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p49^{3F12} Kinase: A Novel MAP Kinase Expressed in a Subset of Neurons in the Human Nervous System

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Summary

Monoclonal antibody 3F12 identifies a cytoplasmic antigen of 49 kDa in human hippocampus and neocortex. The distribution of 3F12 immunoreactive neurons closely matches that of Alzheimer's disease (AD) targeted neurons in these areas. In some hippocampal neurons of AD patients, this antigen colocalizes with ALZ-50, indicating the presence of AD pathology in these neurons. Molecular characterization of the 3F12 cDNA revealed it to be a member of the MAP kinase family, showing 43% amino acid sequence identity to human extracellular related kinase 2 (p42^{mapk}). We have confirmed that p49^{3F12} kinase autophosphorylates both threonine and tyrosine residues, as expected for a MAP kinase. The p49 mRNA is expressed exclusively in the nervous system. In the brain, the distribution of these neurons closely corresponds to 3F12 antigen-bearing neurons. The p49^{3F12} gene maps to the human chromosome 21q21 region, a region that may be important in the pathogenesis of AD and Down's syndrome.

Introduction

The mitogen activated protein (MAP) kinases have emerged as key switch kinases that initiate a cascade of serine/threonine phosphorylation signals (Ray and Sturgill, 1987; Payne et al., 1991). The evolutionarily conserved family of MAP kinases includes at least three vertebrate kinases: p42^{mapk} (extracellular related kinase 2 [ERK2]), p44^{mapk} (ERK1), and p55^{mapk} (ERK3; Boulton et al., 1991). The cascade may also be activated in response to a variety of stimuli, such as those from G protein–coupled receptors (Gardner et al., 1993) and intracellular calcium mobilization (Chao et al., 1992).

Although MAP kinases are expressed ubiquitously in vertebrate tissues, they appear to be enriched in neural tissues. Boulton et al. (1991) have shown that ERK1, ERK2, and ERK3 mRNAs are expressed at highest levels in the nervous system of adult rats, with ERK2 and ERK3 displaying alternative patterns in certain brain regions. The human ERK3 (also known as p63^{mapk}) shows high levels of expression in heart and brain (Gonzalez et al., 1992).

MAP kinases may play a special role in terminally differentiated neurons. ERK1, ERK2, and ERK3 are all expressed in adult neurons, indicating a role for these enzymes other than in cell proliferation. There is also evidence indicating that MAP kinases may be regulated by nerve growth factor (NGF); NGF-dependent stimulation of neurite outgrowth in PC12 cells coincides with a 30-fold increase in MAP kinase activity and translocation of the kinase to the nucleus. Furthermore, MAP kinases may also be involved in the regulation of the NGF-induced G1 arrest of PC12 cells (Rudkin et al., 1989).

Several experiments suggest an involvement in the regulation of cytoskeletal elements by MAP kinases. ERK1 and ERK2 are associated with the microtubule organizing centers during meiotic maturation of mouse oocytes (Verlhac et al., 1993). Disruption of microtubules by colchicine in rat fibroblasts leads to activation of a serine/threonine kinase similar in chromatographic properties to MAP kinases (Shinohara-Gotoh et al., 1991).

Perturbations in the regulation of MAP kinases may be involved in pathological processes of human brain. In Alzheimer's disease (AD), several neuronal cytoskeletal proteins have been shown to be abnormally phosphorylated (Arai et al., 1990). One of these proteins, microtubule-associated protein tau, is abundant in axons, where it stabilizes microtubules (Drechsel et al., 1992). Abnormally phosphorylated tau is the primary constituent of the neurofibrillary tangles (NFT), a pathological hallmark of AD (Goedert et al., 1989). MAP kinase is able to hyperphosphorylate tau in vitro, causing it to acquire abnormal biochemical properties similar to those found in AD NFTs (Lu et al., 1993). The ERK2 protein is immunocytochemically localized to NFTs and dystrophic neurites associated with senile plaques (Trojanowski et al., 1993). In vitro, ERK2 is able to phosphorylate 14–16 proline-directed serine or threonine residues on tau, whereas normal tau in adult brain has 2–3 phosphorylated sites (Drewes et al., 1992; Goedert et al., 1992).

Although some kinase isozymes display regional and cell type specificity in the brain, no neuronal subset specificity for any of the known serine/threonine kinases, including MAP kinases, has yet been reported. We previously described neuronal subset–specific expression of a cytoplasmic protein immunoreactive with MAb 3F12 in a subpopulation of pyramidal neurons in the human hippocampus and neocortex (Miller and Benzer, 1983; Miller et al., 1987; Hinton et al., 1988). Immunoblots of homogenates of human hippocampus detect four immunoreactive bands of 60, 49, 45, and 42 kDa. We also reported the loss of MAb 3F12 immunoreactivity in neurons of AD patients early in the course of the disease. This pattern of loss corresponds, topographically, to the pattern of neuronal degeneration (Miller et al., 1987; Hinton et al., 1988).

Here, we report the cloning and sequencing of the p49^{3F12} cDNA and the identification and partial characterization of its protein product that exhibits significant sequence similarity to members of the MAP kinase family. This kinase is present in a subpopulation of neurons and can colocalize with the ALZ-50 antigen in AD hippocampal sections. Expression of the gene provides a marker for the subset of neurons targeted in AD.

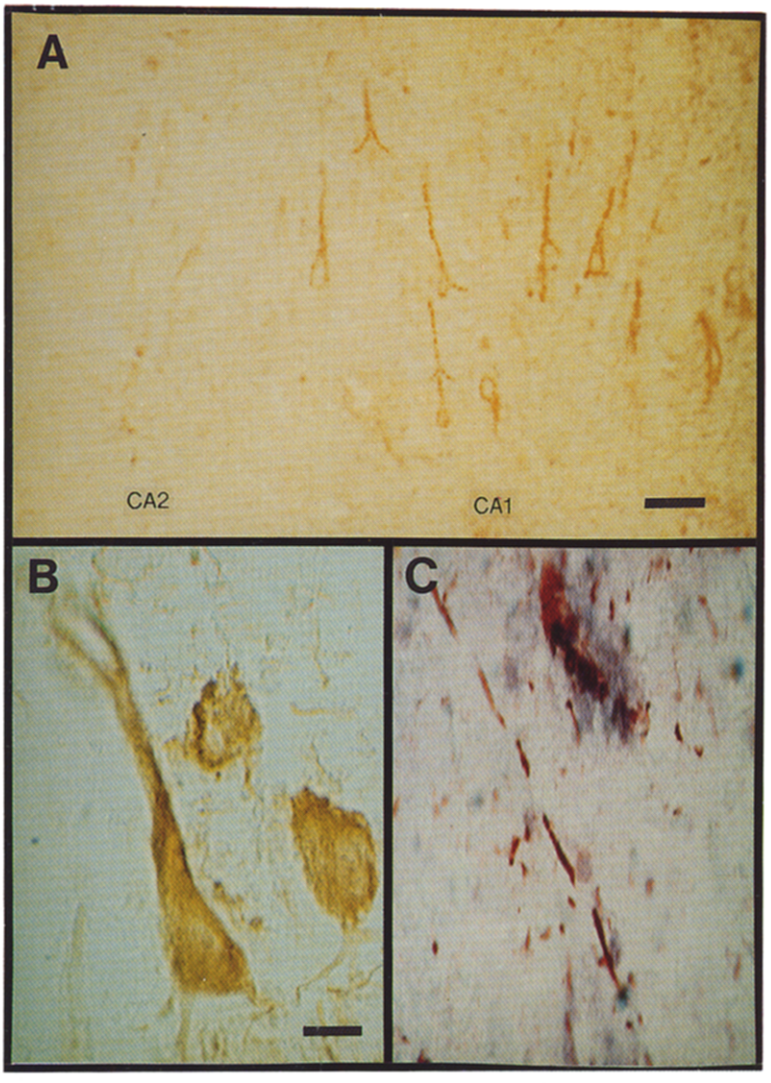


Figure 1. Immunocytochemistry of MAb 3F12 in Neurologically Normal Brain (Immunoperoxidase)

(A) Pyramidal neurons in the CA1 and CA2 regions of the hippocampus are labeled with MAb 3F12. Note the sharp demarcation between staining in the CA1 region and its absence in CA2. Neurons are present in the CA2 region; however, they are not immunolabeled. Bar, 0.1 mm.

(B) The antigen is found in the somal cytoplasm as well as in proximal apical dendrite. Bar, 10 μm.

(C) An axon in a hemotoxylin counterstained section of corpus callosum is densely immunolabeled (red HRP enzymatic product). Bar, 10 μm.

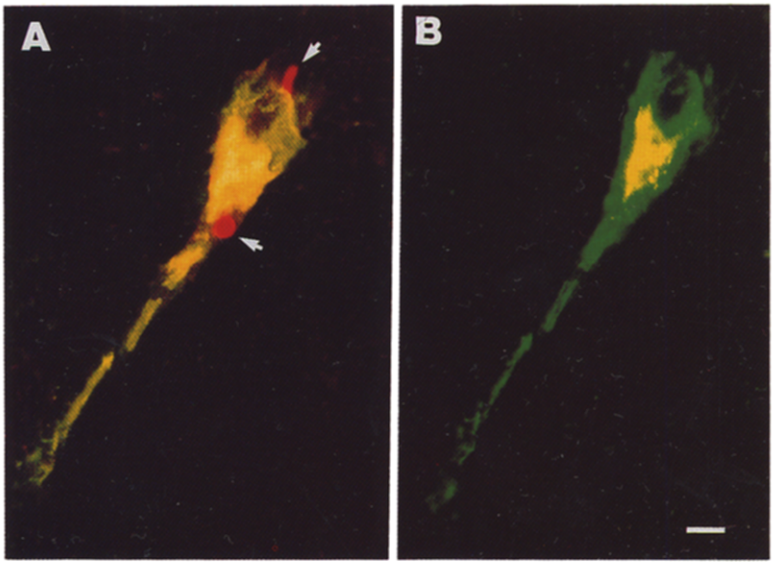


Figure 2. Immunocytochemical Colocalization of $p49^{3F12}$ and ALZ-50 in Hippocampal Pyramidal Neurons of AD Patients

(A) The 3F12 antigen is demonstrated by FITC in the cytoplasm of a pyramidal neuron in the CA1 region of the AD hippocampus. The section was viewed under a single pass filter for FITC. Lipofuschin appears bright yellow.

(B) The 3F12 and ALZ-50 antigens demonstrated by FITC and Texas Red (arrows), respectively, are localized within the cytoplasm of the same AD hippocampal neuron. The section was viewed under a double pass filter for FITC and Texas Red. Lipofuschin appears bright yellow. Bar, 10 μm.

Results

Immunocytochemical Characterization of the Antigen

MAb 3F12 stained a subpopulation of pyramidal neurons in the CA4, CA1, and subiculum regions of the hippocampus and layers 3 and 5 of the neocortex in neurologically normal human brain. Staining was localized to somas and the proximal portions of apical dendrites (Figures 1A and 1B). Some axons were labeled in the white matter of the cerebrum, spinal cord, and the brainstem (Figure 1C).

Hippocampal sections from AD patients were double stained with MAbs 3F12 and ALZ-50. MAb ALZ-50 identifies abnormally phosphorylated tau proteins and is a marker for early neurofibrillary degeneration in AD (Wolozin et al., 1986). The 3F12 and ALZ-50 antigens, labeled with fluorescein isothiocyanate (FITC) and Texas Red, respectively, colocalized in some pyramidal neurons in the CA4, CA1, and subiculum regions. These results confirm that antigen 3F12 is expressed in neurons vulnerable in AD. The 3F12 antigen present in the somal cytoplasm and the proximal apical dendrites was displaced by punctate deposits immunoreactive with MAb ALZ-50 (Figures 2A and 2B). In some neurons, ALZ-50 staining was more extensive and occupied the entire neuronal soma, with little to no remaining 3F12 reactivity.

Molecular Cloning of the 3F12 cDNA

We screened a human hippocampal λgt11 cDNA expression library with MAb 3F12. Among 300,000 plaques, 3 positive clones were isolated with insert sizes of 450, 420, and 351 bp. Each clone was labeled with ^{33}P and hybridized in situ to human hippocampal sections. Only one clone (HH3, 351 bp) hybridized to a subpopulation of pyramidal neurons in a distribution very similar to that of MAb 3F12 immunoreactivity (Figures 3A–3F). DNA sequence analysis of this clone and comparison with sequences in GenBank revealed it to be novel.

To obtain a full length cDNA clone, the ^{32}P-labeled 351 bp clone was used to screen a λZAPII, oligo(dT)-primed human hippocampal library. After screening 150,000 plaques, a 2.4 kb cDNA clone with an exact nucleotide sequence match with clone HH3 was isolated. The low abundance indicated that it was a rare mRNA species, in agreement with the low quantities of MAb 3F12 immunoreactive proteins in hippocampal tissue homogenates. By primer extension, using an antisense primer hybridizing near the 5′ end of the 2.4 kb cDNA, it was determined that this cDNA clone lacked 260 bases from the 5′ untranslated region (UTR; data not shown). Nonetheless, it contained the complete amino acid coding sequence of the p49^{3F12} kinase. The 2.4 kb cDNA clone labeled with ^{33}P also hybridized in situ to the same distribution of neurons as clone HH3.

Sequence Analysis of the cDNA

The nucleotide sequence of the clone revealed it to be 2411 bp in length (Figure 4A). A continous open reading frame encoding 422 amino acids began at position 223. Three additional ATGs in the 5′ UTR were all followed by in-frame stop codons. There was also a 3′ UTR of 918 bp beginning at position 1492. A 34 bp poly(A) tail was present beginning at position 2372, with a possible polyadenylation signal at position 2351. The Kyte–Doolittle hydropathy analysis of the predicted amino acid sequence did not reveal any extended hydrophobic regions that would suggest a membrane-spanning domain. A schematic representation of the p49^{3F12} kinase domains indicates the presence of the ATP-binding domain, two TXY phosphorylation sites separated by two valine residues and the serine/threonine catalytic sequence (Hanks et al., 1988) (Figure 4B).

Nucleotide searches of the GenEMBL database using the blastn program revealed 75% identity with clone HFBCL46 (GenBank accession number M85653) beginning at position 14 and ending at position 373 of the 3F12 cDNA sequence. Swiss-Pro protein database searches with 3F12 amino acid sequence did not match any known proteins; the 3F12 deduced protein sequence is novel. However, significant amino acid sequence homology to MAP serine/threonine kinases was observed. There was a 42% sequence identity between the predicted amino acid sequence of 3F12 cDNA and that of the human ERK2 (p42^{mapk}). A gap alignment of the kinase with human ERK1, ERK2, ERK3, and Saccharomyces cerevisiae LYT2 and FUS3 is shown in Figure 5A. The percent amino acid identities among these kinases are shown in Figure 5B.

Distribution of the mRNA

Northern blots of human hippocampus, primary motor cortex (Brodmann's area 4), frontal association cortex (area 10), primary visual cortex (area 17), cerebellum, spinal cord, and cerebral white matter were probed with a ^{32}P-labeled 1.0 kb cDNA fragment, corresponding to the first 1000 bases of the 2.4 kb cDNA clone (Figure 6A). A 2.7 kb mRNA species was identified in the hippocampus and areas 4, 10, 17, the cerebellum, with a weak signal in the spinal cord. White matter, which lacks neuronal cell bodies, did not show expression of the mRNA, in keeping with the localization of the antigen to neurons. Furthermore, analysis of mRNA from other brain regions revealed expression of the 2.7 kb mRNA in striatum and brain stem (Figure 6B). The expression of the 3F12 mRNA species was also examined in other tissues. In northern blots of poly(A)$^+$ mRNA from heart, placenta, lung, liver, skeletal muscle, kidney, pancreas, and testis, only kidney (Figure 6C) and testis (Figure 6B) showed very weak hybridization to a 2.7 kb species. Other tissues showed no detectable expression (Figure 6C).

Autophosphorylation of 3F12 MAP Kinase

To test for phosphorylation activity of the p49^{3F12} kinase, a plasmid (pTrcHis-3F12) was constructed in which the kinase was expressed as a 49 kDa fusion protein in Escherichia coli after induction by isopropyl β-D-thiogalactopyranoside (IPTG; Figure 7A). The fusion protein was purified to >95% homogeneity for further analysis.

Incubation of the recombinant p49^{3F12} kinase with buffer and [γ-^{32}P]ATP resulted in phosphorylation of the purified protein (Figure 7B). Heat inactivation of the pure protein before autophosphorylation prevented incorporation of

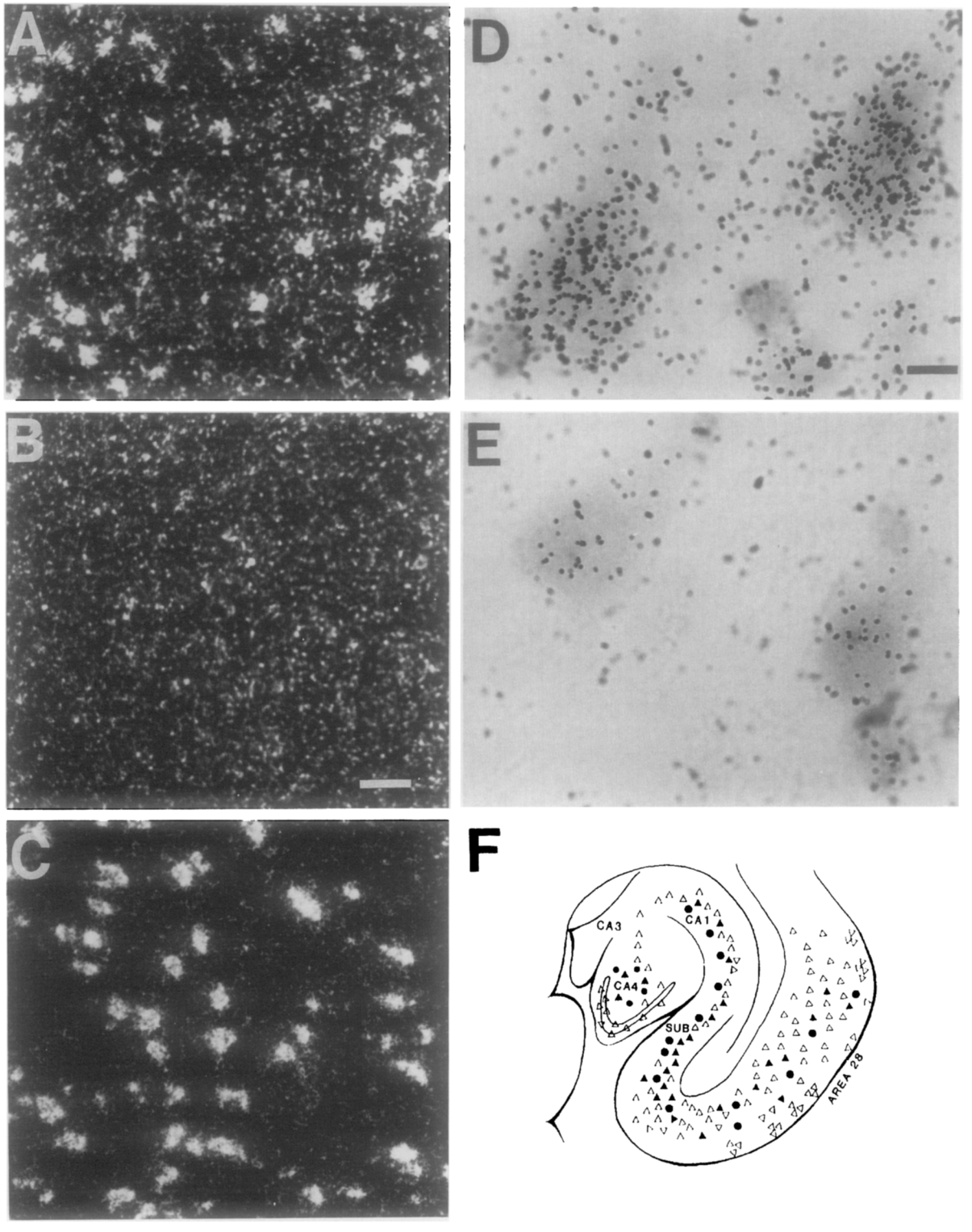


Figure 3. In Situ Localization of the p49^{3F12} Kinase mRNA in Neurologically Normal Hippocampus

Hippocampal sections were probed with ^{33}P-labeled antisense (A and D) and sense (B and E) p49^{3F12} cRNA probes generated from the full-length 2.4 kb cDNA. A subpopulation of pyramidal neurons in the CA4, CA1, and subiculum regions of the hippocampus were labeled. β-APP ^{33}P-labeled antisense cRNA probe was used to determine the integrity of mRNA on tissue sections (C). This probe hybridized to all neurons. Sections were viewed under dark field and light field, respectively. In dark field low magnification (A), silver grains are abundant over neurons in the CA1 region. A concentration of grains over CA1 pyramidal neurons counterstained with Giemsa are seen in light field at higher magnification (D). All slides were exposed to silver emulsion for 6 days at 4°C except β-APP, which was exposed for 3 days (C). A schematic of the human hippocampus delineates regions where neurons immunoreactive with MAb 3F12 (closed triangles) and neurons expressing p49^{3F12} kinase mRNA (closed circles) are located (F). An almost identical distribution of positive neurons is seen in both assays. Open triangles denote nonreactive neurons by immunocytochemistry or in situ hybridization. Note that in (B) and (E), a few grains are present over neurons, owing to background from long exposure times. Bars, 0.1 mm (A, B, and C); 10 μm (D and E).

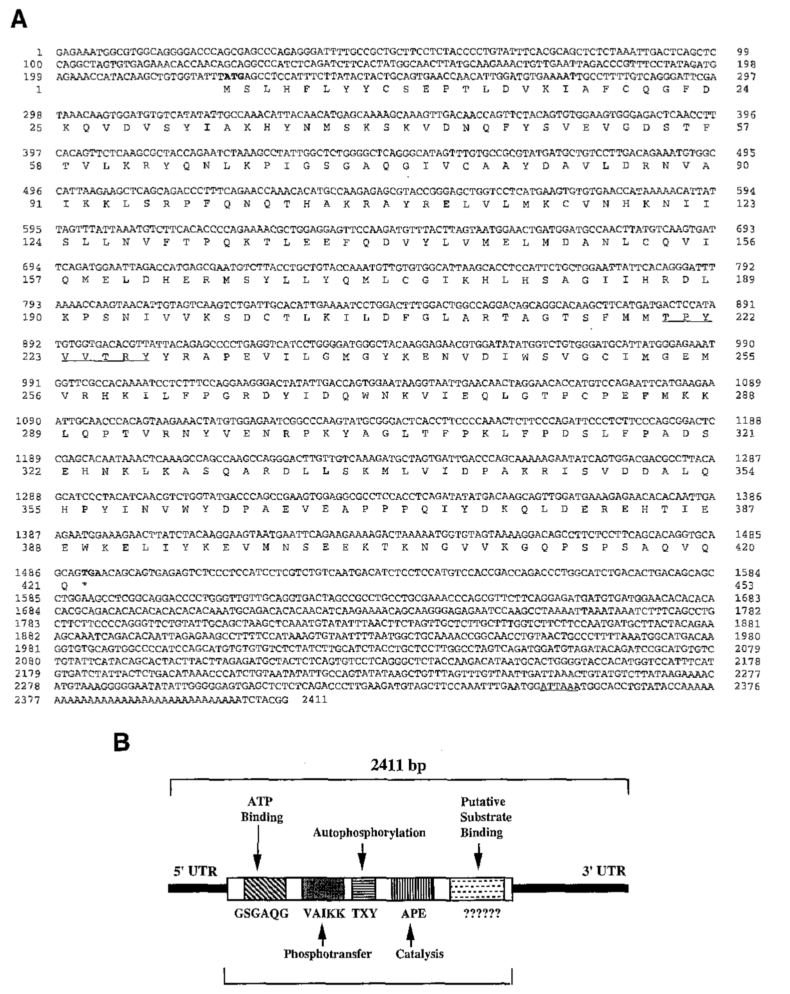


Figure 4. Nucleotide and the Predicted Amino Acid Sequence of the p49^{3F12} cDNA

(A) The open reading frame extends from nucleotide 223 to 1492, encoding a protein of 422 amino acids. ATG of the initiation codon and the TGA of the termination codon are in bold lettering. The two TXY phosphorylation domains are underlined. A possible polyadenylation signal in the 3′ UTR is also underlined.

(B) A domain map of p49^{3F12} kinase showing the amino acid sequences of conserved kinase subdomains.

^{32}P, thus discounting the possibility that tyrosine phosphorylation was due to a nonenzymatic reaction (Seger et al., 1991). Phosphoamino acid analysis of the labeled protein revealed that phosphate was incorporated onto both tyrosine and threonine residues (Figure 7C). The kinase was also autophosphorylated with cold ATP. It showed strong immunoreactivity with antibodies to phosphotyrosine on Western blots. Immunoreactivity could be blocked by the addition of excess phosphotyrosine (Figure 7D).

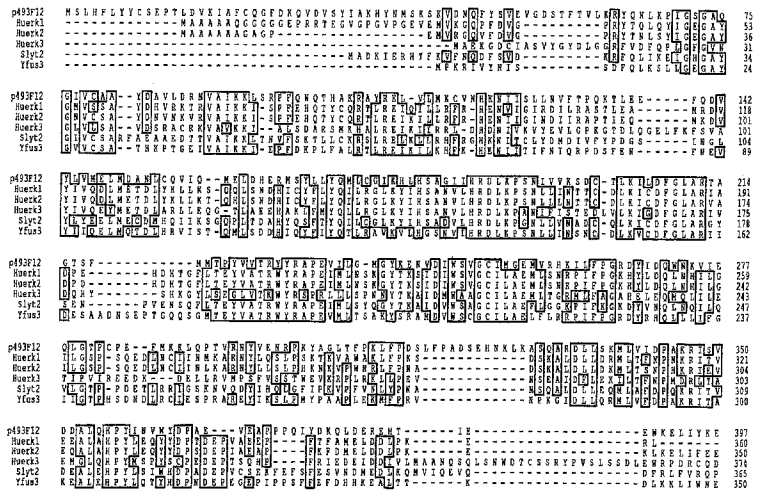
Identification of the Kinase on Immunoblots

Polyclonal antisera generated against the p49 bacterial recombinant protein (anti-p49) identified three immunoreactive proteins of 49, 45, and 35 kDa in Triton X-100 soluble supernatants of human hippocampal homogenates (Figure 8B, lane 1). In a pure preparation of p49 bacterial fusion protein, anti-p49 immunoreactive bands of the same relative mobilities were identified, the band at 49 kDa being the dominant form (Figure 8B, lane 2). MAb 3F12 identified immunoreactive bands at 68, 49, 45, 42 (doublet), and 35 kDa (Figure 8A, lane 1). The immunoreactive bands of 49, 45, and 35 kDa comigrated with those identified by anti-p49. As expected, MAb 3F12 also reacted with the p49 kinase fusion protein (Figure 8A, lane 2). The no-primary-antibody control indicated the presence of a 70 kDa band immunoreactive with the biotinylated secondary antibody (Figure 8C, lane 1). However, this band was clearly distinct from the MAb 3F12 immunoreactive band at 68 kDa. To confirm that anti-p49 and MAb 3F12 recognize the same protein, we purified p49 from hippocampal homogenates by immunoaffinity chromatography using anti-p49. The immunopurified protein was probed on Western blots with MAb 3F12. Both anti-p49 (Figure 8D, lane 1) and MAb 3F12 (Figure 8D, lane 2) recognized the same protein at 49 kDa.

Chromosomal Mapping of the Gene

To determine the chromosomal location of the p49^{3F12} kinase gene, ^{32}P-labeled 2.4 kb cDNA was used to probe Southern blots of hamster somatic hybrid lines. These cell lines harbor at least one human chromosome in addition to the hamster genome. Two unique bands at 5.5 and 2.3 kb (the most intense bands on the blot) were identified in DNA from the cell line containing human chromosome 21 (data not shown). For finer mapping, a 12.5 kb human genomic clone was isolated from a λFixII human genomic library by screening with ^{32}P-labeled 2.4 kb cDNA probe. This clone was then biotin labeled by nick translation and

A



B

	p49^{3F12}	Huerk1	Huerk2	Huerk3	Slyt2	Yfus3
p49^{3F12}	100	41	43	30	41	40
Huerk1		100	87	41	49	52
Huerk2			100	40	49	52
Huerk3				100	33	35
Slyt2					100	47
Yfus3						100

Figure 5. Amino Acid Comparison of p49^{3F12} with Other Proline-Directed Human Kinases

(A) The alignment of the p49^{3F12} protein (top) with human ERK1 and ERK2, ERK3, S. cerevisiae lyt2, and FUS3. p49 displays 41% and 43% amino acid sequence identity to human ERK1 and ERK2, respectively, with the highest identity in the kinase catalytic region.

(B) Comparison of p49^{3F12} kinase deduced amino acid sequence with that of other MAP kinases. Percent identities are shown in the table.

hybridized, along with a chromosome 21 specific cosmid probe, to human metaphase spreads by fluorescence in situ hybridization (FISH). Four fluorescent dots corresponding to areas of probe hybridization were observed on the q arm of chromosome 21 (Figure 9). The two most distal dots hybridized to the telomeric sequences of chromosome 21 as expected, since the cosmid is specific for these sequences. Two other fluorescent dots were observed at the approximate region of 21q21 (Figure 9, inset). These were due to the p49^{3F12} probe, since spreads hybridized with the 21 specific cosmid alone showed only the two telomeric dots. The approximate location of the p49^{3F12} gene was determined by the measurement of the relative distance from the most proximal portion of the

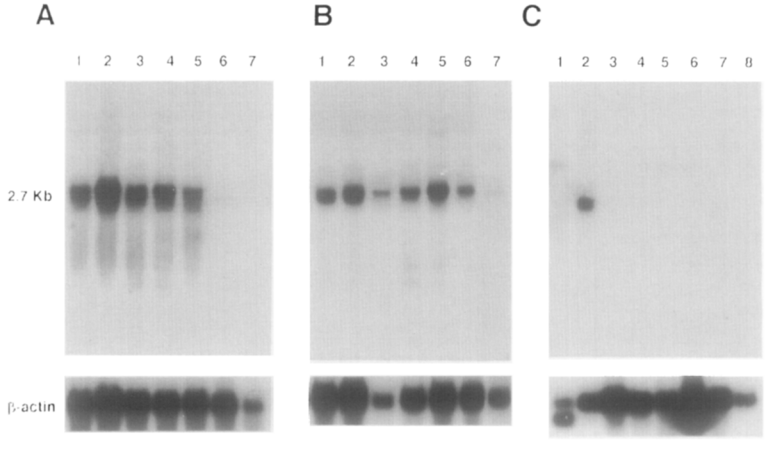


Figure 6. Northern Blot Analysis of p49^{3F12} Kinase mRNA

(A) mRNAs (5 μg) from each CNS site were probed with the 3F12 cDNA and exposed to X-ray film for 3 days. Lane 1, hippocampus; lane 2, area 4; lane 3, area 10; lane 4, area 17; lane 5, cerebellum; lane 6, spinal cord; lane 7, white matter. Note that a single 2.7 kb transcript is seen in the hippocampus and all cortical sites, with none in the white matter. There is markedly lower abundance in the spinal cord.

(B) mRNAs (5 μg) from brain subcortical nuclei, brainstem, and testis were probed with 3F12 cDNA and exposed to X-ray film for 3 days. Lane 1, caudate; lane 2, putamen; lane 3, nucleus basalis; lane 4, thalamus; lane 5, pons; lane 6, medulla; lane 7, testis. A 2.7 kb mRNA was identified in all sites.

(C) mRNAs (2 μg) from various human tissues were probed with ^{32}P-labeled 3F12 cDNA and exposed to X-ray film for 6 days. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. Note that other than in brain, there is hybridization close to threshold only in the kidney. Integrity of the mRNA was established by probing the blots with human β-actin (lower panels).

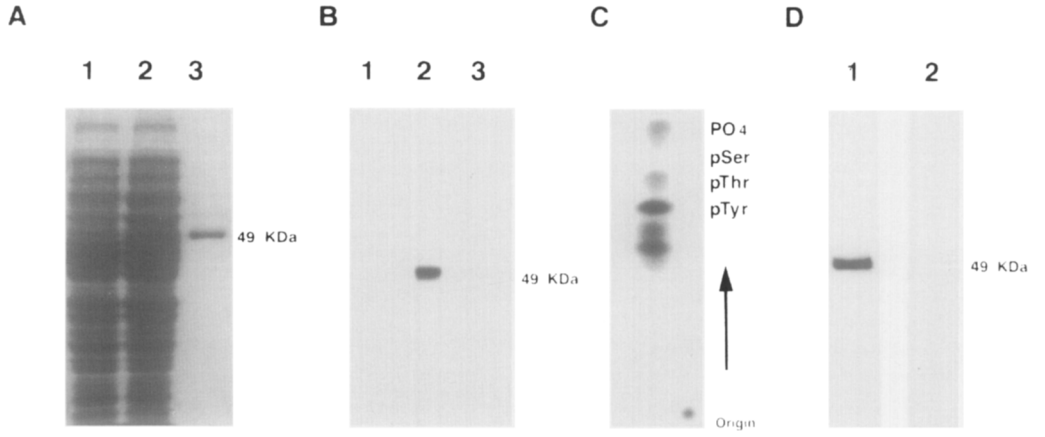


Figure 7. Autophosphorylation of the Recombinant p49[3F12] Kinase Protein

(A) p49[3F12] recombinant protein was produced in bacteria from a construct expressing all but 8 amino acids from the N terminus of the kinase. A 49 kDa protein (containing a histidine tag) was purified from a bacterial lysate by nickel-agarose chromatography. Lane 1, uninduced bacterial lysate; lane 2, induced with IPTG; lane 3, purified p49[3F12] kinase protein. Coomassie blue stain.

(B) The p49[3F12] recombinant protein was incubated with [γ-^{32}P]ATP in kinase buffer, followed by the fractionation of the reaction products on SDS-PAGE and autoradiography. Lane 1, p49[3F12] kinase heat inactivated prior to incubation; lane 2, untreated p49[3F12] kinase; lane 3, bovine serum albumin.

(C) Phosphoamino acid analysis of autophosphorylated p49[3F12] kinase. Autophosphorylated protein was incubated in 6 M HCl and fractionated by thin-layer chromatography. The positions of phosphoserine, phosphothreonine, and phosphotyrosine were marked in an adjacent lane that contained these standards stained with ninhydrin. No ^{32}P-labeled phosphoserine was present in the p49[3F12] hydrolysate. Note that the two lowest bands on the blot represent incompletely hydrolyzed protein. Arrow indicates the direction of migration.

(D) Identification of the autophosphorylated p49[3F12] kinase on Western blot with anti-phosphotyrosine antibodies. For lane 1, the autophosphorylated p49 kinase was fractionated on SDS-PAGE, transferred to nitrocellulose, and probed with MAb anti-phosphotyrosine; for lane 2, MAb anti-phosphotyrosine was preincubated with unlabeled phosphotyrosine.

short arm to the area of probe hybridization and subsequent comparison to a map of human chromosome 21.

Discussion

We have identified the 3F12 neuronal–specific antigen as a novel member of the MAP kinase family, partially confirmed its function, and determined its expression in normal human brain and in various somatic tissues. We have also shown that in brain tissues from patients with AD, MAb 3F12 antigen colocalizes with MAb ALZ-50 in neurons with neurofibrillary pathology. In the human hippocampus, the kinase is distributed in subsets of neurons vulnerable in AD. Furthermore, we have mapped the location of the corresponding gene to the region of 21q21.

In contrast to most MAP kinases described so far, which

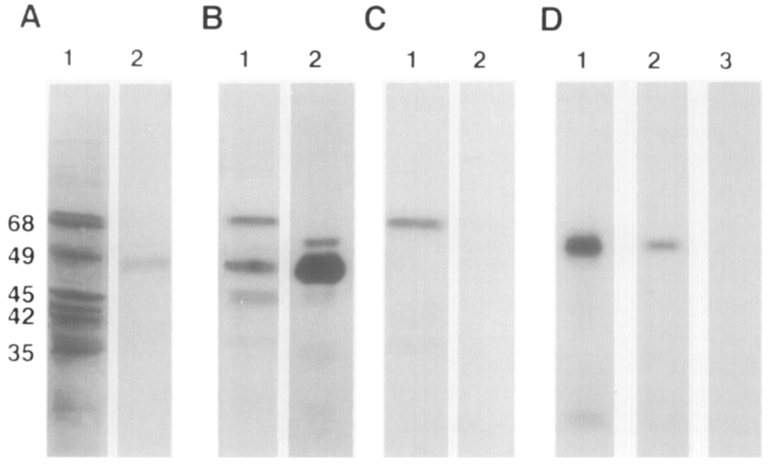


Figure 8. Immunochemical Identification of the p49[3F12] Kinase in Hippocampal Homogenates

One gram of human hippocampus was homogenized in buffer containing 0.1% Triton X-100 and centrifuged at 100,000 × g. The supernatant and a purified preparation of p49[3F12] kinase fusion protein were fractionated by 10% SDS-PAGE and transferred to nitrocellulose. Lane 1, hippocampus supernatant; lane 2, p49[3F12] kinase fusion protein.

(A) MAb 3F12 identifies immunoreactive bands at 68, 49, 45, 42 (doublet), and 35 kDa in hippocampal supernatant (lane 1). MAb 3F12 also identifies the 49 kDa fusion protein (lane 2, arrow).

(B) Anti-p49 identifies immunoreactive bands at 49 (arrow), 45, and 35 kDa in the supernatant and the fusion protein (lanes 1 and 2, respectively).

(C) No primary antibody control indicated that the band at 70 kDa in the supernatant (migrating just above the 68 kDa band labeled by MAb 3F12 in [A]) is due to the nonspecific immunoreactivity of the secondary antibody (lane 1). No immunoreactivity is observed with the p49[3F12] kinase fusion protein (lane 2).

(D) Western blot of immunoaffinity purified p49 from hippocampal homogenates. The blot was probed with the following: lane 1, anti-p49; lane 2, MAb 3F12; lane 3, no primary antibody.

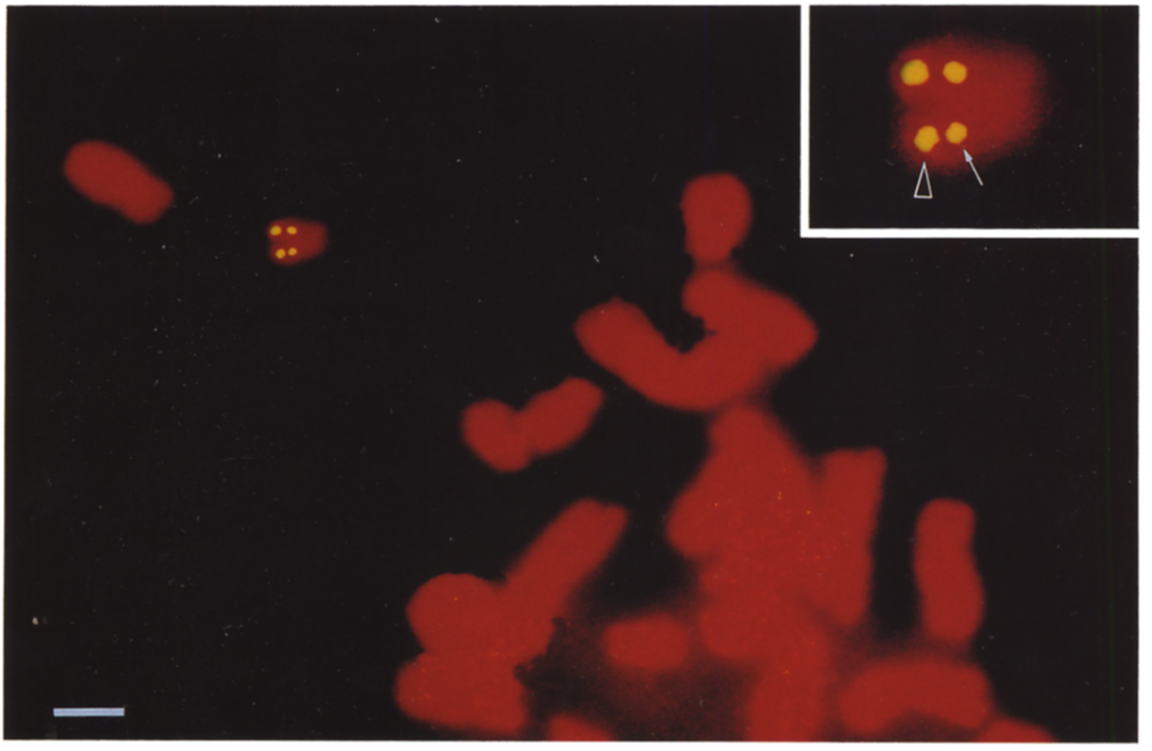


Figure 9. Chromosomal Localization of the p49^{3F12} Kinase Gene by FISH

The 12.5 kb p49^{3F12} human genomic clone and the chromosome 21–specific cosmid probe were biotin labeled by nick translation and hybridized to human metaphase spreads. The probes were detected using streptavidin-FITC on chromosomes that were counterstained with propidium iodide. Low magnification shows specific hybridization of the p49^{3F12} probe (two yellow-green dots) to the long arm of human chromosome 21 (arrow in the inset). The two distal dots are due to hybridization of the human chromosome 21–specific cosmid probe to the telomeric region (arrowhead in the inset).

(Inset) High magnification shows the region of hybridization to be approximately 21q21 (arrow in the inset) as determined by measurement of the distance from the most distal portion of the short arm to the region of hybridization of the p49 probe. Bar, 10 μm.

are ubiquitously expressed, p49^{3F12} kinase expression is restricted to a subset of projection neurons in the nervous system. Analysis of the kinase mRNA by Northern blots and in situ hybridization in various human brain sites revealed expression in a subset of pyramidal neurons in the CA1 and subiculum regions of the hippocampus and in layers 3 and 5 of Brodmann's areas 4, 10, and 17 of the neocortex. Additionally, p49^{3F12} mRNA was detected in subcortical nuclei and brainstem. In nonneural tissues, only the testis and kidney showed markedly lower levels of expression. The lack of any detectable signal in the white matter poly(A)$^+$ mRNA and the weak signal in the spinal cord is in agreement with the regional and neuronal specificity of the 3F12 antigen. To date, the sequence of the p49^{3F12} kinase is novel. However, a previously described cDNA (clone HFBCL46 [EST02170]) from the dBEST library of sequences showed 75% identity with the 3F12 nucleotide sequence. The relatively short clone of 359 bp contains many alignment gaps and nucleotide mismatches. Therefore, it is difficult to determine whether the p49^{3F12} kinase and HFBCL46 are the same clone.

The kinases most closely related to p49^{3F12} are rat ERK1 and human ERK2, which show 41% and 43% amino acid sequence identity, respectively. Both of these kinases have been classified as members of the MAP kinase family and contain a tripeptide (TXY) in the kinase catalytic region. Interestingly, the deduced amino acid sequence of p49 contains two TXY phosphorylation sites separated by two valine residues. This second site may be an additional regulatory element, providing for tighter control of the action of this kinase. Phosphopeptide mapping and site-directed mutagenesis studies have revealed that phosphorylation of threonine and tyrosine residues in this tripeptide are required for maximal kinase activity; even conservative substitution of tyrosine residues reduces activity of the enzyme (Seger et al., 1991). Although Xenopus MAP kinase possesses weak autophosphorylation activity in vitro, deletion of its ATP-binding domain, which abolishes activity of the kinase, does not seem to interfere with phosphorylation of the tripeptide in vivo (Nakielny et al., 1992). To ascertain the functional capacity of p49^{3F12} kinase, we performed autophosphorylation assays with a bacterially produced fusion protein. This kinase possesses weak autophosphorylation activity; the activity is real; it is abolished by exposure to temperatures of 55°C or higher. Incubations of 1 hr or more were required to generate a strong signal. Thus, p49^{3F12} kinase resembles other MAP kinases in that it does not readily autophosphorylate, suggesting that autophosphorylation may not be important for its activation in vivo.

Our studies confirmed that the 49 kDa protein identified by both MAb 3F12 and anti-p49 is the neuronal protein. MAb 3F12 identified two additional proteins of 68 and 42 kDa in hippocampal homogenates. Since these bands are not identified by anti-p49, it is likely that they constitute other related kinases or shared epitopes. The 45 and 35 kDa bands, identified by both MAb 3F12 and anti-p49 in hippocampal homogenates, may be postmortem or preparative degradation products or differentially phosphorylated forms of p49^{3F12} kinase. Anti-p49 also identified bands with the same relative mobilities in the lane containing the purified fusion protein. Moreover, MAb 3F12 identified immunoaffinity purified p49 from hippocampal homogenates. Anti-p49 was not immunoreactive on tissue sections of human hippocampal and frontal cortex. One possible explanation is the masking of epitopes in the native conformation of the kinase in tissue.

ERK1 and ERK2 are expressed in most neurons and reactive astrocytes in the human brain (Hyman, et al., 1994). It is likely that these kinases are also expressed in the subset of neurons expressing p49^{3F12} kinase. ERK1 and ERK2 are regulated by activator kinases such as B-raf and MEK, which are not only expressed in dividing cells, but also in terminally differentiated neurons (Kyriakis et al., 1992). NGF stimulates the action of MAP kinases (Boulton et al., 1991) and the raf-1 protein kinase in PC12 cells (Ohmichi et al., 1992). It would be intriguing to determine whether raf-1 is also capable of activating p49^{3F12} kinase. If so, a mechanism for selective activation of MAP kinases including p49 may exist, allowing targeting of these enzymes to various substrates.

MAP kinases have been implicated in the phosphorylation and activation of transcription factors such as c-myc (Seth et al., 1991). The mechanism of activation of these factors remains controversial; however, activation of MAP kinase in cells expressing these proteins results in increased expression of genes that contain promoter-binding sites for these factors. If p49^{3F12} plays a role in activation of some of these factors, its subset specificity may provide an additional level of regulation, resulting in increased or decreased expression of other genes within the MAb 3F12 immunoreactive subset.

MAP kinases may also be involved in aberrant regulation of the neuronal cytoskeleton; they are capable of abnormally phosphorylating microtubule-associated protein tau at 14–16 proline-directed serine/threonine residues (Drewes et al., 1992). These events may be a precursor for the formation of the NFTs in AD. Localization of the p49^{3F12} kinase to axons and neuronal cell bodies of projection neurons (primarily affected in AD) may provide the opportunity for interaction with tau or other cytoskeletal proteins.

To date, none of the described MAP kinases are restricted in expression to areas containing tangle-bearing neurons. The role of MAP kinases in AD pathology, including the generation of paired helical filaments, has been examined in several laboratories. In biochemical studies, Xenopus MAP kinase converts normally phosphorylated microtubule-associated protein tau into an abnormally phosphorylated form recognizable with antibodies specific for NFTs (Drewes et al., 1992; Goedert et al., 1992; Lu et al., 1993). MAP kinases that recognize proline-directed phosphorylation sites (e.g., ser-pro or thr-pro) seem to be better able to catalyze the transformation of normal tau into an abnormally phosphorylated form than are other kinases, such as Ca^{2+}/calmodulin-dependent kinase (Ca/CaM kinase) or protein kinase C I.

The approximate location of the p49^{3F12} kinase gene to the region of 21q21 may be significant in the pathogenesis of Alzheimer's disease and Down's syndrome. The β-amyloid precursor protein (β-APP) gene has been mapped to the region of 21q11.2-q21 (Tanzi et al., 1987) and the putative Down's syndrome locus to 21q22.3 (Korenberg et al., 1990). Almost all Down's syndrome patients past the age of 35 develop AD-like pathology (NFT and senile plaques) in the brain. One putative locus segregating with the early onset of familial AD phenotype in some pedigrees has also been identified in the region 21q11.2–21q22.2 (St George-Hyslop et al., 1987). The β-APP also undergoes phosphorylation on its C-terminal cytoplasmic domain by protein kinase C and Ca/CaMII kinase, leading to its detachment from cytoskeletal components and possible increased vulnerability to proteolysis (Gandy et al., 1988; Refolo et al., 1991). Speculatively, a gene duplication event involving the p49^{3F12} kinase could lead to its overexpression and abnormal phosphorylation in the brains of Down's syndrome patients. In the case of the familial AD patients, possible mutational events or overexpression of the p49^{3F12} kinase could also result in aberrant function.

We have described the cloning and partial characterization of a novel neuron-specific MAP kinase. The mRNA is expressed in a subset of neurons in the human hippocampus and neocortex. The protein colocalizes with ALZ-50 in AD-vulnerable neurons in the hippocampus, and the gene is located on 21q21, a region that is implicated in AD and Down's syndrome pathogenesis. The kinase may function by phosphorylating neuronal cytoskeletal elements or transcription factors in response to specific stimuli.

Experimental Procedures

Immunohistochemistry

Human tissues were obtained from Alzheimer's Disease Research Consortium of Los Angeles and Orange Counties. Tissue blocks from human hippocampus, neocortex, cerebellum, spinal cord, and white matter from patients ages 35–56 were snap-frozen at autopsy and stored at −90°C. The average postmortem interval was 8 hr for normal and 4 hr for AD patients. Cryostat sections (8–10 μm) were incubated sequentially with the primary antibody, biotinylated goat anti-mouse IgM, avidin-biotin complex (Vector Labs), and diaminobenzidine. The sections were then viewed with an EDGE high resolution microscope (EDGE Instruments).

For double labeling experiments, sections were incubated with the first primary antibody, followed by FITC-conjugated goat anti-mouse IgM (Cappell Labs). Following two washes in 1 × phosphate-buffered solution (PBS), they were incubated sequentially with the second primary antibody, biotinylated goat anti-mouse IgM and Texas Red–conjugated streptavidin (Vector Labs). Following washes, the sections were mounted in vectashield (Vector Labs) and viewed under epifluorescence using a double bypass filter for FITC and Texas Red. MAb 3F12, an IgM, was the generous gift of Dr. Seymour Benzer; MAb ALZ-50, also an IgM, was donated by Dr. Peter Davies.

Immunoblots

Tissues were homogenized 1:5 (g/ml buffer) using a Dounce teflon

homogenizer in 0.1 M phosphate buffer (pH 7.4), 0.1 M NaCl containing 1% Triton X-100, and 2 μm phenyl methyl sulfonyl fluoride (PMSF) and were centrifuged at 100,000 × g for 30 min. The supernatant was then diluted 1:1 with 2× SDS-sample buffer, boiled for 2 min, and fractionated on 10% SDS-polyacrylamide gel. The proteins were electroblotted onto nitrocellulose filters, blocked in 4% dry milk in 0.1% Triton X-100–PBS (TPBS), and incubated overnight with antibody diluted in TPBS at 4°C. Following washes, blots were sequentially incubated with biotinylated secondary antibody, streptavidin-biotin-horseradish peroxidase (HRP) complex and aminoethylcarbazol. For immunoaffinity chromatography, anti-p49 (IgG) was purified on Avid-Al beads (UniSyn Technologies) and bound to Affigel-10 agarose according to the protocol of the manufacturer (BioRad Laboratories). The supernatant from human hippocampal homogenates prepared as above was incubated with beads overnight at 4°C followed by washes in homogenization buffer. The proteins were then eluted from the beads by boiling in 2× SDS sample buffer and analyzed on Western blots.

Construction of Human Hippocampus cDNA Library

RNA from adult human hippocampus was prepared by the guanidine isothiocyanate acid-phenol method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ mRNA was isolated by two rounds of oligo(dT) cellulose chromatography. The construction of an oligo(dT)-primed cDNA library followed the method of Gubler and Hofmann (Sambrook et al., 1989), with some modifications. The cDNA was ligated into dephosphorylated, EcoR1 cut λZAPII arms and packaged in vitro.

cDNA Cloning and DNA Sequencing

MAb 3F12 was used for immunoscreening a random-primed human hippocampal λgt11 expression library (Clontech). Positive clones were plaque purified, and inserts were excised by EcoRI restriction digestion. The insert from clone HH3 was then labeled with ^{32}P by random hexamer priming and used to screen the λZAPII human hippocampal cDNA library under high stringency conditions. Positive clones were plaque purified. To obtain plasmids from isolated phage clones, phagemid rescues were performed using the excision protocol. The DNA sequences of the clones were determined using the sequenase system (United States Biochemical). Both strands were sequenced in their entirety.

The DNA sequence and its deduced amino acid sequence were compared with contents of the GenBank DNA and the Swiss-Pro protein databases using the FASTA and the TFASTA and BLAST programs, respectively. The comparisons were performed using the sequence analysis programs from the University of Wisconsin Genetics Computer Group for the VAX/VMS system (Devereux et al., 1984).

In Situ Hybridization

Human hippocampal sections were cut and fixed in 4% paraformaldehyde, washed in 1× PBS for 15 min at room temperature, and exposed to acetic anhydride (0.25% [v/v] in 0.1 M triethanolamine [pH 8]). The sections were washed twice with 2× SSC, dehydrated through increasing concentrations of ethanol, and air dried. The sections were overlaid with 30–50 ng of p49^{3F12} kinase ^{33}P-labeled cRNA probe in 30 μl of hybridization buffer containing 5× SSC, 5× Denhart's solution, 50% formamide, 100 μg/ml of heat denatured salmon sperm DNA, 20 μg/ml of yeast tRNA (Sigma), and 10% Dextran sulfate. Sections were coverslipped and placed in a humidified incubator overnight at 37°C. Coverslips were removed, and the slides were washed in 0.2× SSC at 50°C and 60°C for 1 hr each. The sections were dehydrated in ethanol, coated with Kodak NTB-2 emulsion, and exposed in the dark at 4°C. After 10 days, the slides were developed and counterstained with Giemsa.

Northern Blot Analysis

Total cellular RNA was prepared from postmortem human tissue by the guanidinium isothiocyanate acid phenol method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ mRNA (5 μg) was fractionated on glyoxal/dimethyl sulfoxide gels and transferred to Nytran membranes (Schleicher and Schuell). RNA was UV cross-linked, and the membrane was air dried. Filters were prehybridized in 6× SSPE, 10× Denhart's, 50% formamide, 1% SDS, and 200 μg/ml of denatured salmon sperm DNA. ^{32}P-labeled p49^{3F12} cDNA (10–50 ng; first 1000 bp) probe was added to the prehybridization mixture, followed by overnight incubation at 37°C. Washes were carried out in 0.1× SSC at 65°C. Filters were placed on enhancing screens (Cronex, Dupont) and exposed to X-ray film at −70°C for 3–7 days for p49^{3F12} and 12 hr for β-actin.

Expression and Purification of 3F12 MAP Kinase

The 3F12 MAP kinase cDNA was cloned into the PstI-KpnI sites of the pTrcHis A expression vector (Invitrogen). The construct was transformed into E. coli, and the fusion protein was expressed and purified on a Ni-NTA column as per the instructions of the manufacturer (Qiagen). The resultant fusion protein contained a 6× histidine tag, an enterokinase cleavage site, and the 3F12 MAP kinase from amino acid 8–422.

Kinase Assays

Autophosphorylation was performed by incubating the 3F12 MAP kinase fusion protein in kinase buffer (25 mM HEPES, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol [pH 7.5], and 100 μM [γ-^{32}P]ATP) at 30°C for 1 hr (Seger et al., 1991). The reaction was terminated by adding SDS electrophoresis sample buffer and heating to 100°C for 3 min. The phosphorylated fusion protein was separated by electrophoresis on a 9% SDS-polyacrylamide gel, blotted onto a polyvinylidine difluoride membrane, and detected by autoradiography on Kodak X-OMAT AR film.

3F12 MAP kinase fusion protein was treated as described above, except unlabeled ATP was substituted for labeled ATP. The membrane was blocked with 5% bovine serum albumin for 1 hr, incubated with a 1:1000 dilution of anti-phosphotyrosine (Sigma), and washed with PBS/0.1% Tween 20. The membrane was then incubated with horseradish peroxidase–conjugated anti-mouse IgG, washed, and visualized with 1-chloro-naphthol. Phosphoamino acid analysis was done as previously decribed (Crews et al., 1991).

Genomic Cloning and FISH

A human λFixII (Stratagene) placenta genomic library was screened with ^{32}P-labeled 2.4 kb cDNA probe. A clone with an insert size of approximately 12.5 kb with multiple internal EcoRI sites was isolated. The EcoRI fragments were gel purified on low melt agarose and labeled with biotin by nick translation.

For FISH, slides with metaphase spreads were denatured in 2× SSC, 70% formamide at 70°C for 2 min and dehyrated in 70% and 95% ethanol for 2 min each (Trask, 1991). The slides were then hybridized overnight at 37°C in buffer containing 2× SSC, 50% formamide, 200 μg/ml of sheared salmon sperm DNA, 200 ng of biotin-labeled 3F12 genomic DNA, and 100 ng of a biotinylated cosmid probe specific for human chromosome 21 telomeric sequences (Oncor). After hybridization, slides were washed at 72°C in 2× SSC for 5 min, equilibrated in 1× PBS, and incubated with streptavidin-FITC (Zymed) for 1 hr at room temperature. Following washes in 1× PBS, slides were mounted in 50 μl of antifade solution containing 25 mg/ml of triethylenediamine, 50% glycerol, and 200 ng/ml of propidium iodide in 1× PBS. The slides were viewed under fluorescence and photographed using the Oncor FISH image analysis system. The location of the p49^{3F12} gene was determined by the measurement of the distance from the most proximal portion of the short arm of chromosome 21 to the location of the hybridized probe. The relative location of the hybridized probe was then determined on a map of human chromosome 21 from human gene map 10.

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GenBank Accession Number

The GenBank accession number for the sequence reported in this paper is U07620.

Note Added in Proof

The kinase SAPkb reported by Kyriakis et al. (1994) is probably the rat homolog of the $p49^{3F12}$. This kinase displays a 98% amino acid identity with the human $p49^{3F12}$ kinase. It also shows 98% amino acid identity with the mouse homolog of p49 (unpublished data). JNK-1 is also a member of this subfamily (Dérijard et al., 1994).