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A novel brain-specific 25 kDa protein (p25) is phosphorylated by a Ser/Thr-Pro kinase (TPK II) from tau protein kinase fractions

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A novel brain-specific 25 kDa protein (p25) was purified from a bovine brain extract. The protein was phosphorylated by Ser/Thr-Pro kinase (TPK II) in tau protein kinase fractions at the Ser residues of Ser-Pro sequences. Using immunoblot analysis, the protein was found only in brain extracts, and was most abundant in the brain regions such as cerebrum and hippocampus, but less abundant in cerebellum, medulla oblongata and olfactory bulb. The protein was detected in rat, bovine and human brain extracts, indicating that this protein specifically exists in mammalian brain tissues.

Brain-specific protein; Phosphorylation; Protein kinase; Immunoblot analysis

1. INTRODUCTION

Tau is known as one of the microtubule-associated proteins (MTPs). Recently, it is reported that paired helical filaments accumulate in aged human brains, particularly in ones of Alzheimer's disease. The paired helical filaments contain phosphorylated tau [1,2]. We are therefore interested in the kinases which phosphorylate tau.

A protein kinase which phosphorylates tau (TPK), was partially purified from a rat brain extract and the phosphorylation of tau was stimulated in the presence of tubulin [3]. Then by the analysis of the phosphorylated tau, the tau protein kinase fraction (TPKs) was found to contain a Ser/Thr-Pro kinase (TPK II) [4]. Recently, there have been found a few Ser/Thr-Pro kinases, which require X-Ser/Thr-Pro-X sequences as a minimal recognition site [5–8]. The TPK II from tau protein kinase fractions is considered as a member of this enzyme family.

During further purification of the enzyme from the bovine brain extract, a 25 kDa protein (p25) was detected. This protein was novel and found to be a substrate for TPK II. Antibodies of p25 were prepared and as a result of immunoblot analysis, p25 was further found to be a brain-specific protein.

Abbreviations: PBS, phosphate-buffered saline; MTPs, microtubule-associated proteins; BSA, bovine serum albumin.

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2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP was purchased from Amersham. Most chemicals, except where noted, were obtained from Sigma.

Tau was purified from MTPs by the method of Grundke-Iqubal et al. [9]. Tubulin was purified from MTPs by the method of Kumagai and Nishida [10]. Protein concentrations were determined by the method of Bradford [11] with the reagent of Bio-Rad. Wistar rats (5 weeks of age) were obtained from Sankyo Lab., and adult bovine brains from the Nakashibetsu preparation center, Mitsubishi Kasei Co.

2.2. *Preparation of various tissue extracts*

Various tissues from rat and bovine brains were homogenized in buffer A (100 mM MES, pH 6.5, 0.5 mM Mg-acetate, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin) and centrifuged at 14 000 rpm for 1 h at 4°C. The supernatants were mixed with one third volume of glycerol and served as tissue extracts. Human brain extracts were kindly provided by Dr. Y. Ihara (University of Tokyo). A normal brain extract (an adult female) and an Alzheimer brain extract (a female, 67 years old) were prepared as 20% homogenate in Tris-saline.

2.3. *Assay for kinase activity*

Phosphorylation of tau was measured as described by Morofushi [12]. Tau (0.15–0.2 mg/ml) and the kinase were mixed in buffer A and the reaction was started by the addition of [γ-^{32}P]ATP (5000 Ci/mmol) at a final concentration of 0.1 mM at 37°C. In the case of synthetic peptides, the reaction mixture contained 100 mM Tris-HCl, pH 7.6, 10 mM Mg-acetate, 50 μM peptide and 100 μM [γ-^{32}P]ATP (5000 Ci/mmol) in a total volume of 50 μl. The assay was carried out as described by Roskoski [13].

One unit of the kinase was defined as the amount of the enzyme required to incorporate 1 pmol of ^{32}P into the substrate (tau or peptide) in 1 min at 37°C.

2.4. *Analysis of amino acid sequences of p25 and oligopeptide synthesis*

The sequence of the amino acids of p25 was determined by a pulse-liquid phase protein sequencer (Applied Biosystems 477A). Partial peptides of p25 were chemically synthesized by solid phase method [14], using Biosearch model 9500 peptide synthesizer.

2.5. *Preparation of antibodies to p25 and to the synthetic peptides*

Partially purified p25 (by gel filtration) was separated in SDS/PAGE and was transferred onto nitrocellulose paper. A fine powder suspension of p25-nitrocellulose in PBS was injected into rabbit with adjuvant.

The synthetic oligopeptide (F3) was covalently crosslinked to the carrier protein, keyhole limpet hemocyanin, through the cysteine residue attached to the N-terminal of the peptide, according to the method of Liu et al. [15]. Rabbits were immunized by multiple intramuscular injection of peptide solution or p25 nitrocellulose suspension with Freund's complete adjuvant. Booster immunizations were given at 4-week intervals with the same quantity of antigens in Freund's incomplete adjuvant.

A monoclonal antibody of p25 was prepared with purified p25 from SP-Sephadex according to the same methods as described previously [16].

2.6. *SDS-polyacrylamide slab gel electrophoresis (SDS/PAGE) and Western immunoblotting*

SDS/PAGE was carried out with a 3% stacking gel and a 12.5% resolving gel. Gels were stained with Coomassie brilliant blue R250 or 2D-silver stain II (Daiichi Pure Chemicals). For autoradiography, the gels were dried in vacuum and exposed to Kodak Omat RPI 6 film at −80°C. For Western immunoblotting, electrophoretic transfer onto nitrocellulose paper (Schleicher and Schuell) was carried out by the method of Tang et al. [17]. Immunodetection employed the Vectastain ABC system (Vector Laboratories).

3. RESULTS AND DISCUSSIONS

3.1. *Preparation of p25 and TPK II*

To prepare p25 and TPK II, bovine brain was homogenized in buffer A and the supernatant was treated with assembly–disassembly cycles to prepare MTPs according to the method of Ihara et al. [18]. After ammonium sulfate fractionation (0.33–0.50 saturation) of MTPs, phosphocellulose column chromatography (0.1–0.8 M NaCl linear gradient) was carried out. The tau-rich fraction was eluted with 0.3 M NaCl and the p25-rich fraction was eluted with 0.4 M NaCl in buffer A. Kinase activity was detected in both fractions. These fractions were applied on a G3000 SW gel filtration column (Toso Co., 7.5 × 600 mm), equilibrated with 0.5 M NaCl-buffer A. Both the kinase activity and p25 were eluted together at the molecular mass of about 50 kDa. The kinase activity and p25 were finally separated through SP-Sephadex column chromatography. The results are shown in Fig. 1. The kinase was eluted at pH 7.6 with a gradient of pH 6.6–8.5 of buffer B. Subsequently, p25 was eluted at 0.05 M NaCl with a linear gradient of 0–0.1 M NaCl in buffer B, pH 8.5. The kinase fraction (Fr. 22–33) and p25 fraction (Fr. 81–92) were concentrated with YM 10 Diaflo membrane (Amicon) and served as the enzyme and the substrate respectively. Before using p25 as the substrate, the protein was treated with trichloroacetic acid to remove contaminating kinase activity. Although another tau kinase activity (TPK I) was detected around Fr. 60, p25 was not phosphorylated by this kinase.

3.2. *Properties of p25*

Although p25 was isolated from bovine MTPs during the purification of TPK, the protein did not specifically bind to MTPs, but was evenly detected in the tissue extract itself. This was confirmed by the immunoblotting method. The supernatant, after the removal of MTPs, contained p25 at the same ratio to total protein as that in MTPs (data not shown). As a simple method of preparation of p25, it was found possible to recover it in the supernatant by heat-treating the tissue extract, according to the method of tau preparation [9]. The amount of p25 in bovine brain extracts was estimated as 0.5–1.0 μg/mg (total protein) by the immunoblotting method. p25 was a found to be a basic protein, with an isoelectric point of pH 9.9 determined by isoelectric focusing column chromatography at pH 3.5–10.0 with Ampholine (LKB-Produkter AB). The molecular mass of p25 was estimated to be about 50 kDa on gel filtration of G 3000 SW, while the result on SDS-PAGE showed that the molecular mass was 25 kDa. These results suggest that p25 may exist in a dimeric form.

3.3. *Distribution of p25 in various mammalian tissues*

The distribution of p25 was determined in various tissues of mammals using monoclonal and polyclonal antibodies to p25. Figure 2a shows the result of Western blotting with a polyclonal antibody to p25 of various tissue extracts from rats. p25 was detected only in brain extracts. A faint band of a larger molecular mass was detected in a kidney extract, but this reaction appeared even in the presence of excess p25, therefore, it is not a p25 related protein.

Figure 2b shows the distributions of p25 in different regions of rat brains using a polyclonal antibody to F3. Figure 2c shows the distributions of p25 in various regions of bovine brains using a monoclonal antibody to p25. These results indicated that p25 was widely distributed in various regions of mammalian brains such as cerebrum and hippocampus, but was less abundant in the cerebellum, olfactory bulb and medulla oblongata.

The p25 was detected in human as well as rat and bovine brain extracts, as shown in Fig. 2d. The results indicate that this protein exists in mammalian brains. The only monoclonal antibody obtained from mouse hybridoma had no cross-reactivity with rat brain extracts. Similarly, the monoclonal antibody from bovine p25 had a common epitope with that of human, but not of rat. The result may indicate that p25 of various mammalian brains contains both constant and variable amino acid regions. Polyclonal antibodies obtained using p25 or synthetic partial peptides of the p25 showed cross-reactivity with these three mammals.

It seemed that the amount of p25 in Alzheimer's disease brain was slightly less than that in normal human brain extract (Fig. 2d). However, the number of samples are too limited to speculate the relation between the amount of p25 and the aging effect. To elucidate the biological significance of p25, the developmental and

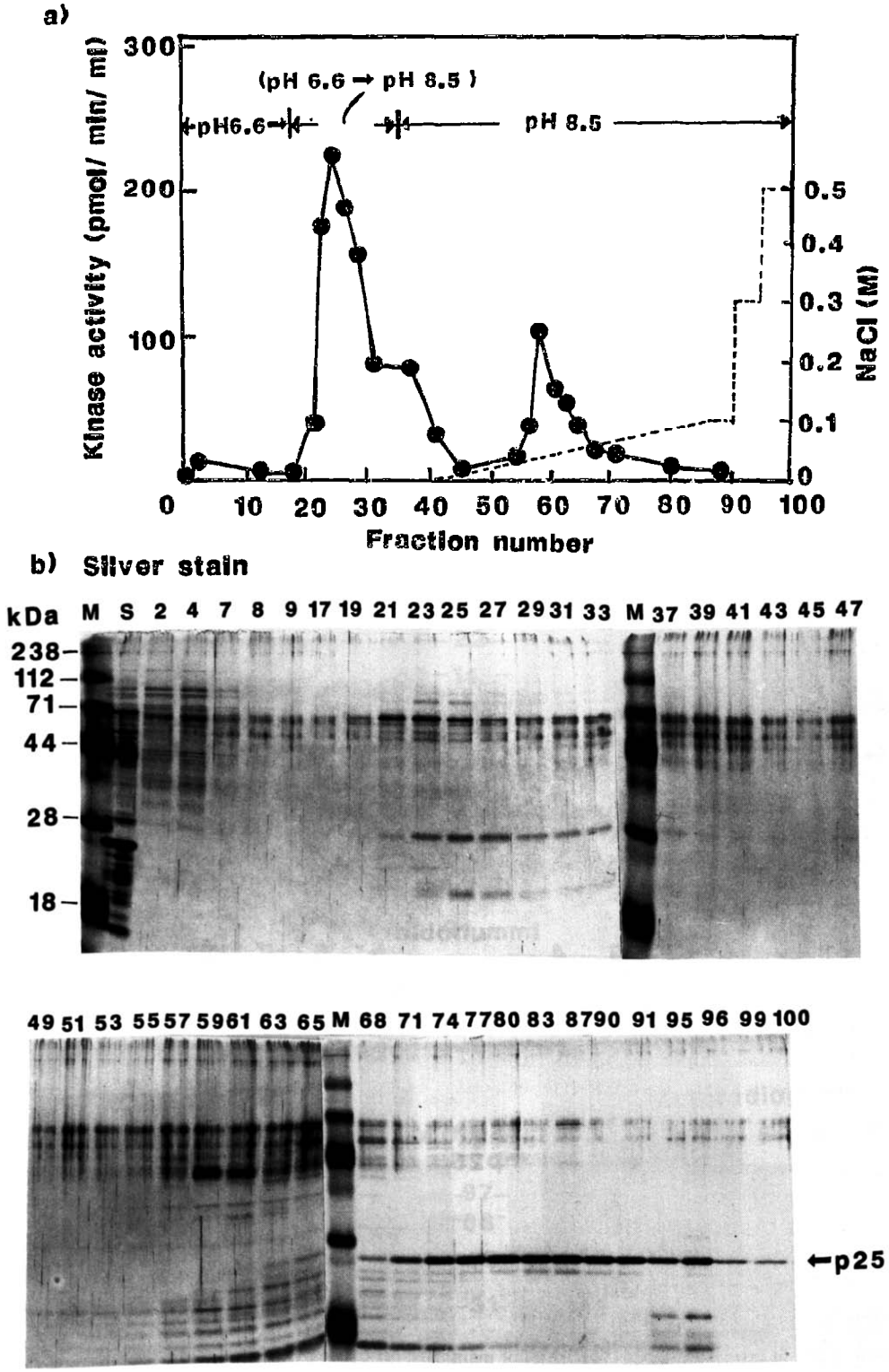

Fig. 1. SP-Sephadex column chromatography of p25 and TPK II. (a) The G -3000 SW fraction (7.6 ml, 1.75 mg) containing p25 and the kinase activity was loaded onto a 2 ml SP-Sephadex column equilibrated in buffer B (50 mM Tris-acetate, pH 6.6, containing 10% glycerol, 0.5 mM Mg-acetate, 1 mM EGTA, 0.05% Tween 20, 20% glycerol, and protease inhibitors). The column was prepared through a series of washing and development; the first washing was with the equilibration buffer, pH 6.6, followed by a development with a linear pH gradient from pH 6.6 to pH 8.5 (10 ml each), the second washing with the buffer, pH 8.5 and then with a linear salt gradient, 0 to 0.1 M NaCl (20 ml each), and the final series of stepwise washing with 0.1 M, 0.3 M and 0.5 M NaCl. Elution fractions (45 drops/tube) were collected. The kinase activity was assayed with tau (●) as described in Section 2. (b) SDS/PAGE of SP-Sephadex eluates. Aliquots (6 μl) were withdrawn from each tube and subjected to SDS/PAGE and the gels were stained with silver stain. Fraction numbers are indicated above each lane. M, pre-stained molecular weight markers (BRL); S, charged sample.

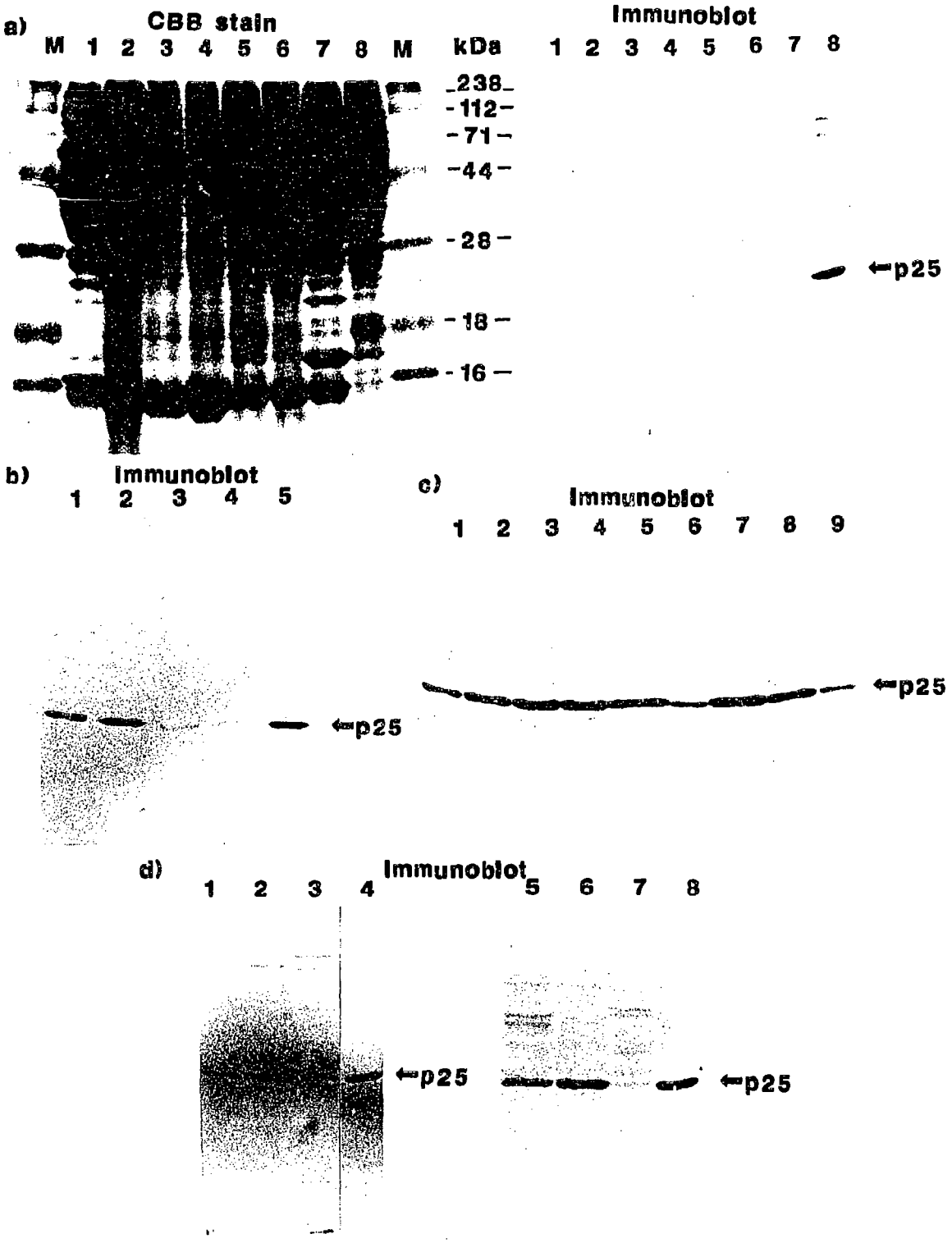

Fig. 2. Immunodetection of p25 in extracts from various tissues of mammals. (a) Crude extracts were prepared from rat muscle (lane 1), kidney (lane 2), lung (lane 3), spleen (lane 4), thymus (lane 5), liver (lane 6), heart (lane 7), brain (lane 8). The proteins (120 μg) from the different tissues were subjected to SDS-PAGE. The separated proteins were either stained with CBB or electrotransferred onto nitrocellulose paper followed by immunoreacting with anti-p25 antiserum. Lane M, pre-stained molecular standard markers. (b) The tissue extracts of rat brains were prepared from cerebrum (lane 1), hippocampus (lane 2), cerebellum (lane 3), olfactory bulb (lane 4), and the rest of the brain (lane 5). These proteins (120 μg) were subjected to SDS-PAGE. The separated proteins were electrotransferred onto nitrocellulose paper followed by immunoreacting with rabbit antiserum directed against a synthetic p25 peptide, F3. (c) The tissue extracts of bovine brains were prepared from cerebrum (anterior lane 1, intermediate lane 2, posterior lane 3), septal area (lane 4), hippocampus (lane 5), cerebellum (lane 6), corpora quadrigenia (lane 7), pons (lane 8), medulla oblongata (lane 9). These proteins (120 μg) were separated as described in (b), and immunoreacted with anti-p25 monoclonal antibody. (d) Brain extracts were prepared from human (Alzheimer's disease) (lanes 1,5), human (normal) (lanes 2,6), rat (lanes 3,7), and bovine (lanes 4,8). The proteins (60 μg) of various brain extracts were separated as described in (b) and were immunoreacted with the anti-F3 antiserum (lanes 1–4) or with monoclonal antibody raised against bovine p25 (lanes 5–8).

Table I
K_m and V_{max} of synthetic peptides

Protein	Peptides name	Animo acid residues sequence	K_m (mM)	V_{max} (mmol/h/mg)
25kP	F3	KAISSPTVSRLTD	1.00	0.80
25kP	F5a	PANKTPPKSPGEPAKDKAAK	0.63	2.66
25kP	F5b	MADSRPKPANKTPPKSPGEPAKDPAAK	0.31	0.80
tau	K1	VAVVRTPPKSPSSAK	0.56	1.29
tau	K2	SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK	0.25	4.70
tau	K3	SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK	0.19	1.60

The phosphorylated serine is underlined. The common 6 amino acid sequences are marked with broken lines.

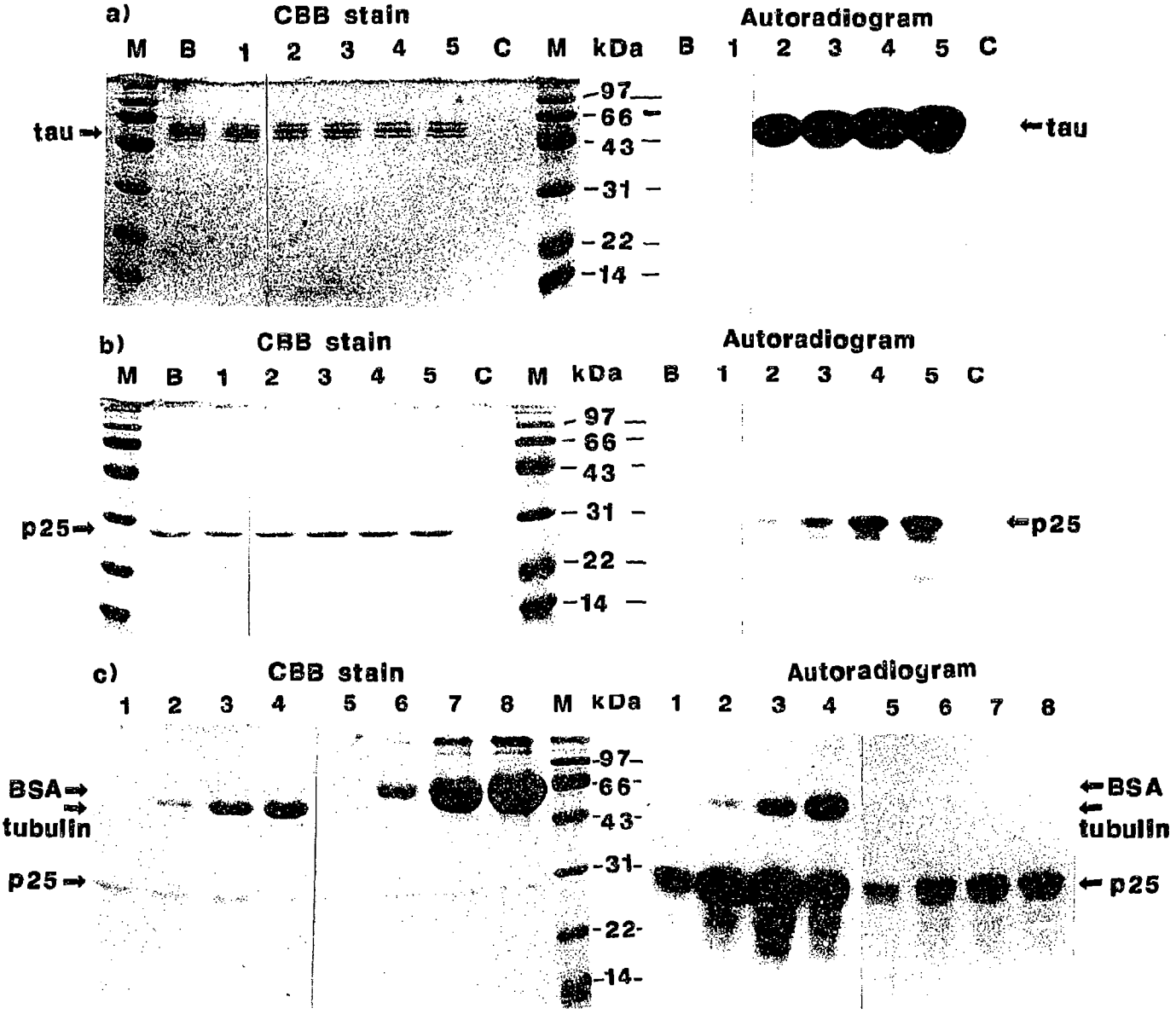

Fig. 3. Time course of phosphorylation of tau and p25, and stimulation with tubulin. (a) Tau (1.5–2 μg) or (b) p25 (1 μg) were incubated with the kinase (0.5 unit for tau, 1 unit for p25) at 37°C for 1 min (lane 1), 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), 4 h (lane 5); lane B, incubated for 4 h without the enzyme; lane C, incubated for 4 h without the substrate; lane M, standard markers (Bio -Rad). (c) p25 was incubated with the kinase for 1 h at 37°C without tubulin (lanes 1,5); with tubulin, 1.3 μg (lane 2), 5.3 μg (lane 3), 10.6 μg (lane 4); with BSA, 2.5 μg (lane 6), 10 μg (lane 7), 20 μg (lane 8). The reaction products were subjected to SDS/PAGE, and the gels were stained with CBB. The radiolabeled products were revealed by autoradiography.

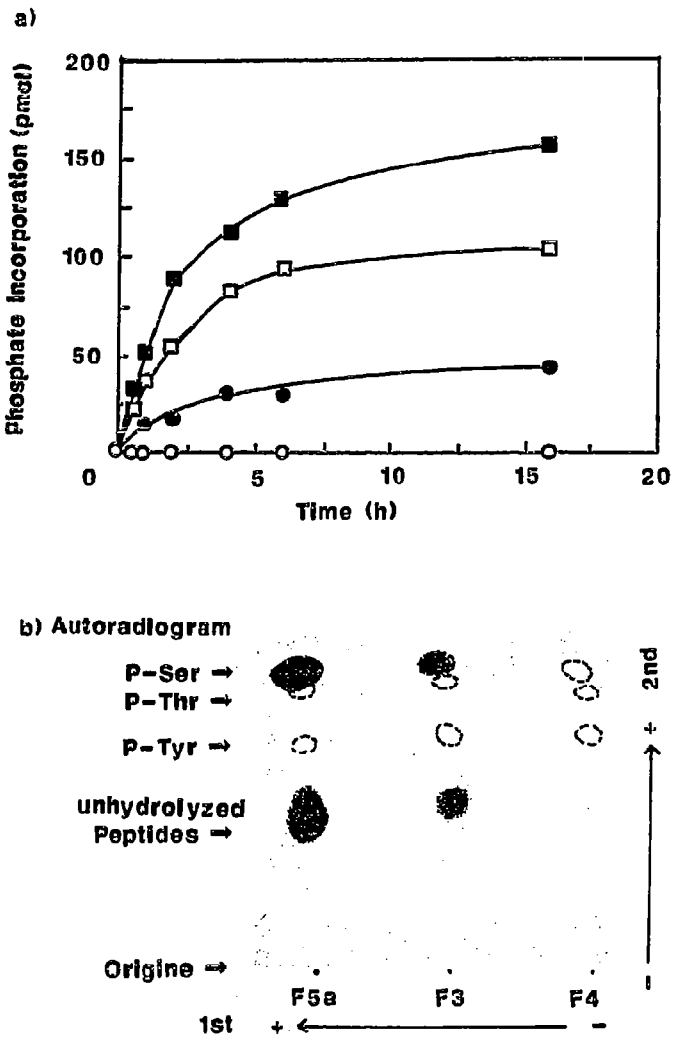

Fig. 4. Phosphorylation of synthetic peptides of p25. (a) The phosphorylation of peptides by the kinase was monitored as a function of time. The substrates were, ●, F3 (KAISSPTVSRLTD); ○, F4 (KERFDPSGRGK); ■, F5a (PANKTPPKSPGEPAKDKAAK); □, F5b (MADSRPKPANKTPPKSPGEPAKDKAAK). (b) Phosphoamino acids of ^{32}P-labeled peptides were hydrolyzed and analyzed with two-dimensional electrophoresis. First dimension, acetic acid/ formic acid/H_2O (78:25:897), pH 2.0 at 450 V for 1 h ; 2nd dimension, acetic acid/pyridine/H_2O (50:5:945), pH 3.5, at 450 V for 3 h [19,20]. The radiolabeled amino acids were detected by autoradiography. The positions of the phosphorylated amino acid standards were visualized by ninhydrin staining as shown with dotted circles.

immunohistological distribution in rat brain tissues are presently under investigation.

3.4. *Phosphorylation of p25 and tau*

It has been reported that TPK II phosphorylates Ser or Thr of Ser/Thr-Pro sequences of tau [4]. Partial amino acid sequences of p25 were determined and the results showed this to be a novel protein. The complete cDNA sequence will be published elsewhere. p25 has two Ser-Pro sequences, one of which has six amino acids identical to the K1 site of tau ([4] and Table I). These observations suggested that p25 might also be phosphorylated by TPK II. As shown in Fig. 3, p25 and tau were phosphorylated by TPK II but p25 was not as extensively phosphorylated as was tau. The phosphorylated protein of about 23 kDa seen in Fig. 3b (lane c) was observed even without addition of p25 to the reactions. Other smaller phosphorylated proteins are probably partially degraded p25. Phosphorylation of tau was shown to be stimulated by tubulin [3]. Results shown in Fig. 3c demonstrate that p25 phosphorylation was also stimulated by tubulin but not by BSA, indicating that the stimulation was not simply stabilization of the proteins. The significance of this stimulation remains unknown.

3.5. *Phosphorylation of synthetic peptides*

Three synthetic peptides (Table I) containing the Ser/Thr-Pro sequences found in p25 were made and shown to be substrates for TPK II (Fig. 4). A control peptide similar to peptide F3 but with the Ser-Pro changed to Pro-Ser was not phosphorylated. The site of phosphorylation (Table I) was identified as Ser (Fig. 4b). A single Ser residue was phosphorylated on peptide F3. The identification was established by sequence analysis as PTH-dehydroalanine, the main product of Edman degradation of phosphoserine [4]. Phosphorylation sites in tau were found to be limited to K1, K2 and K3 ([4] and Table I). The K_m and V_{max} values were determined for all of the peptides listed in Table I. It was interesting to note that peptide F5 from p25 and peptide K1 of tau have six amino acid identities and had very similar K_m values.

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REFERENCES

[1] Ihara, Y., Nukina, N., Miura, R. and Ogawara, M. (1986) J. Biochem. 99, 1807–1810.
[2] Grundke-Iqubal, I., Iqubal, K., Tung, Y.-C. Quinlan, M., Wisniewski, H.M. and Binder, L.I. (1986) Proc. Natl. Acad. Sci. USA 83, 4913–4917.
[3] Ishiguro, K., Ihara, Y., Uchida, T. and Imahori, K. (1988) J. Biochem. 104, 319–321.
[4] Ishiguro, K., Omori, A., Sato, K., Tomizawa, K., Imahori, K. and Uchida, T. (1991) Neurosci. Lett., in press.
[5] Vulliet, P.R., Hall, F.L., Mitchell, J.P. and Hardie, D.G. (1989) J. Biol. Chem. 264, 16292–16298.
[6] Wible, B.A., Smith, K.E. and Angelides, K.J. (1989) Proc. Natl. Acad. Sci. USA 86, 720–724.
[7] Cisek, L.J. and Corden, J.L. (1989) Nature 339, 679–684.
[8] Woodford, T.A. and Pardee, A.B. (1986) J. Biol. Chem. 261, 4669–4676.
[9] Grundke-Iqubal, I., Iqubal, K., Quinlan, M., Tung, Y.-C., Zaidi, M.S. and Wisniewski, H.M. (1986) J. Biol. Chem. 261, 6084–6089.
[10] Kumagai, H. and Nishida, E. (1979) J. Biochem. 85, 1267–1274.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[12] Murofushi, H. (1973) Biochim. Biophys. Acta 327, 354–364.
[13] Roskoshi, R. (1983) Methods Enzymol. 99, 3–6.
[14] Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149–2154.
[15] Liu, F.-T., Zinnecker, M., Hamaska,T. and Katz, D.H. (1979) Biochemistry 18, 690–697.

[16] Takahashi, M. and Tomizawa, K. (1990) Eur. J. Biochem. 192, 735–740.
[17] Tang, V.C.W., Peralta, J.M. and Simons, A.R.(1983) Methods Enzymol. 92, 377–391.
[18] Ihara, Y., Fujii, T., Arai, T., Tanaka, R. and Kaziro, Y. (1979) J. Biochem. 86, 587–590.
[19] Cooper, J.A., Sefton, B.M. and Hunter, T. (1983) Methods Enzymol. 99, 387–402.
[20] Mitsui, K. and Iwashita, S. (1990) Seikagaku 62, 457–461 (in Japanese).