 cells. Band intensities were quantitated by densitometric scanning using a phosphorimager. The β-catenin values were normalised against that of G3PDH. β-catenin levels in the particulate fraction were unchanged upon transfection of cos-7 cells with APPswe, indicating that mutation of APP-FL at positions 670, 671 was enough to abolish it's ability to downregulate the particulate pool of β-catenin (values are expressed as Mean ± S.E.M).

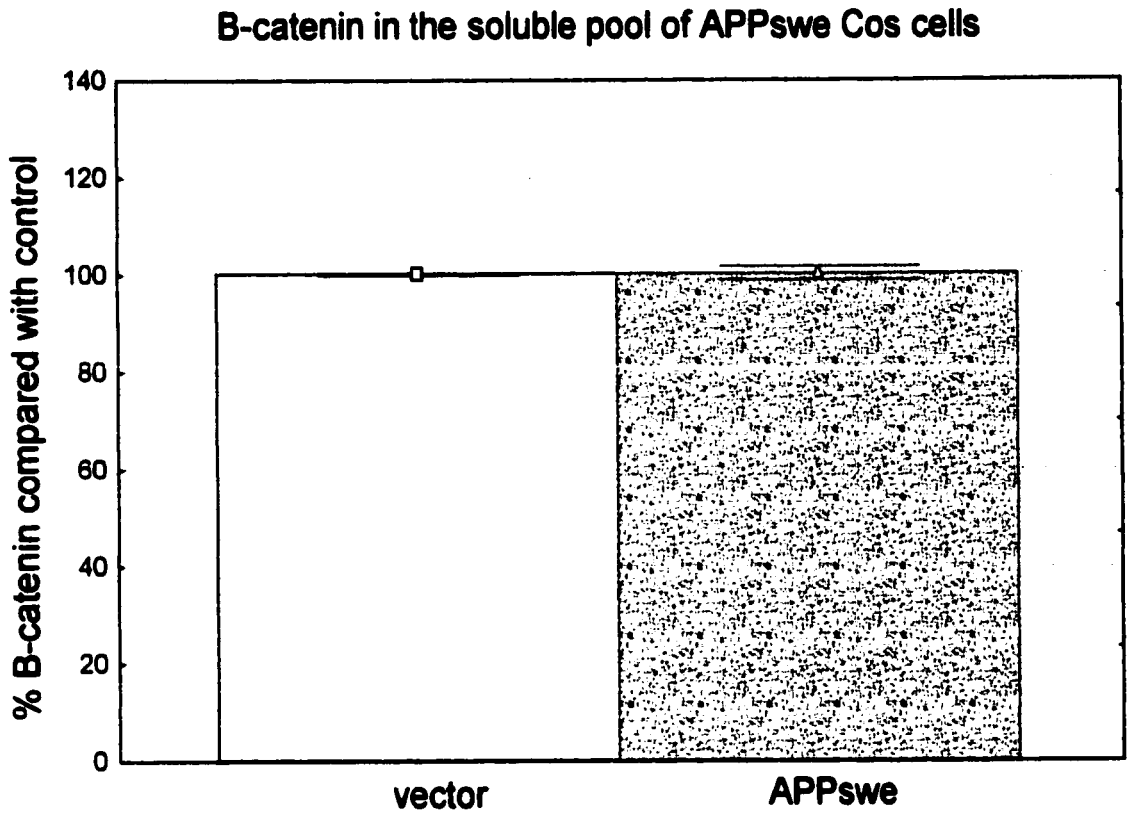


Fig. 26-B. Quantitation of β-catenin in the cytosolic fraction of APPswe transfected cos-7 cells. Band intensities were quantitated by densitometric scanning using a phosphorimager. The β-catenin values were normalised against that of G3PDH. β-catenin levels in the cytosolic fraction were unchanged upon transfection of cos-7 cells with APPswe. (values are expressed as Mean ± S.E.M).

β-catenin is downregulated by full length APP. When the cytoplasmic tail 44 amino acids are deleted as in $\Delta APP_{708-751}$, the ability of APP to downregulate β-catenin is lost suggesting that the downregulating effects of APP is mediated through its cytoplasmic tail. Upon mutation of just two amino acids (670 and 671, as seen in APPswe), the ability of APP to downregulate β-catenin is abolished. It is plausible that

these mutations may alter the functional properties of the cytoplasmic tail of APP such that it mimics ΔAPP 708-751, leading to it's inability to downregulate β-catenin. Whether APP mediates these effects by direct association with β-catenin is being investigated by co-immunoprecipitation experiments. It is our educated guess that APP's effect on β-catenin is perhaps mediated through other molecules inside the cell because previous investigations into β-catenin interacting proteins did not reveal the presence of APP, whereas on the other hand, several proteins were identified that are now accepted to be directly associated with β-catenin inside the cell. This question will be answered with the results of co-immunoprecipitation experiments.

Thus, our experiments with cos-7 cells have demonstrated that normal APP downregulates β-catenin levels. When it is mutated (APPswe), the levels of β-catenin cannot be downregulated. In other words, the steady state levels of β-catenin is increased if APP functions are altered as in the case of cytoplasmic tail truncation or APPswe mutation. In sporadic cases of AD, there is no mutation in APP. Inspite of that we have observed that β-catenin levels are increased. This may be possible if APP in the cytoplasmic tail region is cleaved such that it is now unable to downregulate β-catenin. Alternatively, there must be protein-protein interactions in the cytoplasmic tail of APP, whose functions are altered in sporadic cases of AD such that the ability to downregulate β-catenin is lost resulting in an increase in the levels of β-catenin.

Discussion

This study was conducted to determine whether the abnormal hyperphosphorylation of tau in AD reported previously (Grundke-Iqbal et al., 1986a, 1986b, Iqbal, K et al., 1986, 1989) is a product of a protein phosphorylation/dephosphorylation imbalance which can result in non-tau phosphoproteins also being abnormally hyperphosphorylated in brains of AD patients. We found that two non-tau proteins, β-tubulin and β-catenin are also abnormally hyperphosphorylated in AD brains, suggesting a general imbalance in the protein phosphorylation-dephosphorylation system in brains of AD patients.

Microtubule associated protein tau is abnormally hyperphosphorylated in AD brain (Grundke Iqbal et al., 1986 a ; 1986b ; Iqbal et al., 1986;1989 ; Kopke et al., 1993). Phosphorylation of any protein is a net result of the activities of kinases / phosphatases that act upon them. Therefore, hyperphosphorylation of tau might be due to an increase in kinase(s) or a decrease in phosphatase(s) activities or a combination of both. Tau can be phosphorylated by several kinases, both PDPKs and non-PDPKs *in vitro* (Ishiguro et al., 1992 ; Baudier et al., 1987 ; Roder et al., 1991 ; Drewes et al., 1992 ; Ledesma et al., 1992 ; Littersky et al., 1992 ; Singh et al., 1994). Most of these kinase activities are not found to be upregulated in AD. However, recently, Patrick et al., 1999, demonstrated upregulation of cdk5 activity in AD brains. To be activated, cdk5 requires to be associated with the regulatory subunit p35. They found that p25, a truncated form of p35 accumulates in the brains of AD patients. This accumulation correlates with the increase in cdk5 activity. In vivo, the p25/Cdk5 complex hyperphosphorylates tau, which reduces tau's ability to associate with microtubules.

On the other hand, the phosphorylation of tau in mammalian brain appears to be regulated mainly by protein phosphatase-2A (Gong et al., 2000). The activity of this enzyme is compromised in AD brain (Gong et al., 1993 ; Gong et al., 1995). All these data with reference to the activities of kinases and phosphatases point to a general imbalance in the protein phosphorylation-dephosphorylation system in AD brain.

In our study, we investigated the presence of hyperphosphorylated non-tau proteins. Assaying for phosphate bound to proteins is one way to determine phosphorylation status of proteins (Hess et al., 1975). We found that the 100,000 x g pellet of gray matter homogenates from AD cases revealed a significant increase (~11.0 pmoles phosphate per ug protein) in the phosphate bound to proteins compared with that from age-matched controls. This is a significant difference because hyperphosphorylated tau can only account for ~3.0 pmoles phosphate per ug protein. These data indicate presence of other abnormally hyperphosphorylated proteins in AD brain.

Western blot analyses with P-Ser antibodies revealed hyperphosphorylation of several proteins. All, except a ~54 kD protein, were also labeled with antibodies to tau, indicating hyperphosphorylation of a non-tau protein.

We purified the ~54 kD protein by preparative SDS-PAGE. MS-MALDI analysis revealed the identity of the ~54 kD protein as β-tubulin (X 79535). We confirmed the identity of the purified protein by Western blots developed with anti-β-tubulin antibodies. Since MS-MALDI and Western blot analyses does not reflect on the purity of the protein, N-terminal amino acid sequencing was carried out to investigate the purity of the preparative electrophoresis-purified ~54 kD protein. This technique also is useful in the identification of the protein. Amino terminal sequencing revealed that not only was the protein β-tubulin, but also that it was pure. Based on Western blots with anti-phospho-Ser antibodies, MS-MALDI analyses, Western blotting with anti-β-tubulin antibodies and amino terminal amino acid sequencing, it appears that β-tubulin (X 79535) is probably abnormally hyperphosphorylated in AD brain. However, conclusive evidence for the abnormal hyperphosphorylation of of β-tubulin in AD brain will require an assay of the P-Ser levels in purified of β-tubulin. These studies are currently underway. β-tubulin is one of the two tubulin monomers (other being α-tubulin) that forms the microtubules (MT) with the help of microtubule associated proteins. In the affected neurons of AD brain, the microtubules are rarely seen (Terry. et al., 1972). Large body of evidence has established that hyperphosphorylated tau contributes to the microtubule disassembly. Tau is required for MT assembly, maintenance of MTs and stability of MTs (Weingarten et al., 1975). It has been demonstrated that unlike normal tau, AD P-tau cannot promote MT assembly, cannot stabilize MT or maintain the MT structure (Alonso et al.,1994 ; Alonso et al.,1996 ; Iqbal K. et al., 1994). Also, AD P-tau sequesters normal tau and other MAPs and makes them unavailable for MT assembly (Alonso et al., 1997).

But, the possible role of tubulin in AD brain towards the disassembly of microtubules has never been considered. A study has shown that bovine brain tubulin when phosphorylated, by a serine-threonine kinase - CaM Kinase, loses its ability to assemble microtubules and when dephosphorylated, tubulin regains its ability to assemble microtubules (Wandosell et al., 1986). Moreover, phosphorylation enhances its interaction with the membrane (Hargreaves et al., 1986).

The hyperphosphorylated β-tubulin observed by us may be either from the membrane pool or cytoplasmic pool. If it is from the cytoplasmic pool then it may have aggregated as a result of hyperphosphorylation into the particulate pool. β-tubulin (X 79535) is not a major isotype of β-tubulin in the neurons. β-tubulin-III is the predominant isotype of β-tubulin in neurons. Therefore, a slight increase in the specific isotype may remain undetected considering its relatively small contribution to the total pool. Although, the contribution of β-tubulin (X 79535) in MT assembly in a young, healthy neuron may not be very significant, it may play a significant role in the AD brain for the following reasons. In the brains of AD patients, normal tau is sequestered by AD P-tau. This suggests a decrease in available normal tau for MT assembly. Under these conditions, even a small fraction of hyperphosphorylated β-tubulin (presumably in a compromised functional state) may contribute significantly to the disassembly of MTs.

If β-tubulin belongs to the insoluble pool, it would be hard to attribute any function, since this area of research remains unexplored.

β-catenin is another protein that we have observed to be hyperphosphorylated at threonine residues in AD brain. β-catenin is a very promiscuous protein because of it's capacity to interact with a large number of proteins inside the cell (Rubinfeld et al., 1993 ; Ikeda et al., 1998 ; Stahl et al., 1999 ; Tago et al., 2000 ; Kawajiri et al., 2000). Therefore, it might play very important roles in maintaining the homeostasis of the cell. There are two major known functions of β-catenin depending on which cellular pool it belongs to viz. the cytosolic pool and the particulate pool. The cytosolic pool is involved in the Wnt signalling pathway (Kirkpatrick et al., 1995 ; Miller et al., 1996). When this pathway is activated, inactivation of GSK-3β-catenin (Cook et al., 1996) leads to the stabilization and accumulation of hypophosphorylated β-catenin (Peifer et al., 1994a,b), which then interacts with members of the TCF family of transcription factors to bring about gene expression (Behrens et al., 1996 ; Huber et al., 1996b ; Molenaar et al., 1996). The particulate pool of β-catenin on the other hand, forms a part of the adherens junctions, mediating cell-cell adhesion as well as cell-cell communication (Nagafuchi et al., 1989 ; Ozawa et al., 1992). It serves as a bridge between cadherins and the actin cytoskeleton. Therefore, hyperphosphorylation of β-catenin may have significant consequences. If the hyperphosphorylated β-catenin belongs to the cytosolic pool, then abnormal hyperphosphorylation may dysregulate its normal functions in gene expression. It has been reported that loss of β-catenin function can lead to apoptosis (Neo et al., 2000 ; Weihl et al., 1999). Conversely, if the hyperphosphorylated β-catenin is from the particulate pool then, it might interfere with cell-cell adhesion or cell-cell communication. The cell-cell adhesion and / or cell cell communication may be hampered such that the cells get detached from its neighbouring

cells leading to its death and ultimately contributing to the pathogenesis of AD. Detachment of β-catenin from the cell adherens junctions has been previously shown to induce apoptosis (Vallorosi et al., 2000) Interestingly, in the blood brain barrier (BBB) in AD, there is less β-catenin immunoreactivity (A.Vorbrodt, personal communication). This may be a result of hyperphosphorylation of β-catenin. Hyperphosphorylation of β-catenin at tyrosine residues in epithelial cells transfected with Src and Ras leads to detachment of β-catenin from the adherens junctions and loss of cell-cell attachment. Whether similar events might follow hyperphosphorylation of β-catenin at threonine, is currently not understood. The interactions of β-catenin and cadherin, and consequently cell adhesion, may be regulated in part through phosphorylation of β-catenin (Matsuyoshi et al., 1992 ; Behrens et al., 1993 ; Hamaguchi et al., 1993 ; Hoschuetzky et al., 1994 ; Peifer et al., 1994b ; Shibamoto et al., 1994 ; Kinch et al., 1995 ; Yost et al., 1996). When epithelial cells were transformed via the expression of activated Src and Ras, it resulted in tyrosine phosphorylation of β-catenin and a coincident decrease in cell adhesion, disassembly of adherens junctions and conversion of epithelial cells to a mesenchymal phenotype (Matsuyoshi et al., 1992 ; Behrens et al., 1993 ; Hamaguchi et al., 1993 ; Shibamoto et al., 1994 ; Kinch et al., 1995 ; Takeda et al., 1995). Phosphorylation of particulate pool of β-catenin also occurs on Ser / Thr residues. It has been reported that membrane or particulate pool of β-catenin is more highly phosphorylated on both Ser / Thr and on Tyr residues than souble β-catenin (Peifer et al., 1994b), though it is unclear which kinases are responsible for this phosphorylation.

Our data revealed that hyperphosphorylation of β-catenin is at threonine residues. In order to understand the regulation of these abnormal hyperphosphorylation sites on β-catenin, we are currently attempting to map the phosphorylation sites by mass spectrometry in collaboration with the laboratory of Don Hunt at the University of Virginia. Knowledge of these abnormal phosphorylation sites on β-catenin from AD brains will enable us to speculate on the candidate enzymes that might be responsible for the hyperphosphorylation. This information will be of great utility in coming to an understanding about what kinases / phosphatases underlie the imbalance in the protein phosphorylation - dephosphorylation system in AD brain.

Thr41 on β-catenin is a consensus site for phosphorylation by a GSK-3β (Yost et al., 1996) It has been reported (Aberle H et al., 1997) that upon phosphorylation at this site, β-catenin gets tagged for ubiquitin mediated proteosome degradation, thereby regulating the levels of β-catenin and ultimately the signaling mediated through β-catenin. We took advantage of the commercial availability of this antibody. Upon Western blotting the 100,000 x g pellet with this antibody and normalising the immunoreactivity with that of β-catenin, we found no observable difference between AD and controls. These results are consistent with our observation that there is an increase in the levels of β-catenin in AD brain. Therefore, there should be either a decrease or no change in phosphorylation at Thr^{41} in AD brain. These data also suggest that the hyperphosphorylation is at some other threonine site(s).

We have also found that the levels of β-catenin in the AD brain 100,000 x g particulate fraction (particulate pool of β-catenin) was significantly higher than age-matched controls. Whereas, the cytosolic pool of β-catenin showed no observable differences. The data from the cytosolic pool of β-catenin corroborated with Yankner's observation that the cytosolic pool of β-catenin remained unchanged in brains of sporadic AD patients. β-catenin levels are known to be regulated in several ways. At the cellular level, Adenomatous Polyposis Coli (APC), a tumor suppressor protein and GSK-3β negatively regulates β-catenin. Wnt signaling upregulates β-catenin by inhibiting degradation through GSK-3β mediated phosphorylation and ubiquitin-proteosome mediated degradation. PS-1 (gene involved in some of the familial cases of AD) can also regulate the levels of β-catenin. Mutations of PS-1 associated with familial Alzheimer disease can stabilize β-catenin resulting in an increase in its level (Kang et al., 1999). Although, contradictory observations were reported from other laboratories, ie. cytoplasmic β-catenin levels were decreased by mutations in PS-1 (Zhang et al., 1998 ; Murayama et al., 1998), it can be safely said that PS-1 regulates β-catenin levels in different cellular pools. According to the study by Kang et al.,1999, inducible overexpression of PS-1 led to increased association of β-catenin with GSK-3β, a negative regulator of β-catenin, and accelerated the turnover of endogenous β-catenin. In support of the finding, the β-catenin half-life was dramatically longer in fibroblasts deficient in PS-1, and this phenotype was completely rescued by replacement of PS1, demonstrating that PS1 normally stimulates the degradation of β-catenin. In contrast, overexpression of FAD-linked PS1 mutatns (M146L & ΔX9) failed to enhance the association between GSK-3B and β-catenin and interfered with the

constitutive turnover of β-catenin. These effects of PS1 were also demonstrated in vivo in the brains of transgenic mice in which the expression of the M146L mutant PS1 correlated with increased steady state levels of endogenous β-catenin. PS-1 alters APP processing. PS-1 and APP are associated physically as shown by coimmunoprecipitation experiments (Takashima et al., 1998). Both PS-1 and APP when mutated cause AD. Due to the inter-relationship between PS-1 and APP, we asked the question if APP can regulate the levels of β-catenin.

We transiently transfected cos-7 cells (which have very little endogenous APP), with various constructs of APP – (1) APP-FL (APP full length ie. APP751), (2) APPswe (APP M670N,K671L) and (3) APPΔC (APP $\Delta_{708-751}$)

Upon transfection of cos-7 cells with APP-FL, we found that the levels of β-catenin in the cell lysates decreased by ~40%. The ability of APP FL to downregulate β-catenin levels was lost when the cytoplasmic tail was deleted from APP, indicating this part of APP is important for mediating the downregulation of β-catenin. APPswe was also unable to downregulate β-catenin indicating the mutation interfered with the ability of APP to downregulate β-catenin.

β-catenin is found in two major pools – cytoplasmic and particulate. Having observed APP's ability to regulate β-catenin levels in the cell lysates, we investigated whether there was a particular pool of β-catenin that is selectively regulated by APP. Therefore, we measured the levels of β-catenin in the particulate and cytosolic pools from cos-7 cells transfected with the above mentioned APP constructs. We found that only the particulate pool of β-catenin is influenced by APP. The particulate pool is known to be an integral component of the adherens junction and is involved in cell-cell adhesion as well as cell-cell communication.

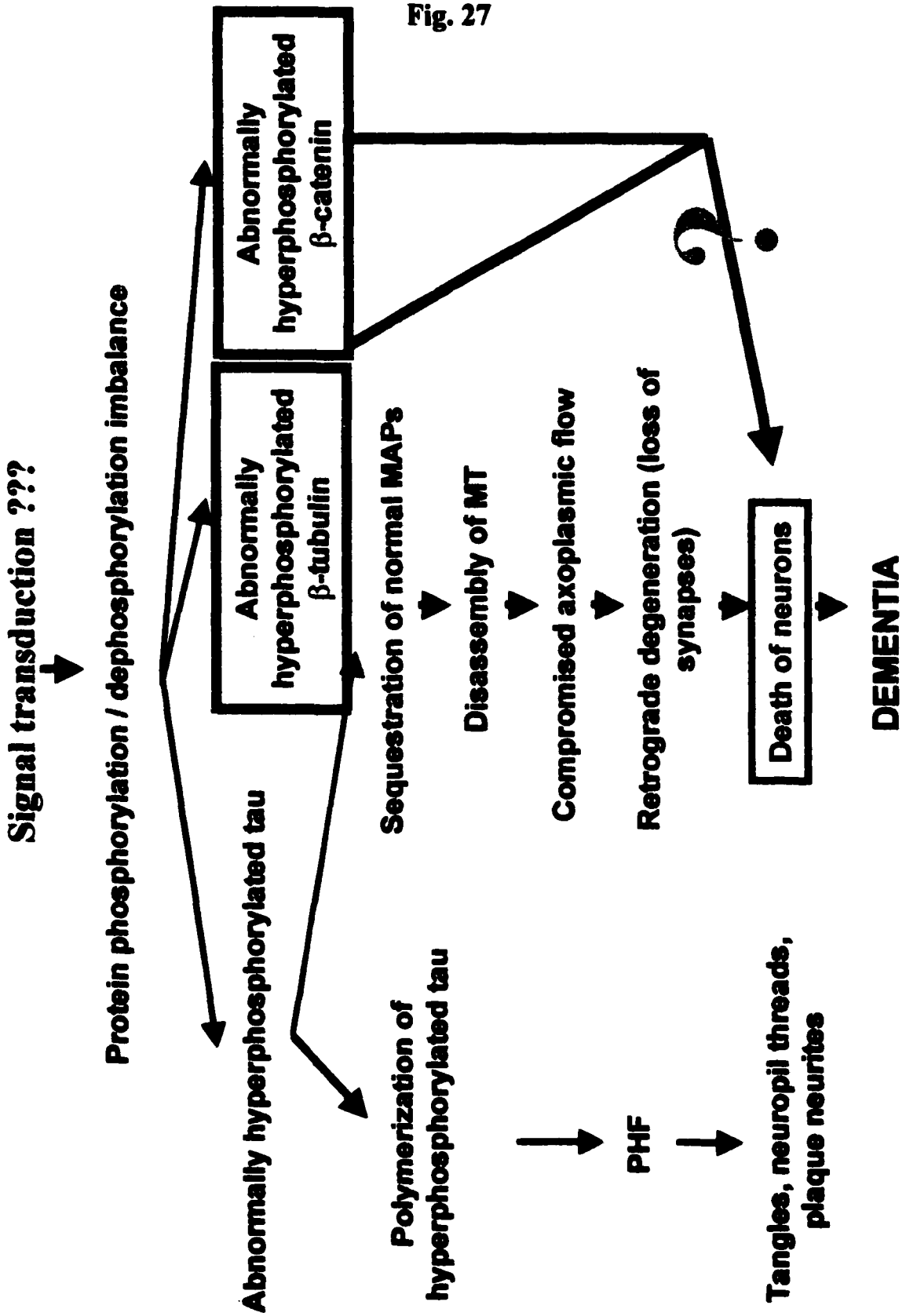
We propose that events similar to those associated with changes in APP function (such as is the case with APPswe) may be responsible for the elevation or accumulation of β-catenin in the brains of sporadic AD patients.

In summary, we have demonstrated hyperphosphorylation of β-tubulin as well as β-catenin, two non-tau proteins, indicating that a general imbalance in protein phosphorylation-dephosphorylation exists in the brains of AD patients (fig. 27). To further support this theory, other proteins have also been reported to be hyperphosphorylated in AD brains. CRMP-2 is hyperphosphorylated at serine as well as threonine in the brains of AD patients (Gu et al., 2000). Dynein, a motor protein is also hyperphosphorylated in AD brain (Kopec et al., 1997). The level of β-catenin is elevated in the particulate fraction of AD brain. This may be as a result of events that are similar to those associated with mutations in APP or PS-1 or may be a result of hyperphosphorylation which then leads to accumulation of β-catenin.

In conclusion, a general imbalance in protein phosphorylation-dephosphorylation system in AD brain leads to the hyperphosphorylation of several non-tau proteins including β-tubulin and β-catenin. Hyperphosphorylated β-tubulin and β-catenin may contribute to the pathogenesis of AD.

Fig. 27. Model for the role of hyperphosphorylated β-tubulin and β-catenin in the pathogenesis of Alzheimer disease.

Fig. 27



MATERIALS AND METHODS:

Brain tissue:

The brains of 15 AD patients with a mean age of 71.8 yrs (± 8.3 yrs) was chosen for the present study. All the AD brains had been histopathologically confirmed and contained the hallmark lesions specific for AD viz. plaques and tangles. Post-mortem delays were 4.0 ± 1.5 hrs. The mean age of the non-AD brains were 79.4 yrs (± 4.8 yrs.). Post-mortem delays were 4.5 ± 1.0 hrs. The human tissues were obtained from the Brain tissue Resource Center McLean Hospital, Belmont, MA and and the Netherlands Brain Bank (Dr. Rivka Ravid), The Netherlands and from IBR Brain Bank (Dr. Pete Kozlowski), Staten Island, NY.

Chemicals and Reagents:

Sodium Vanadate, Sodium desoxycholate and trichloroacetic acid were from Fisher Scientific Co. Dithiothreitol was from Calbiochem. Chelating Sepharose Fast Flow was from Pharmacia Biotech. Polyclonal antibodies to phosphoserine and phosphothreonine and monoclonal antibodies to phosphotyrosine were from Zymed Labs, San Francisco, CA. [^{125}I]anti-mouse IgG and [^{125}I]anti-rabbit IgG were from Amersham. Anti-mouse-HRP and anti-rabbit-HRP were from Santa Cruz Biotechnology labs, California. Monoclonal anti-β-catenin antibodies were from transduction laboratories and anti-β-tubulin antibodies were from Calbiochem. All remaining reagents were obtained from Sigma.

Congo Red Staining

To estimate the extent of amyloid plaques and neurofibrillary tangles, a speck of tissue was taken on a glass microslide(1" x 3" x 1 mm) and a smear made out of it with 2 microliters of 0.1% Congo red in 1% SDS and 50% glycerol. The smear was covered with a glass coverslip before viewing under polarised light.

Separation of phosphoproteins

Immobilised metal ion affinity chromatography (IMAC) exploits a molecule's affinity for chelated metal ions. The amino acid histidine present in many proteins forms complexes with transition metal ions such as Fe^{3+}. Chelating Sepharose Fast flow with immobilized Fe^{3+} will therefore selectively retain proteins with exposed histidine. Phosphorylation increases the binding of histidine to Fe^{3+}. The strength of binding is also affected by the pH of the buffer used. This technique has been successfully used to isolate phosphoproteins differing in as little as one phosphate group(Andersson, and Porath,., 1986). The exact principle behind the affinity of phosphoproteins to Fe^{3+}, chelated on to agarose gel via a spacer arm - iminodiacetic acid (IDA), is not well understood. However, it has been observed that phosphate ions change the color of the IDA- Fe^{3+} gels, indicating complex formation. Also, phosphoaminoacids, strongly bind to IDA- Fe^{3+} at acidic pH and are readily recovered from IDA- Fe^{3+} columns by increasing the pH of the buffer or with 20 mM phosphate.

Chelating Sepharose was packed in a column (1 x 4 cm ; 2 ml bed volume) and 2.5 bed volumes of 50 mM $FeCl_3$ was passed through the column. This was followed by

extensive washing of the column with distilled water, until the eluent becomes colorless. The column was then equilibrated with 0.1 M MES pH 5.7, containing 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF. After loading the sample, the proteins were eluted by step elution, first by using 0.1 M MOPS, pH 7.0, containing 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 1 M NaCl and then by 0.1 M Tris, pH 9.0, containing 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 1 M NaCl. The flow rate was 15 ml / hr. throughout the procedure ((Andersson and Porath, 1986).

Removal of non-protein phosphates

To an aliquote of the sample, 0.0125% Na-desoxycholate and 6% trichloroacetic acid (TCA) were added. After centrifugation in a Beckman Model GS-6R centrifuge at 3600 r.p.m. for 30 min., ether : ethanol (1 : 1) mixture was added to the pellet and mixed thoroughly before centrifugation. The pellet obtained was then boiled in 16% TCA for 20 min. and centrifuged as above. The pellets were washed with 10% TCA before resuspending in 0.1 N NaOH. Protein was assayed by modified Lowry method (Bensadoun, and Weinstein, 1976) and required amounts of protein dried using a Jouan RC 10.22 vaccum concentrator and the dried samples then used for phosphate analysis.

Phosphate analysis

For total phosphate estimation, to the dry samples, 60 microliter of 5% $MgNO_3$ in 95 % ethanol was added. The samples were heated on a heating block for 20 min., followed by ashing over open flame, until white powder was formed. The powder was dissolved in 40 microliters of 1 N H_2SO_4, followed by centrifugation to get rid of any

precipitates that may interfere with the assay. The supernatant was taken for analyses. A 25 microliter aliquot was taken on a microtiter plate to which 50 microliters of dye mixture (see below for composition) was added to the samples. After 15 min. incubation, optical density values were read at 660 nm. The values for protein-bound phosphate were calculated by subtracting the values of inorganic phosphate from the total phosphate (Hess, and Derr, 1975).

Dye mixture composition:

0.045 % Malachite green, 4.2 % ammonium molybdate in 4 N HCl, 3:1 mixed with 100 microliters of 2% Tween-20.

Western blot analyses

The 100, 000 x g pellet or supernatant of the tissue homogenate was mixed and heated in 1 X Laemmli's sample buffer for 5 min. After protein determination by modified Lowry's method (Bensadoun, and Weinstein, 1976) equal amounts of protein were separated by electrophoresis on a 5- 15 % gradient SDS-PAGE gels (Laemmli, 1970). After transferring proteins to PVDF membrane, the membranes were blocked with 5 % BSA-TBST (bovine serum albumin -50 mM Tris buffer, pH 7.6, 0.9% NaCl, 0.05 % tween-20), and incubated with the appropriate primary antibody, followed by incubation with [^{125}I] labeled or HRP labeled secondary antibody. After the membranes were washed adequately with 0.05 % Tween-20 in 50 mM Tris buffer, pH 7.6, the membranes were incubated with ECL reagents(1 : 1), whenever HRP-labeled secondary antibody were used. The membranes were exposed to a phosphorimager screen or a

kodak x-ray film. The immunoreactivity was quantitated using a phosphorimager (Fuji film BAS-1500, Japan).

Preparative Electrophoresis

Preparative electrophoresis was carried out using Model 491 prep cell (Bio-rad Laboratories). Protein samples (1 mg) was heated for 5 min in 1 X Laemmli buffer before loading on top of the stacking gel of the Prep Cell. Fractions (2 ml total volume) were collected at a flow rate of 60 ml / hr. Electrophoresis was carried out at 100 volts - constant voltage. The fractions were concentrated using Amicon concentrators before loading on an analytical 10 % SDS- PAGE.

RNA Isolation

RNA was extracted by homogenizing tissue samples in TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.) using a polytron homogenizer. The sample volume never exceeded 10 % of the volume of TRI reagent used for homogenization. The homogenized samples were stored for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. After adding 0.2 ml chloroform per ml of TRI reagent, the samples were covered tightly, shook vigorously for 15 seconds, after which they were stored at room temperature for 2 - 3 minutes. The resulting mixture was centrifuged at 12,000 x g (max.) for 15 minutes at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, interphase, and the colorless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the intephase and organic phase.

The aqueous phase was transferred to a fresh tube for precipitation of RNA by mixing with isopropanol. For 1 ml TRI reagent used for initial homogenization, 0.5 ml of isopropanol was used. Samples were stored at room temperature for 5 - 10 minutes and centrifuged at 12,000 x g (max.) for 10 minutes at 4°C. RNA precipitate, which forms a gel-like pellet on the side and bottom of the tube was then washed once with 1 ml 75 % ethanol by vortexing and subsequent centrifugation at 7500 x g (max.) for 5 minutes at 4°C. RNA pellet was then dried by air drying or under vacuum (5 - 10 min.). The RNA pellet was then dissolved in small volumes of nuclease-free water. Concentrations and amounts recovered were calculated from $O.D_{260}$ nm readings.

Northern blot analysis

RNA (10 ug / lane) was run on 1% agarose / 0.66 M formaldehyde gels in MOPS buffer. Following electrophoresis, RNA was transferred to Immobilon Ny+ (Fisher Scientific, Springfield, NJ, U.S.A.) membranes using downward capillary transfer. Membranes were cross-linked (UV Stratalinker 1800, Stratagene, La Jolla, CA, U.S.A.) and incubated in prehybridization solution (5 X saline-sodium phosphate-EDTA buffer, 5 X Denhardt's, 1% sodium dodecyl sulfate, 10% dextran sulfate, 50 % formamide, 100 ug / ml calf thymus DNA) for 1 - 4 hrs at 42°C. The membranes were hybridized with [^{32}P]-random prime-labeled cDNA probe overnight at 42°C with fresh prehybridization solution that contained ~10^9 dpm of probe per membrane.

cDNA Probe

The cDNAs were made by RT-PCR with AD brain RNA as template and primers (Integrated DNA Technologies, Inc., Coralville, IA, U.S.A.) listed below. RT-PCR was performed using the Epicentre technologies MasterAmp RT-PCR Kit according to the manufacturer's instructions. The probe was prepared by incubating the template RNA (100 ng), 0.25 uM each primer, 400 uM each dNTP, RT-PCR buffer { 50 mM Tris-HCl (pH 9.0, 25°C), 20 mM (NH4)2SO4, 12.5 mM NaCl }3 mM MgCl2, 5 ul MasterAmp 10 X PCR enhancer, 0.5 mM MnSO4 and 2.5 units of RetroAmp RT DNA Polymerase on ice immediately before starting the reaction. Mineral oil was added and centrifuged briefly . First strand was synthesised (reverse transcription) in a thermal cycler preheated at 60°C for 20 minutes. 25 cycles of PCR were performed : Denaturing at 92°C for 30 seconds and annealing and extending the primers at 68°C for 60 seconds. 5 ul reaction sample was analyzed on a 1% agarose gel. A single PCR product of 454 bp was obtained as expected. After PCR products were purified (Wizard PCR purification kit, Promega) and labeled (Prime-a-Gene kit, Promega) according to the manufacturer's instructions with [α-32P]dCTP (Amersham Life Sciences Corp., Arlington Heights, IL, U.S.A.). Labeled products were purified on MicroSpin S-300 HR columns (Amersham-Pharmacia Biotechnology, Piscataway, NJ, U.S.A.).

Cell culture and Transfection

Cos-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5% CO2. Transfections were carried out using LipofectAMINE PLUS Reagent (Life Technologies, U.S.A.). The day before

transfection, cells were trypsinized and counted before plating them so that they are 50 - 80- % confluent the day of transfection. To 1 ug of each DNA, 4 ul of PLUS reagent was added and incubated for 15 minutes at room temperature to precomplex the DNA with the reagent. In a second tube, LipofectAMINE reagent (4 ul) was diluted with serum-free dilution medium (100 ul). Pre-complexed DNA and diluted LipofectAMINE reagent mix were combined and incubated for 15 minutes at room temperature. While complexes are forming, medium on the cells were replaced with serum-free transfection medium (0.8 ml). The DNA-PLUS - LipofectAMINE reagent complexes were added to each dish containing fresh medium on cells. The complexes were mixed into the medium gently and incubated at 37°C at 5% CO2 for 3 hrs. After 3 hrs incubation, the volume of medium was increased to normal volume and serum was added to bring the final concentration to that of normal growth medium. After 48 hrs, of transfection cells were harvested for analyses.

APP constructs

The APP constructs that were inserted into the vector are as shown below.

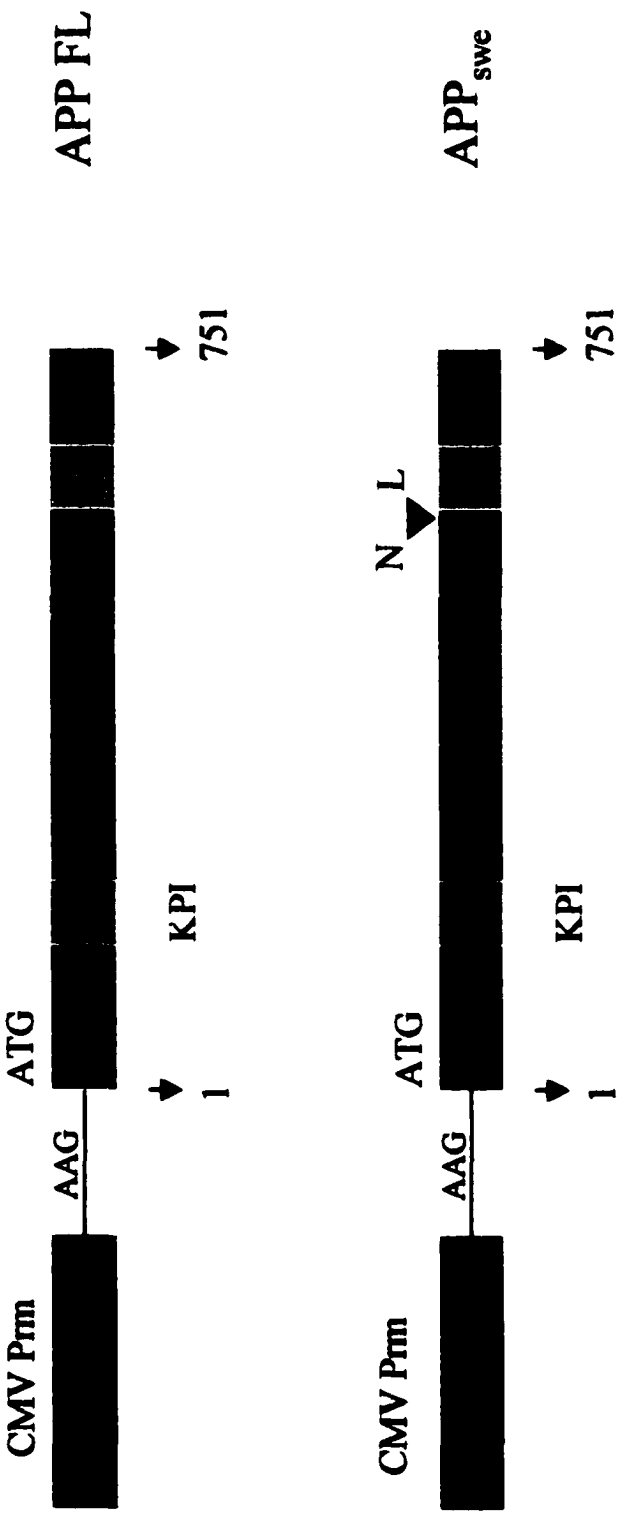


Fig. 28. APP constructs used to transfect cos-7 cells

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