Volume 217, number 2, 237-241 FEB 04834 June 1987

Protein kinase C phosphorylates tau and induces its functional alterations

Minako Hoshi, Eisuke Nishida, Yoshihiko Miyata, Hikoichi Sakai, Tomoko Miyoshi*, Hiroshi Ogawara* and Tetsu Akiyama*+

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113 and **Department of Biochemistry, Meiji College of Pharmacy, Nozawa, Setagaya-ku, Tokyo 154, Japan*

Received 13 April 1987

We found that tau, one of the major microtubule-associated proteins, is a good substrate for protein kinase C. The phosphorylation occurred mainly on serine residues and the sites phosphorylated by protein kinase C were largely different from those phosphorylated by cAMP-dependent protein kinase as analyzed by phosphopeptide mapping. The protein kinase C-mediated phosphorylation of tau reduced its abilities to promote tubulin polymerization and to cross-link actin filaments. The reduction in its abilities was in proportion to the number of phosphates incorporated into tau.

Phosphorylation; Protein kinase C; Actin cross-linking; Microtubule assembly; Microtubule-associated protein

1. INTRODUCTION

Protein kinase C, whose activity is stimulated by unsaturated diacylglycerol or by tumor promoters, would be one of the major routes to pass the information of extracellular signals into the cell interior [1]. We have investigated the actions of purified protein kinase C on cytoskeletal proteins [2], because treatment of cells with the potent tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate induces a rapid redistribution of the cytoskeleton [3,4].

Tau is one of the major protein factors first identified as proteins which copurify with brain microtubules and promote tubulin polymerization in vitro [5-7] and thus thought to be essential as a regulator of microtubule assembly [8]. Recent biochemical studies have shown that tau has the ability to interact with actin and intermediate filaments in addition to microtubules [9-11]. These studies suggest that tau plays an important role in controlling the intracellular organization of cytoskeleton. Here, we show that tau is a good substrate for the activated protein kinase C and that phosphorylation of tau by protein kinase C inhibits the abilities of tau to promote tubulin polymerization and to cross-link actin filaments.

Correspondence address: M. Hoshi, Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

+ Present address: Department of Pathology, Institute of Virus Research, Kyoto University, Shogoin, Sakyo-ku, Kyoto 606, Japan

2 MATERIALS AND METHODS

2.1. *Preparation of protein kinase C and cyto-skeletal proteins*

Protein kinase C was purified from rabbit kidney or brain as in [2,12]. Microtubule protein was isolated from porcine brains by three cycles of temperature-dependent assembly and disassembly [13], tubulin from microtubule protein by phosphocellulose chromatography [5,9], and tau from the heat-stable microtubule-associated protein fraction by DEAE-cellulose chromatography

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

[9–11]. Rabbit skeletal muscle actin was prepared according to Spudich and Watt [14] and further purified by gel filtration on Sephadex G-100 [15]. Catalytic subunit of cAMP-dependent protein kinase (bovine heart) was purchased from Sigma.

2.2. *Phosphorylation of tau by protein kinase C*

To analyze the kinetics of phosphorylation, purified tau (10 μg) was incubated with purified protein kinase C (0.1 μg) in the presence of [γ-^{32}P]ATP at 25°C for various times. Detailed assay conditions are given in the figure legends. The reaction was terminated by addition of Laemmli sample buffer [16] and boiling the mixture for 2 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. For estimation of the number of phosphates incorporated into tau, the bands containing tau were excised from the gels and the radioactivity was counted with a scintillation counter. For investigation of functional alteration of tau by protein kinase C-mediated phosphorylation, we prepared phosphorylated and unphosphorylated tau by incubating 500 μg/ml of tau with 1 μg/ml of protein kinase C at 25°C in the presence and absence of ATP, respectively. Because tau is heat-stable, the reaction was terminated by boiling the mixture for 2 min. When the incubation was performed in the absence of ATP, ATP was added to the sample after boiling. Then, these samples were assayed for their abilities to induce tubulin polymerization and to cross-link actin filaments.

2.3. *Microtubule assembly*

The phosphorylated or unphosphorylated tau was mixed with phosphocellulose-purified tubulin (1.5 mg/ml) at 0°C in a solution of 70 mM Mes, 10 mM Hepes, 0.6 mM $CaCl_2$, 2.2 mM EGTA, 4 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 0.2 mM ATP, 0.02 mM PMSF, 10 μg/ml of phosphatidylserine, 0.1 μg/ml of diolein and 1 mM GTP (pH 6.7). Microtubule assembly was initiated by elevating the temperature of the mixture from 0 to 35°C. The assembly process was monitored by measuring turbidity at 350 nm using a Gilford 260 spectrophotometer.

2.4. *Falling ball assay*

Low-shear falling ball viscometry was performed as in [17]. Various concentrations of the phosphorylated or unphosphorylated tau were mixed with monomeric actin (0.15 mg/ml) in a solution of 50 mM Mes, 5 mM Hepes, 3.6 mM $MgCl_2$, 0.6 mM $CaCl_2$, 0.6 mM EGTA, 15 mM KCl, 0.8 mM DTT, 0.02 mM PMSF, 5 μg/ml of phosphatidylserine, 0.05 μg/ml of diolein and 0.15 mM ATP (pH 6.4). The samples were then drawn up into capillary tubes and incubated for 30 min at 20°C before viscosities were measured.

3. RESULTS AND DISCUSSION

Tau, one of the major brain microtubule-associated proteins, consists of four or five closely related polypeptides with molecular masses between 55 and 62 kDa [6,7]. We found that tau was markedly phosphorylated by purified protein kinase C in the presence of Ca^{2+} and phosphatidylserine (fig.1A). The incorporation of ^{32}P was near-

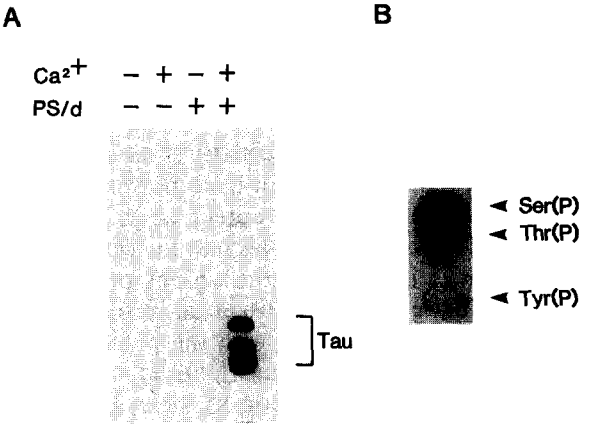

Fig.1. (A) Phosphorylation of tau by protein kinase C. Tau (10 μg) was phosphorylated by protein kinase C (0.1 μg) for 5 min at 25°C in the presence or absence of phosphatidylserine (50 μg/ml) and diolein (0.5 μg/ml) and/or Ca^{2+} (0.5 mM) in a solution of 20 mM Hepes, 10 mM $MgCl_2$, 20 μM ATP ($1–2 \times 10^6$ cpm [γ-^{32}P]-ATP) and 0.4 mM EGTA (pH 6.9). Samples were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. The autoradiogram is shown. (B) Phosphoamino acid analysis of tau phosphorylated by protein kinase C. Tau was phosphorylated for 30 min in the presence of Ca^{2+}, phosphatidylserine and diolein under the same conditions as in (A). Phosphoamino acid analysis was performed as in [2].

ly linear for 20 min and continued to increase further in the presence of Ca^{2+} and phosphatidylserine (fig.2A). When 10 μg tau was phosphorylated for 90 min by 0.1 μg protein kinase C in the presence of 0.5 mM ATP, 3.8 mol phosphate were incorporated into 1 mol tau. The apparent K_m for tau was 6 μM from the Lineweaver-Burk plot (fig.2B).

Phosphoamino acid analysis revealed that tau was phosphorylated by protein kinase C mainly on serine and faintly on threonine residues (fig.1B). Since it has been reported that cAMP-dependent protein kinase also phosphorylates tau on serine residues [18,19], we compared the site specificity of phosphorylation of tau by these two kinases by two-dimensional tryptic phosphopeptide mapping. Fig.3 shows that the sites phosphorylated by protein kinase C are clearly distinct from those by cAMP-dependent protein kinase. On the other hand, it was also reported that Ca^{2+}/calmodulin-dependent protein kinase, unlike these two kinases, phosphorylates tau on threonine as well as serine residues [19]. Thus, these three kinases may phosphorylate tau differently.

We investigated whether the protein kinase C-mediated phosphorylation of tau alters its function. Fig.4 shows that the unphosphorylated tau (89 μg/ml) promoted tubulin polymerization effec-

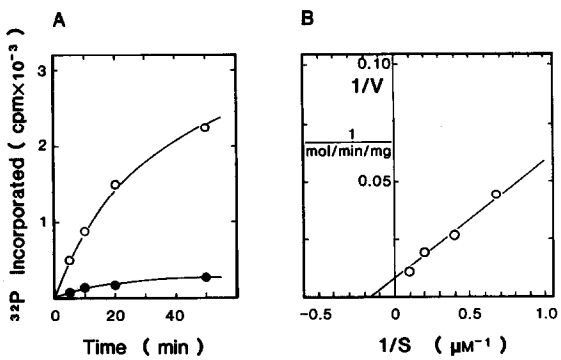

Fig.2. (A) Time course of phosphorylation of tau by protein kinase C. Tau was incubated with protein kinase C for the indicated times in the absence (●) or presence (○) of Ca^{2+}, phosphatidylserine and diolein as in fig.1A. Samples were analyzed as described in section 2. (B) Lineweaver-Burk plot for phosphorylation of tau by protein kinase C. The initial velocity of phosphorylation of tau was estimated after 5 min incubation.

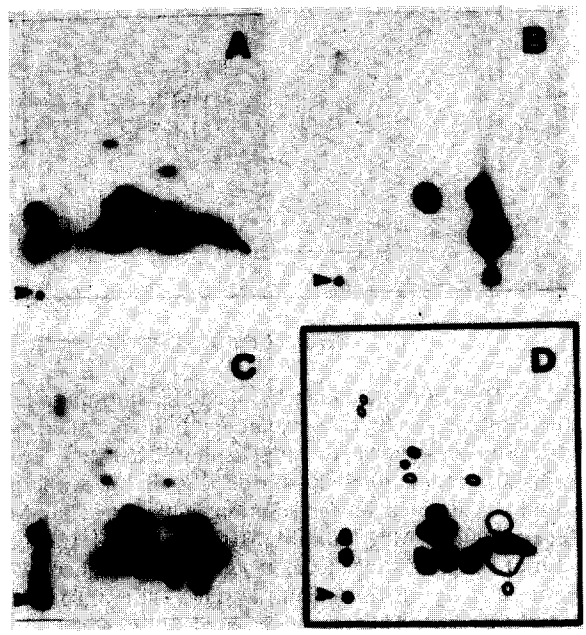

Fig.3. Phosphopeptide maps of tau phosphorylated by protein kinase C or by cAMP-dependent protein kinase. Tau was phosphorylated by protein kinase C or catalytic subunit of cAMP-dependent protein kinase for 30 min under the conditions in [2]. Bands containing phosphorylated tau were excised from the dried gels and two-dimensional tryptic phosphopeptide mapping of tau was performed [2]. Arrowheads point to the origin of sample application. (A) Tau phosphorylated by protein kinase C, (B) tau phosphorylated by cAMP-dependent protein kinase, (C) mixture of tau phosphorylated by each kinase separately, (D) schematic drawing of phosphopeptides in A–C. Phosphopeptides derived from tau phosphorylated by protein kinase C (◍) or by cAMP-dependent protein kinase (○) and co-migrating phosphopeptides (●) are shown.

tively when mixed with phosphocellulose-purified tubulin (1.5 mg/ml), while tau phosphorylated by the activated protein kinase C induced less tubulin polymerization. Under the conditions used, 0.3 mol phosphate were incorporated into 1 mol tau after incubation for 5 min with the activated protein kinase C, 0.9 mol phosphate at 25 min, and 3.0 mol phosphate at 90 min. The initial rates of tubulin polymerization with these phosphorylated tau samples were 75, 41 and 18% of that induced by the unphosphorylated one, respectively (fig.4). Control experiments indicated that incuba-

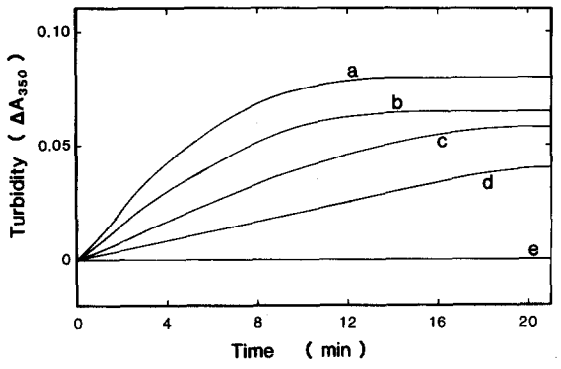

Fig.4. Effect of protein kinase C-mediated phosphorylation of tau on its ability to promote tubulin polymerization. Phosphorylated or unphosphorylated tau was prepared as described in section 2 with or without 0.5 mM ATP in a buffer solution containing 20 mM Hepes, 40 mM Mes, 10 mM $MgCl_2$, 1.6 mM $CaCl_2$, 1.3 mM EGTA, 50 mM KCl, 25 μg/ml of phosphatidylserine, 0.25 μg/ml of diolein, 2.4 mM DTT, 0.05 mM PMSF (pH 6.9). This phosphorylated or unphosphorylated tau (89 μg/ml) was mixed with tubulin (1.5 mg/ml) and the time course of microtubule assembly was monitored by measuring the turbidity at 350 nm. (a) + unphosphorylated tau, (b) + tau phosphorylated for 5 min, (c) + tau phosphorylated for 25 min, (d) + tau phosphorylated for 90 min, (e) tubulin alone.

tion of tau in the absence of protein kinase C induced no loss in activity of tau. Furthermore, it was found that tau lost its ability to induce tubulin polymerization to a greater extent when the phosphorylation by protein kinase C was performed in the presence of Ca^{2+} and phosphatidylserine than in their absence (not shown). Thus, there was a good correlation between the number of phosphates incorporated into tau and the extent of inhibition of its tubulin-polymerizing activity.

The ability of tau to interact with actin filaments was also found to be inhibited by protein kinase C-mediated phosphorylation. Table 1 shows that unphosphorylated tau (80 μg/ml) increased markedly the viscosity of actin filaments, whereas the same concentration of tau phosphorylated by the fully activated protein kinase C (2.0 mol phosphate incorporated per mol tau) did not increase the viscosity at all. Tau phosphorylated in the absence of Ca^{2+} and phosphatidylserine induced an intermediate increase in actin viscosity (table 1).

Table 1

Effect of the protein kinase C-mediated phosphorylation of tau on its ability to cross-link actin filaments

Phosphorylation			Fold increase in low-shear viscosity
Incubation conditions		Phosphate incorporated/tau (mol/mol)	
ATP	PS/Ca^{2+}		
−	+	0	44
+	−	0.4	31
+	+	2.0	1

Tau was incubated at 25°C for 90 min with protein kinase C in the presence or absence of Ca^{2+} and phosphatidylserine as in fig.4. Then, the ability of phosphorylated or unphosphorylated tau (80 μg/ml) to cross-link actin filaments (0.15 mg/ml) was assayed by falling ball viscometry as described in section 2. The data are normalized to the viscosity of actin alone. PS, phosphatidylserine

Although the values of the viscosity attained varied somewhat from experiment to experiment, protein kinase C-mediated phosphorylation reproducibly decreased the ability of tau to cross-link actin filaments dependent on the extent of phosphorylation by protein kinase C.

These results clearly indicate that tau is a good substrate for activated protein kinase C and that protein kinase C-mediated phosphorylation of tau reduces its abilities to induce tubulin polymerization and to cross-link actin filaments. Recent studies have shown that tau is likely to be a predominant species integrated into paired helical filaments in Alzheimer disease and that tau in Alzheimer brain is an abnormally phosphorylated form [20,21]. Furthermore, preliminary data suggest a defect in microtubule assembly in Alzheimer disease/senile dementia of the Alzheimer type [22]. Although the types of kinases involved in the phosphorylation of tau in living brain remain unknown, it is possible that the phosphorylation catalyzed by protein kinase C is important in regulation of the function of tau.

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan (60065005).

REFERENCES

[1] Nishizuka, Y. (1984) Nature 308, 693–698.

[2] Akiyama, T., Nishida, E., Ishida, J., Saji, N., Ogawara, H., Hoshi, M., Miyata, Y. and Sakai, H. (1986) J. Biol. Chem. 261, 15648–15651.

[3] Rifkin, D.B., Crowe, R.M. and Pollack, P. (1979) Cell 18, 361–368.

[4] Schliwa, M., Nakamura, T., Porter, K.R. and Euteneuer, U. (1984) J. Cell Biol. 99, 1045–1059.

[5] Weingarten, M.D., Lockwood, A.H., Hwo, S.-Y. and Kirschner, M.W. (1975) Proc. Natl. Acad. Sci. USA 72, 1858–1862.

[6] Cleveland, D.W., Hwo, S.-Y. and Kirschner, M.W. (1977) J. Mol. Biol. 116, 207–225.

[7] Cleveland, D.W., Hwo, S.-Y. and Kirschner, M.W. (1977) J. Mol. Biol. 116, 227–247.

[8] Drubin, D.G. and Kirschner, M.W. (1986) J. Cell Biol. 103, 2739–2746.

[9] Nishida, E., Kotani, S., Kuwaki, T and Sakai, H. (1982) in: Biological Functions of Microtubules and Related Structures (Sakai, H. et al. eds) pp. 285–295, Academic Press, Tokyo.

[10] Miyata, Y., Hoshi, M., Nishida, E., Minami, Y. and Sakai, H. (1986) J. Biol. Chem. 261, 13026–13030.

[11] Kotani, S., Nishida, E., Kumagai, H. and Sakai, H. (1985) J. Biol. Chem. 260, 10779–10783.

[12] Uchida, T. and Filburn, C.R. (1984) J. Biol. Chem. 259, 12311–12314.

[13] Nishida, E., Kumagai, H., Ohtsuki, I. and Sakai, H. (1979) J. Biochem. 85, 1257–1266.

[14] Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.

[15] Nishida, E., Maekawa, S. and Sakai, H. (1984) Biochemistry 23, 5307–5313.

[16] Laemmli, U.K. (1970) Nature 227, 680–685.

[17] MacLean-Fletcher, S.D. and Pollard, T.D. (1980) J. Cell Biol. 85, 414–428.

[18] Pierre, M. and Nunez, J. (1983) Biochem. Biophys. Res. Commun. 115, 212–219.

[19] Yamamoto, H., Fukunaga, K., Goto, S., Tanaka, E. and Miyamoto, E. (1985) J. Neurochem. 44, 759–768.

[20] Ihara, Y., Nukina, N., Miura, R. and Ogawara, M. (1986) J. Biochem. 99, 1807–1810.

[21] Grundke-Iqbal, I., Iqbal, K., Tung, Y.-C., Quinlan, M., Wiseniewski, H.M. and Binder, L.I. (1986) Proc. Natl. Acad. Sci. USA 83, 4913–4917.

[22] Iqbal, K., Merz, P.A. and Wiseniewski, H.M. (1982) J. Neuropathol. Exp. Neurol. 41, 364 (abstr.).