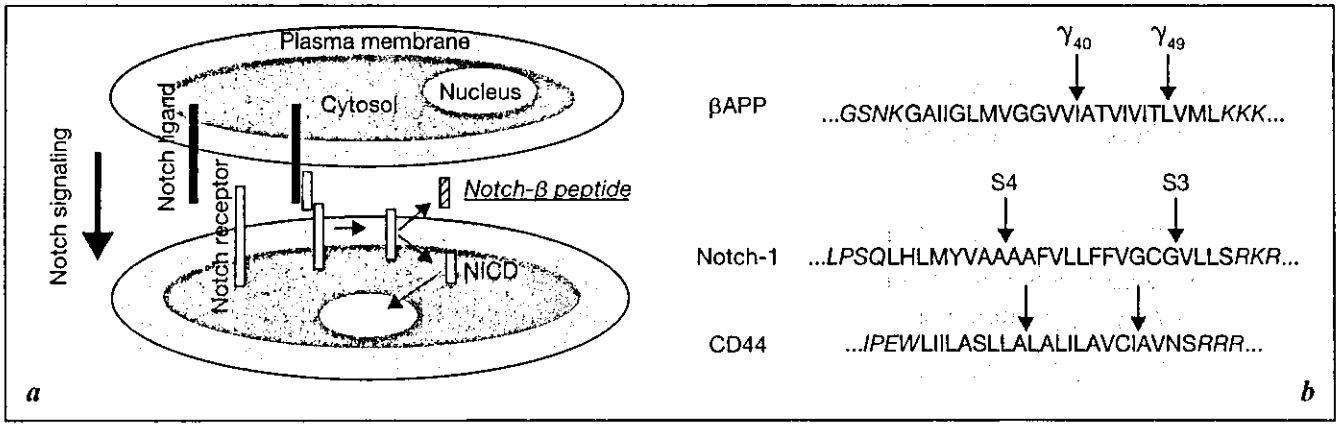

Fig. 4. ***a*** Notch signaling accompanies secretion of Notch-β peptide. By sequential endoproteolysis of Notch-1 shown in figures 2b, 3, an Aβ-like peptide, Nβ, is released. ***b*** PS-dependent intramembranous proteolysis, which we termed the 'dual cleavage' mechanism. Arrows indicate proteolytic cleavage sites. Small transmembrane peptides between 2 cleavage sites (arrows) have not yet been identified.

but are formed by distinct proteolysis upstream of N-termini of CTFs (fig. 4b). Thus, intramembranous endoproteolysis, which liberates an Aβ-like peptide, essentially consists of a distinct dual endoproteolysis, which we have termed 'dual cleavage' mechanism (fig. 4b) [10, 11]. These findings seem to indicate that 'dual cleavage' is necessary to degrade and liberate transmembrane peptides from membrane.

An important finding is that, similar to the pathological cut of βAPP, the precision of the γ-cleavage-generating C-terminus of Nβ is affected by familial-AD-associated PS1/2 mutations (fig. 5) [10]. These mutations were found to cause a partial shift in the cleavage site that generates increased levels of Nβ species whose C-termini are elongated by 2–4 amino acids [10]. This means that the level of secretion of Nβ1733-35 compared to that of Nβ1731, the most abundant Nβ species, is upregulated in the mutant-expressing cells [10]. We therefore suggest that secretion of Aβ-like peptides such as Nβ share the same γ-secretase mechanism as that of Aβ (see also fig. 2b, 3, 4b, 5).

Level of an Elongated Aβ-Like Peptide as a Substitute for Aβ May Reflect AD-Associated Pathological Impairment of γ-Secretase

Although the findings are only preliminary, Nβ, like Aβ, did not seem to aggregate, fibrillate nor accumulate in AD brains [Okochi and Arai,

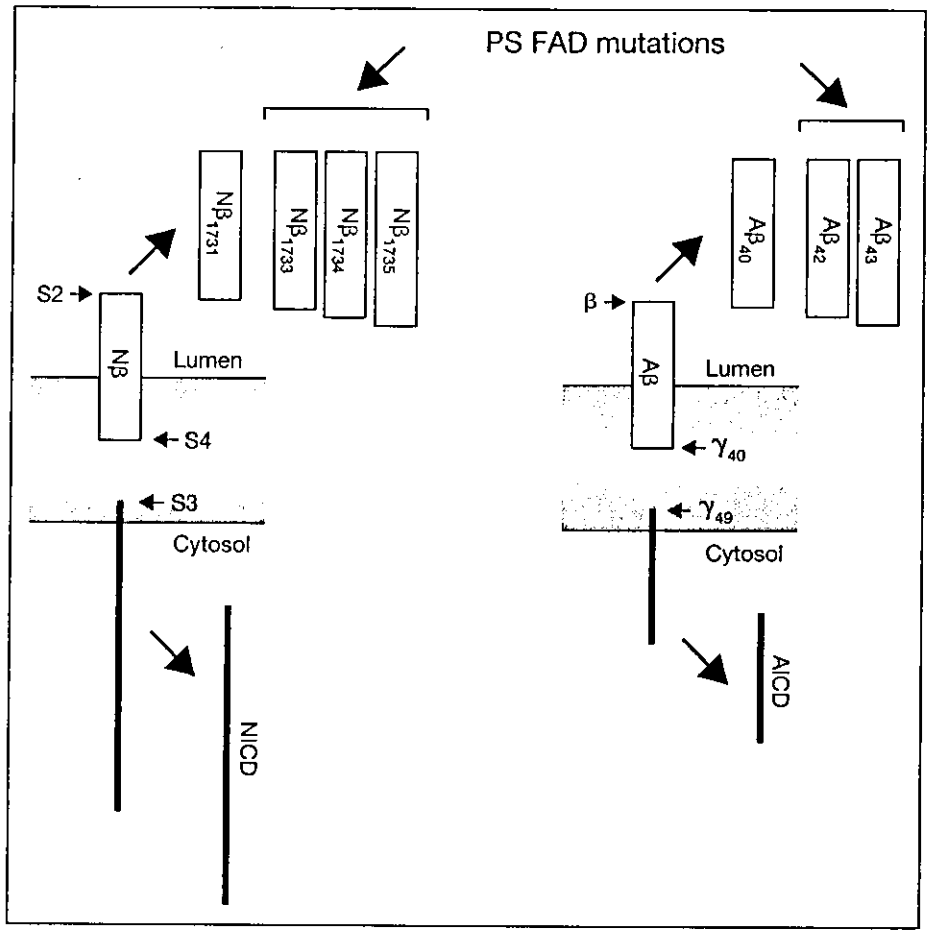

Fig. 5. Familial-AD-associated PS mutations affect γ-cleavage and elongate Nβ. These mutants showed a very similar effect on the precision of the γ-cleavage for the two distinct substrates. The magnitude of the effect, as analyzed so far, was not dependent on the substrates but on mutations of PS. In other words, PS mutants, while dramatically upregulating Aβ42 generation, simultaneously increase the level of elongated Nβ. This seems to indicate that mutations affect direct interaction between PS and their substrates.

unpubl. obs.]. Therefore, by measuring Nβ or the level of Nβ1733-35 relative to that of Nβ1731 in CSF or peripheral, it may be possible to determine the level of γ-secretase activity or Aβ42/40 generation ratio in patients with sporadic AD (see also fig. 5). Aβ deposition leading to AD may gradually take place over a number of years. It is likely that, in the process, γ-secretase activity is upregulated [28] or the precision of γ-cleavage in the brain is affected [1]. Therefore, by measuring the level of Aβ-like peptide or the relative level of elongated species in healthy individuals, it may be possible to diagnose those who are likely to develop AD before they show symptoms.

It Is Worthwhile Studying Whether Secreted Nβ Peptide Level Is Upregulated in Cells of Human Malignancies

Various kinds of examinations have demonstrated that expression levels of Notch receptors including Notch-1 are strongly upregulated in tumor cells, which indicates that Notch signaling is promoted in human malignancies [29]. Very recent evidence indicates a novel mode of cross-talk between the epidermal growth factor receptor/Ras/mitogen-activated protein kinase cascade and the Notch pathway [30]. Moreover, there are indications that oncogenic mutants of Ras observed in 25–50% of human cancer perform an oncogenic function through Notch signaling [29]. One might therefore argue that Notch signaling, a local cell signaling which suppresses cell differentiation and promotes proliferation, may be involved in tumor genesis itself. However, since no one knows how local cell signaling can be monitored, no attempts have been made so far, to measure the signaling level or to evaluate the level in relation to diagnosis or therapy for human tumor in vivo. We have discovered that, for each Notch-signaling fragment produced, a kind of 'peptide evidence' of the signaling event is definitely secreted extracellularly (see also fig. 4a). By measuring this Nβ peptide level, therefore, the level of Notch signaling may be assessed.

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Activated Protein Kinases and Phosphorylated Tau Protein in Alzheimer Disease

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Neurofibrillary tangles of paired helical filaments (PHF) are neuropathological hallmarks of Alzheimer disease (AD), and abnormally hyperphosphorylated tau protein is the major protein subunit of PHF [1–4]. Several kinases and phosphatases are thought to be involved in the process [5–11]. However, the mechanisms of phosphorylation of tau protein and neurodegeneration in AD are still unclear.

In this review, phosphorylation of tau protein and the recently found activated protein kinases in AD are overviewed and their involvement in the formation of phosphorylated tau protein and in the neurodegenerative process of AD are discussed.

Phosphorylation of Tau Protein

Tau protein has six isoforms which derive from a single gene by an alternative splicing mechanism, and the longest isoform is composed of 441 amino acids [12] (fig. 1). An alternative splicing mechanism produces tau with two, one and no N-terminal insertion(s); it also produces tau with and without an insertion in microtubule binding domains. Isoforms with an insertion in microtubule binding domains are called 4-repeats tau, and the others are called

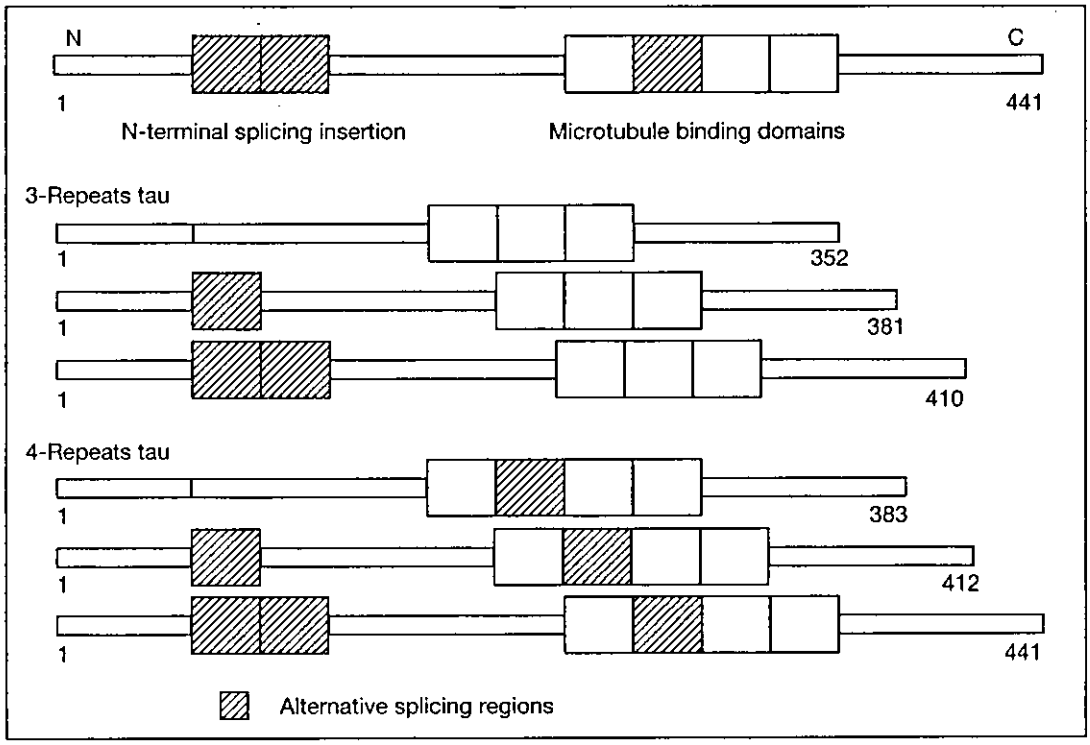

Fig. 1. Structure of tau protein. Tau protein consists of six isoforms which derive from a single gene by an alternative splicing mechanism; the isoforms are composed of 352, 381, 383, 410, 412, and 441 amino acids, respectively. An alternative splicing mechanism produces tau with two, one and no N-terminal insertion(s); it also produces tau with and without an insertion in microtubule binding domains. Isoforms with an insertion in microtubule binding domains are called 4-repeat tau, and others are called 3-repeat tau. Hatched squares indicate alternative splicing regions.

3-repeat tau. The expression of these isoforms is developmentally regulated; fetal tau consists of only 3-repeat tau and adult tau consists of all six isoforms [13]. Tau protein is one of the microtubule-associated proteins and has an ability to promote microtubule assembly. This activity is regulated by phosphorylation, which impairs the ratio of microtubule assembly [14].

Tau protein in normal brain contains 1–2 mol of phosphate(s) per 1 mol of tau protein; however in AD brains, tau protein contains 5–9 mol of phosphates [15]. This abnormal phosphorylated tau protein is present in the affected neurons as amorphous aggregates [16]. Phosphorylation of many Ser/Thr sites on tau protein has already been reported (fig. 2). Among 27 phosphorylated Ser/Thr sites on tau protein in AD, 11 sites are proline-directed (-Ser/Thr-Pro-) sites [17]. Therefore proline-directed protein kinases have been thought to be important in abnormal phosphorylation of tau protein in AD.

Among proline-directed kinases, glycogen synthase kinase-3 (GSK-3) is one of the important kinases in the phosphorylation of tau protein. GSK-3

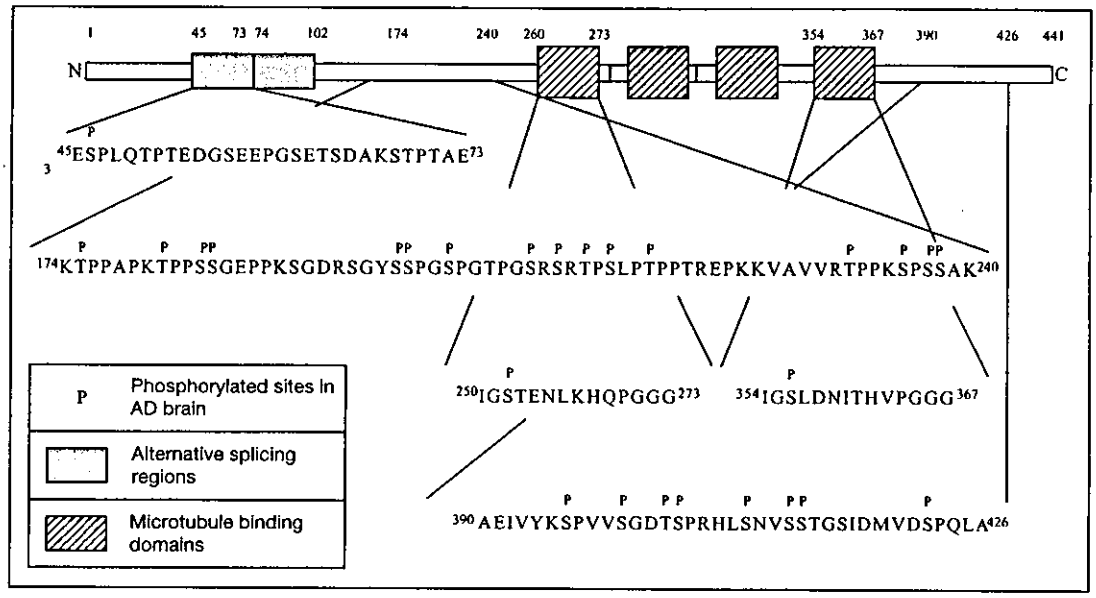

Fig. 2. Phosphorylated sites in an AD brain. The structure of tau is the longest isoform, and P associated with Ser/Thr indicates a phosphorylated site. Gray and hatched boxes indicate alternative splicing regions and microtubule binding domains, respectively.

strongly phosphorylates tau protein in vitro, and is localized in axons [18, 19]. And in fact, previously reported tau protein kinase-I was identified as GSK-3β [20, 21].

Regulation of Activity of GSK-3

GSK-3 is a calcium- and cyclic nucleotide-independent kinase that phosphorylates glycogen synthase, a regulatory enzyme of glycogen. The molecular weights of the α- and β-isoforms are 51 and 46 kD, respectively, and both are known to be able to phosphorylate tau protein [20, 21]. Protein phosphatase-I, G-subunit, the RII subunit of cyclic AMP-dependent protein kinase, phosphatase inhibitor-2, myelin basic protein and neurofilaments are known as substrates for GSK-3 [22–25]. The phosphorylation of GSK-3 regulates its activity and it has been reported that phosphorylation at Tyr 216 and nonphosphorylation at Ser9 are necessary for the activation of GSK-3β [26–28]. The consensus sequence of GSK-3 for phosphorylation is not only proline-directed Ser/Thr, but also Ser-X-X-X-Ser(p) [Ser(p) is prephosphorylated Ser] [29]. Glycogen synthase, β-catenine and tau protein contain Ser/Thr-X-X-X-Ser/Thr sequences and this implies that these proteins have potential sites for phosphorylation by GSK-3 after prime phosphorylation by other kinases [29–31].

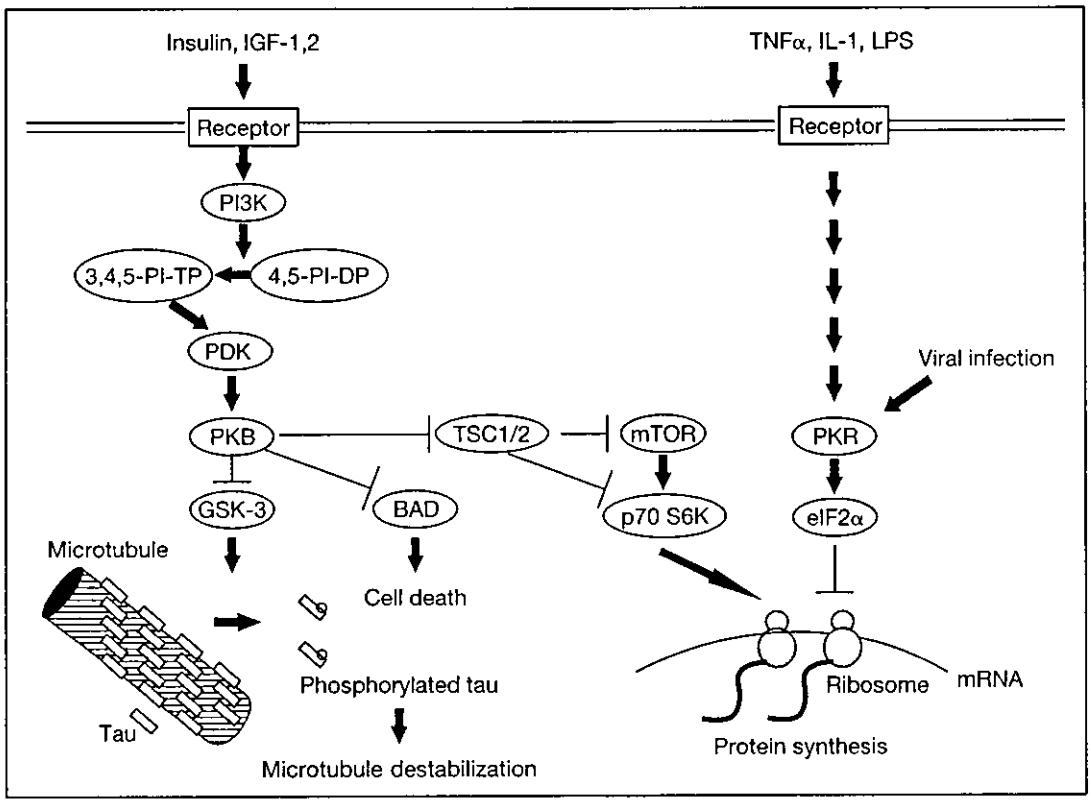

Fig. 3. Intracellular signal transduction on the activated kinases in an AD brain. The PI3K pathway is shown on the left. Binding of insulin, IGF-1 or 2, to the receptor leads to autophosphorylation of the receptor, and PI3K is activated by its binding to the receptor. Activated PI3K converts 4,5-PI-DP to 3,4,5-PI-TP. PDK is activated by its binding with 3,4,5-PI-TP and phosphorylates PKB. Activated PKB (by its phosphorylation) phosphorylates GSK-3 and inhibits the kinase activity of GSK-3. GSK-3 strongly phosphorylates tau protein and phosphorylated tau protein is detached from microtubules, leading to destabilization of microtubules. Then active PKB inhibits tau phosphorylation and microtubule destabilization. In addition, active PKB phosphorylates BAD, a proapoptotic protein, and phosphorylated BAD leads to inhibition of apoptosis. Further active PKB phosphorylates components of TSC, TSC1 and TSC2, and phosphorylation of TSC1/2 inhibits its inhibitory potency to activation of p70 S6K by mammalian target of rapamycin (mTOR). Therefore active PKB accelerates the activation of p70 S6K by mTOR, leading to activation of protein synthesis. On the right, regulation of eIF2α and PKR, and dsRNA-activated protein kinase are shown. TNFα, IL-1 and lipopolysaccharide (LPS) bind their receptors, and induce the expression of PKR; viral infection also activates PKR. Activated PKR phosphorylates eIF2α, and phosphorylated eIF2α inhibits the translational machinery.

The regulation of the signal transduction cascade has been studied, and regulatory mechanisms of GSK-3 have already been identified (fig. 3). The predominant regulatory system of the activity of GSK-3 is the phosphatidylinositol-3 kinase (PI3K) pathway, which is stimulated by insulin, IGF-1 or -2 [32]. The binding of insulin, IGF-1 or -2, to the receptor leads to autophosphorylation of

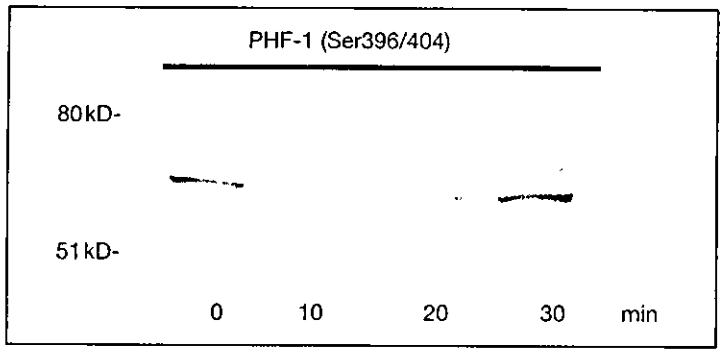

Fig. 4. Phosphorylation levels of tau protein in SH-SY5Y neuroblastoma cells treated with insulin. SH-SY5Y human neuroblastoma cells were treated with 250 μg/ml of insulin and the levels of phosphorylation of tau protein were investigated employing PHF-1 antibody that reacted with tau phosphorylated at Ser396/404. The intensities of bands decreased within 10–20 min, suggesting that dephosphorylation of tau protein was induced. The effect of insulin disappeared after 30 min.

the receptor, and PI3K is activated by its binding to the receptor. Activated PI3K converts 4,5-phosphatidylinositol diphosphate (4,5-PI-DP) to 3,4,5-phosphatidylinositol triphosphate (3,4,5-PI-TP). Phosphatidylinositol-dependent kinase (PDK) is activated by its binding with 3,4,5-PI-TP, and phosphorylates protein kinase B (PKB). Activated PKB (by its phosphorylation) phosphorylates GSK-3, and inhibits the kinase activity of GSK-3 [32]. In addition, active PKB phosphorylates BAD, a pro-apoptotic protein, and phosphorylated BAD is sequestrated from mitochondria, leading to inhibition of apoptosis [33, 34]. Therefore activation of PKB plays an important role in inhibition of apoptosis. Taken together insulin, IGF-1 or -2, is able to reduce the phosphorylation of tau protein through inhibition of GSK-3 and to lead to cell survival.

The level of phosphorylation of tau protein was regulated by this pathway. SH-SY5Y human neuroblastoma cells were treated with 250 μg/ml of insulin and the levels of phosphorylation of tau protein was investigated employing PHF-1 antibody that reacted with tau phosphorylated at Ser396/404 (fig. 4). Insulin is thought to activate the PI3K pathway that reduces the activity of GSK-3. Dephosphorylation of tau protein was induced with 10–20 min, and after 30 min the effect of insulin disappeared.

To observe the role of PI3K in the regulation of tau phosphorylation, wortmannin, an inhibitor of PI3K, was employed in our previous investigation [35]. And increased phosphorylation levels of tau protein at the PHF-1 (Ser 396/404) site in the early phase (1–3 h) were observed, suggesting that PI3K is involved in the regulation of phosphorylation of tau protein [35].

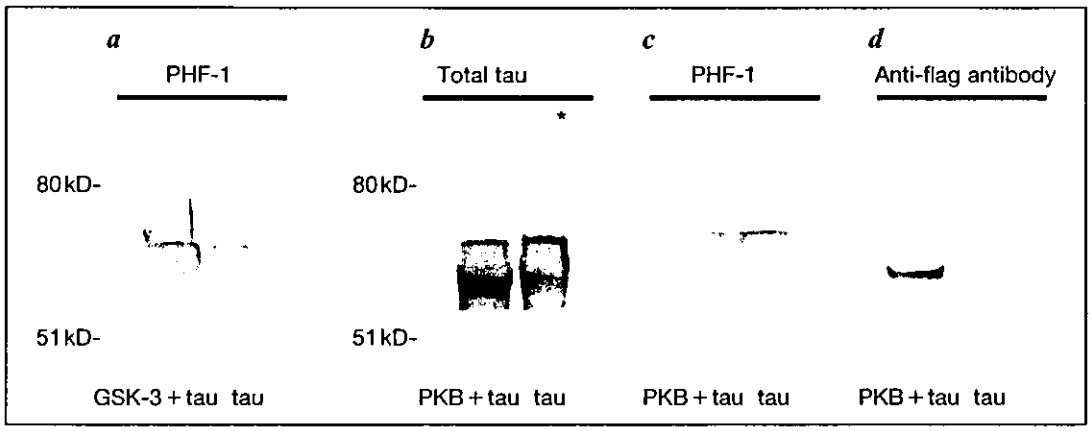

Fig. 5. Phosphorylation of tau protein in 293T cells transfected with tau, GSK-3, and PKB. 293T cells were transfected with tau, GSK-3 and PKB genes. The vectors containing tau (the longest isoform), GSK-3β, and PKBα, were constructed by insertion into pcDNA3.1 (Invitrogen). 293T cells were cotransfected by lipofectamine (Invitrogen) with tau + mock genes, tau + GSK-3 genes, and tau + PKB genes; sufficient expression of gene products was observed 48 h after the transfection. ***a*** The cells were cotransfected with tau + GSK-3 genes and tau + mock genes. The PHF-1 antibody (Ser396/404) revealed more increased phosphorylation of tau protein in cells cotransfected with tau + GSK-3 genes than in cells cotransfected with tau + mock genes (***b–d***) The cells were c-transfected with tau + PKB genes and tau + mock genes. ***b*** Prior to application of the first antibody, membrane was treated with alkaline phosphatase, to observe total tau protein. After treatment, the Tau-1 antibody that reacted with dephosphorylated tau protein at Ser198/199/202 revealed similar expression of tau protein in cells cotransfected with tau + PKB genes and with tau + mock genes. ***c*** The PHF-1 antibody revealed decreased phosphorylation of tau protein in cells co-transfected with tau + PKB genes than in cells co-transfected with tau + mock genes, suggesting that phosphorylation of tau protein by GSK-3 is attenuated by overexpressed PKB. ***d*** Anti-Flag antibody staining was observed only in cells cotransfected with tau + PKB genes, suggesting the PKB gene was appropriately expressed in this experiment.

To confirm the involvement of PKB and GSK-3 in the regulation of phosphorylation of tau protein, 293T cells were transfected with GSK-3 and PKB (fig. 5). The vectors containing tau (the longest isoform), GSK-3β, and PKB-α (generous gifts from M. Goedert, Medical Research Council, UK, A. Takasima, Riken, Japan, and U. Kikkawa, Kobe University, Japan, respectively) were constructed by insertion into pcDNA3.1 (Invitrogen). 293T cells were cotransfected with tau + mock genes, tau + GSK-3 genes, and tau + PKB genes, and sufficient expression of gene products was observed 48 h after the transfection. The PHF-1 antibody (Ser396/404) revealed more increased phosphorylation of tau protein in cells cotransfected with tau + GSK-3 genes than in cells cotransfected with tau + mock genes (fig. 5a). In the membrane treated with alkaline phosphatase, the Tau-1 antibody that reacted with total tau protein revealed a similar expression of tau protein in cells cotransfected with tau + PKB genes,

and with tau + mock genes (fig. 5b). Anti-Flag antibody staining was observed only in cells co-transfected with tau + PKB genes, suggesting the PKB gene is appropriately expressed in this experiment (fig. 5d). Then the PHF-1 antibody revealed decreased phosphorylation of tau protein in cells cotransfected with tau + PKB genes than in cells cotransfected with tau + mock genes, suggesting that phosphorylation of tau protein by GSK-3 is attenuated by overexpressed PKB (fig. 5c). Taken together, the PI3K pathway regulates tau phosphorylation in cultured cells.

Activated Kinases Colocalized with Phosphorylated Tau Protein in AD

As described above, GSK-3 might be a major kinase involved in the formation of abnormally phosphorylated tau protein in AD. Biochemical analysis revealed that the levels of GSK-3, as determined by indirect ELISA, are increased by approximately 50% in the postsynaptosomal supernatant from AD brains as compared with the controls [36]. Immunohistlogical analysis revealed that GSK-3 is prominently present in neuronal cell bodies and their processes and colocalizes with neurofibrillary changes in AD brain [36]. Furthermore, active GSK-3β, phosphorylated at Tyr 216, was found to initially accumulate in the cytoplasm of pretangle neurons [37]. And these active GSK-3-positive neurons appear initially in the pre-alpha layer of the entorhinal cortex and extend to other brain regions, coincident with the sequence of the development of neurofibrillary changes [37]. It was also reported that total GSK-3α, total GSK-3β, and active GSK-3β were found to colocalize with the granulovacuolar degeneration and to be associated with the granules of the granulovacuolar bodies [38]. Further, GSK-3β was expressed in neurons containing neurofibrillary tangles, but only a small proportion of intracellular neurofibrillary tangles was observed to be GSK-3β-immunoreactive [38]. It suggests that neurons developing granulovacuolar degeneration sequester an active form of GSK-3 in this compartment or that activation of GSK-3 is involved predominantly in the early stages of neurodegeneration.

As to the intracellular signal transduction pathway, the upstream regulator of GSK-3 was also investigated in AD brains. As mentioned above, PKB is an important intermediate in the PI3K-signaling cascade that acts to phosphorylate GSK-3β at its serine 9 residue, thereby inactivating it. The amount of activated PKB phosphorylated at Thr308 increased in correlation to the progressive sequence of neurofibrillary changes assessed according to Braak's criteria [39]. This activated PKB was found to appear in particular in neurons that are known to later develop NFTs in AD [39]. Western blotting showed that activated PKB

was increased by more than 50% in the 16,000 *g* supernatants of AD brains as compared with normal aged controls [39]. This increase in PKB levels corresponded to a several-fold increase in the levels of total tau and abnormally hyperphosphorylated tau. Apparently, it is difficult to explain co-localization of the phosphorylated tau with both active GSK-3 and active PKB, because active PKB is thought to inactivate GSK-3. Presumably, more complex mechanisms are involved in the neurodegenerative process in AD; a decreased activity of protein phosphatases might be able to explain these situations.

In addition to active GSK-3 and PKB, other kinases related to the protein translation system have also been reported to be active in AD brains. Eukaryotic initiation factor-2α (eIF2α) is a regulator of protein translation and a phosphorylated form of eIF2α terminates global protein translation [40]. This eIF2α is phosphorylated by several kinases including protein kinase R (PKR), double-stranded RNA (dsRNA)-activated protein kinase, that is a ubiquitously expressed serine/threonine protein kinase induced by interferon and activated by dsRNA, TNF, IL-1 and lipopolysaccharide, or viral infection [41] (fig. 3). Chang et al. [42] reported that in AD brains both phosphorylated eIF2α and PKR were observed in affected neurons, while they were rarely observed in age-matched control brains.

The ribosomal S6 protein kinase p70 S6 kinase is known for its role in modulating cell size and cell survival [43]. Activated p70 S6 kinase upregulates ribosomal biosynthesis and enhances the translational capacity of the cell. Signal transduction cascade that activates p70 S6 kinase has been investigated, and the mammalian target of rapamycin (mTOR) and tuberous sclerosis complex (TSC) were reported to be important regulators in this translational machinery [44] (fig. 3). Recent reports revealed that phosphorylation of p70 S6 kinase by mTOR induced activation of p70 S6 kinase, leading to activation of translation, and that mTOR and p70 S6 kinase were inhibited by TSC [44]. On the other hand, the inhibitory potential of TSC to mTOR was reported to be downregulated by its phosphorylation by PKB [45, 46]. Therefore, p70 S6 kinase is located down stream of the PI3K pathway. An et al. [47] reported that the levels of phosphorylated p70 S6 kinase (at Thr389 or at Thr421/Ser424) were increased in accordance with the progressive sequence of neurofibrillary changes according to Braak's criteria [47]. Both PKR and p70 S6 kinase Both eIF2α and p70 S6 kinase are working to regulate translation, but the former inhibits translation and, on the other hand, the latter enhances it. Therefore activation of PKB and p70 S6 kinase might be an auto-feedback response to inhibition of translation induced by activated eIF2α and PKR. Until now only little evidence on the involvement of translational machinery in AD pathology has been reported. On the translational system, we have previously reported that peptidyltransferase inhibitors induced the phosphorylation of tau protein and

neuronal cell death in SH-SY5Y cells; dysfunction of ribosome in AD was also reported by others [48, 49]. More investigations on this matter will be required to clarify the neurodegenerative mechanisms including AD.

Conclusion

The mechanisms of phosphorylation of tau protein are still unclear; however, the signal transduction pathway of GSK-3, a strong candidate that phosphorylates tau abnormally, was overviewed. The PI3K pathway has an important role in both the regulation of phosphorylation of tau and cell survival. In addition PKB, which participates in the PI3K pathway, also has an important role in the regulation of p70 S6 kinase activity that regulates protein translation. From the reports on the phosphorylation of p70 S6 kinase, eIF2α and PKR in AD brains, both the PI3K pathway and the protein translational machinery might be closely involved in AD pathology, and more investigations will be required to understand tau phosphorylation in neurodegeneration.

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Genetic Analysis of Familial Alzheimer's Disease in a Japanese Population

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Alzheimer's disease (AD) is one of the most common neurodegenerative disorders in the elderly population. The pathological hallmarks (amyloid plaque, neurofibrillary tangle and neuronal cell loss) have been well characterized. The causal genes for early-onset familial Alzheimer's disease (FAD) are the presenilin 1 (PS-1) gene on chromosome 14 [1], the presenilin 2 (PS-2) gene on chromosome 1 [2] and the amyloid precursor protein (APP) gene on chromosome 21 [3]. In addition, apolipoprotein E (APOE) allele 4 (ε4), located on chromosome 19, is a well-established genetic risk factor for sporadic AD [4]. Among these genes, mutations in PS-1 seem to be the most common genetic factor underlying the development of early-onset FAD. To date, over 100 missense mutations for PS-1, 8 mutations for PS-2 and 16 mutations for APP are cited in an online database (AD mutation database; http://molgen-www.uia.ac.be/ADMutations/). Several PS-1 mutations and only an APP mutation (V717I) were previously described in the Japanese population (table 1) [2, 5–19]. Eighteen missense mutations in the PS-1 gene were reported in the Japanese familial AD (FAD) pedigrees. No pathogenic mutation of the PS-2 gene has been identified in the Japanese population. In this chapter, we report the results of our most recent studies of these three genes in FAD and sporadic AD patients in a Japanese population.

Subjects and Methods

Patient Samples

Twenty-two Japanese patients were selected from 5 early-onset (<65 years old) FAD patients (mean age of onset: 58.2 years), 7 late-onset (>65 years old) FAD patients

Table 1. APP, PS-1 and PS-2 gene mutations in Japanese FAD and sporadic AD

	Exon	Mutation	Reference
PS-1	5	V96F	5
		E123K	6
	6	H163R[1]	5, 7, 8, 9
	7	E184D	10
		G209R	11
		I213T	5
		G217D	12
		F237I[2]	13
	8	A260V	2, 14
		S266G	15
	8	R269H	9
		E273A	9
		E280A	8
		A285V	2
	9	S290C	16
	11	G384A	9
		N405S	17
	12	A431V	18
APP	17	V717I	19

[1]This mutation was reported in early-onset FAD and early-onset sporadic AD.

[2]This mutation was reported in early-onset FAD with spastic paraparesis.

(mean age of onset: 70.3 years old), and 10 early-onset sporadic AD patients (mean age of onset: 55.4 years). All patients fulfilled the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorder Association (NINCDS-ADRDA) criteria for probable and possible AD [20]. These diagnoses were assisted by MRI or CT imaging studies. We defined patients as having FAD if at least 2 members were affected in a family and the difference in age of onset was less than 20 years. Genomic DNA was extracted from peripheral leukocytes using the standard phenol-chloroform method and subjected to PCR amplifications.

Primers and PCR Amplification

Intronic primers were generated to amplify exons 16, 17 and 18 of the APP gene, exons 3–12 of the PS-1 and PS-2 genes. The primer sequences are provided in table 2. In brief, 50–100 ng DNA was amplified using PCR in each 15 μl reaction mixture using 1 mmol of specific primers and 0.8 units of Taq DNA polymerase (TaKaRa, Tokyo, Japan) in supplied 1 × PCR buffer for 35 cycles of 30 s at 94°C for denaturing, 30 s at 58°C for annealing, and 40 s at 72°C for extension.

Table 2. PCR primers (5′→3′)

APP		Size, bp
APP Ex16-F	TAGAAAGAAGTTTTGGGTAGGCTTT	260
APP Ex16-R	AGAGTTAATAGGTCATTTGGCAAGACA	
APP Ex17-F	AATGAAATTCTTCTAATTGCGTTT	258
APP Ex17-R	TTCTCTCATAGTCTTAATTCCCACTT	
APP Ex18-F	TTACTGCTTCTCCATGTTCACC	249
APP Ex18-R	TTCTATAAATGGACACCGATGG	
PS-1		
PS1Ex-3F	TCTTTCCCTTTTCAGAACCTCA	222
PS1-Ex3R	CAGAGGTGAGGGGAGATGATAA	
PS1-Ex4F	TCATAGTGACGGGTCTGTTGTT	423
PS1-Ex4R	CTTCAAGGTGATGATGACATGC	
PS1-Ex5F	TTCTGTGTTGGAGGTGGTAATG	287
PS1-Ex5R	CTGTGACAAGAATACCCAACCA	
PS1-Ex6F	AGTCTGGGCGACAAAGTGAG	237
PS1-Ex6R	TGATAGCTACACAGCACAAAGG	
PS1-Ex7F	GTTTGGGAGCCATCACATTATT	373
PS1-Ex7R	AGATGAGGAAAGAAAACACTCCA	
PS1-Ex8F	CCTTCGTTAATTCCTCCCTACC	248
PS1-Ex8R	CATGTGCTTCAGTTCCGATAAA	
PS1-Ex9F	GGAGAAATGATGGCTTGTTGTT	266
PS1-Ex9R	GGAGTCTATGACCAAAGAAAGACG	
PS1-Ex10F	CAATGACAGCTAGTTACTGTTTCCA	346
PS1-Ex10R	TTCATTTTATTCTCAAAAAGGTTGA	
PS1-Ex11F	ATTCATTGTGGGGTTGAGTAGG	294
PS1-Ex11R	GAACTGCCTTAAAGGGACTGTG	
PS1-Ex12F	CTTGTGATTGAGTTTTGCCTGA	294
PS1-Ex12R	TGTCCTCCCCAGATTTTGTTAT	

PS-2		Size, bp
PS2-Ex3F	GTCTCACAGGAAAGTGGAACAAG	284
PS2-Ex3R	GAATGTCTGGTTTTCATCTGTAAGG	
PS2-Ex4F	TGGAAAGCAACATTCAAACTTC	456
PS2-Ex4R	TTCTTCATCCCTGCTCTTTACC	
PS2-Ex5F	TAGCAGGTCCAGAATCACTCAAG	318
PS2-Ex5R	GTTTTTCTAAAGGCGGCTGTTT	
PS2-Ex6F	GCTTGGGTATCAGTCTCAGGAT	258
PS2-Ex6R	GAGCTCGATGGTCATCTTTCC	
PS2-Ex7F	GAATGGTGGTAAACTGCTAGGC	407
PS2-Ex7R	CTCTGCAGAAAGGGATGCAAG	
PS2-Ex8F	GCTACAGGGCAGGCTCTTCT	206
PS2-Ex8R	ACCCCGAGTCAGGCAGAG	
PS2-Ex9F	GCTTTCTGGGACGCAGACT	311
PS2-Ex9R	TGGTTTTCAACGGACCTTTC	
PS2-Ex10F	ACCCCTTCTTGGAGCTTTGT	282
PS2-Ex10R	GAGCCTCCACCCTCTGTCTC	
PS2-Ex11F	GGGCCAGAGTTTCTCTTCTTTT	281
PS2-Ex11R	CCCTAGGGATCCTGAGACCTG	
PS2-Ex12F	CCCAGGGACTAGACCATGACT	290
PS2-Ex12R	AACTGCATCCAATGAAAATTCC	

Table 3. Identified mutation and polymorphism

	Exon (PCR region)	Polymorphism	Amino acid	NCBI SNP cluster ID
APP	Exon 16	2032 G/A	D678N	–
	Exon 18	IVS17-10 T/C	intron	–
PS-2	Exon 3	69 T/C	A23A	rs11405
		129 C/T	N43N	rs6759
	Exon 4	IVS3-42 G/A	intron	rs1295644[1]
		IVS3-29 T/C	intron	rs1295643
		260 T/C	H87H	rs1046240[1]
	Exon 5	IVS5+30 G/C	intron	rs2236910
	Exon 8	861 C/T	P287P	–
	Exon 9	IVS8-24 G/A	intron	rs2802267
	Exon 11	IVS11+24 G/A	intron	rs2855562

[1]These are linked polymorphisms in our samples.

Single-Strand Conformation Polymorphism Analysis and Sequence Analysis

PCR products of AD samples for screening were subjected to single-strand conformation polymorphism (SSCP) analysis. One microliter of PCR product was denatured in formamide-containing buffer at 95°C for 8 min, quickly chilled on ice, and electrophoresed on a 12% polyacrylamide gel with 10% glycerol at 4°C for 24 h at 200 V. DNA bands were visualized using silver staining. The mobility-shifted band was directly cut from the gel using a freshly prepared razor blade. The eluted band was re-amplified under identical PCR conditions for 45 cycles. The purified PCR product derived from the extra band was subjected to direct sequencing using a Big Dye cycle sequence kit (Amersham Bioscience Japan, Tokyo, Japan) and the ALF automated luminescent sequencer (Applied Biosystems Japan, Tokyo, Japan). APOE genotyping was carried out according to standard procedures [22].

Results

In PCR-SSCP analysis, 2 extra conformers in the APP gene (exon 16, exon 18), none in the PS-1 gene and 9 in the PS-2 gene were observed. Table 3 shows the identified mutation and polymorphisms identified by sequence analysis. No missense mutations in the PS-1 and PS-2 gene were detected in any samples. Except for the IVS17-10 T/C polymorphism of the APP gene and the 861 C/T (P287P) polymorphism of the PS-2 gene, the identified polymorphisms were previously reported in the NCBI SNP database (the APP gene; http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId = 35, the PS-1 gene; http://www.ncbi.nlm.