Properties of a microtubule-associated cofactor-independent protein kinase from pig brain

Clay W SCOTT,* Claudia B. CAPUTO and Andre I. SALAMA

Department of Pharmacology, ICI Pharmaceuticals Group, ICI Americas, Wilmington, DE 19897, U.S.A.

A protein kinase activity was identified in pig brain that co-purified with microtubules through repeated cycles of temperature-dependent assembly and disassembly. The microtubule-associated protein kinase (MTAK) phosphorylated histone H1; this activity was not stimulated by cyclic nucleotides, Ca^{2+} plus calmodulin, phospholipids or polyamines. MTAK did not phosphorylate synthetic peptides which are substrates for cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, Ca^{2+} /calmodulin-dependent protein kinase II, protein kinase C or casein kinase II. MTAK activity was inhibited by trifluoperazine [IC_{50} (median inhibitory concn.) = $600\text{ }\mu\text{M}$] in a Ca^{2+} -independent fashion. Ca^{2+} alone was inhibitory ($\text{IC}_{50} = 4\text{ mM}$). MTAK was not inhibited by heparin, a potent inhibitor of casein kinase II, nor a synthetic peptide inhibitor of cyclic AMP-dependent protein kinase. MTAK demonstrated a broad pH maximum (7.5–8.5) and an apparent K_m for ATP of $45\text{ }\mu\text{M}$. Mg^{2+} was required for enzyme activity and could not be replaced by Mn^{2+} . MTAK phosphorylated serine and threonine residues on histone H1. MTAK is a unique cofactor-independent protein kinase that binds to microtubule structures.

INTRODUCTION

Microtubules are dynamic structures composed of parallel protofilaments arranged to form a hollow cylinder about 25 nm in diameter. These cytoskeletal elements are involved in a variety of cellular functions, including organelle transport, formation of the spindle apparatus in mitosis, and maintenance of cell morphology. The microtubule protofilaments are complex biochemical structures composed of tubulin, a heterodimer of α and β subunits each with an M_r of 50000, and an assortment of other proteins known collectively as microtubule-associated proteins (MAPs; for review see Dustin, 1984; Olmsted, 1986). MAPs bind to microtubules and co-purify with tubulin at constant stoichiometry during repeated cycles of microtubule assembly–disassembly. The most abundant and best characterized MAPs consist of two groups: the high- M_r proteins (> 180000), which includes MAPs 1A, 1B, 1C and 2, and a family of microheterogeneous proteins of M_r 55000–65000 called tau proteins (Olmsted, 1986; Matus, 1988). Although the precise functions of MAPs are not understood, they are known to promote the assembly of microtubules *in vitro* (Weingarten *et al.*, 1975; Cleveland *et al.*, 1977) and stabilize microtubules *in vivo* (Drubin *et al.*, 1986). They also appear to act as coupling agents between microtubules and actin, secretory vesicles, neurofilaments and other intermediate filaments (Vallee *et al.*, 1984a,b; Griffith & Pollard, 1982).

Many studies have demonstrated that MAPs are phosphorylated *in vivo* (Theurkauf & Vallee, 1983; Burns *et al.*, 1983; Shaw *et al.*, 1988) and that phosphorylation of certain MAPs alters their assembly-promoting effect *in vitro* (Jameson *et al.*, 1980; Murthy & Flavin, 1983).

Both cyclic AMP-dependent protein kinase (cAMP-kinase) and Ca^{2+} /calmodulin-dependent protein kinase II (CaM-kinase II) are present in microtubule preparations and can phosphorylate various MAPs (Vallee *et al.*, 1981; Vallano *et al.*, 1985). Each enzyme phosphorylates MAP2 *in vitro*, decreasing the ability of MAP2 to promote microtubule assembly (Yamamoto *et al.*, 1985). Since cAMP and Ca^{2+} plus calmodulin effect the number and organization of microtubules in intact cells (Kram & Tomkins, 1973; Nath *et al.*, 1978; Keith *et al.*, 1983), it is possible that phosphorylation of MAPs may be one mechanism for modulating microtubule function.

Investigators have examined the role of phosphorylation in microtubule biology by studying the protein kinases which associate with microtubules and the substrates for these kinases which coexist on the cytoskeletal structures. The identification of cAMP-kinase as a microtubule-associated enzyme and the physical interaction of this enzyme with microtubules have been well established (Vallee *et al.*, 1981; Theurkauf & Vallee, 1982). The type II cAMP-kinase holoenzyme is bound to microtubules through an interaction of the cAMP-kinase regulatory dimer with MAP2. cAMP binds to the regulatory dimer, resulting in the release of cAMP-kinase catalytic subunits while the cAMP-regulatory-dimer·MAP2 complex remains attached to the microtubule structure. The released catalytic subunits are active and phosphorylate appropriate substrate proteins.

In contrast with cAMP-kinase, the nature of the association of CaM-kinase II with microtubules is not yet understood. CaM-kinase II is found attached to cold-stable microtubules (Larson *et al.*, 1985) as well as microtubules purified through repeated cycles of temperature-dependent assembly and disassembly

Abbreviations used: MAPs, microtubule-associated proteins; cAMP, cyclic AMP; cAMP-kinase, cAMP-dependent protein kinase; CaM-kinase II, Ca^{2+} /calmodulin-dependent protein kinase II; MTAK, microtubule-associated protein kinase; IC_{50} , median inhibitory concn.

* To whom correspondence should be addressed.

(Vallano *et al.*, 1985). It is not known how this enzyme associates with two distinctly different populations of microtubules.

In addition to cAMP-kinase and CaM-kinase II, reports exist identifying cofactor-independent protein kinase activities in microtubule preparations (Piras & Piras, 1974; Sheterline, 1977; Burns & Islam, 1984). It is not known whether these activities represent novel protein kinases or are cofactor-independent forms of cAMP-kinase or CaM-kinase II. We have begun to investigate these various protein kinases and their interaction with microtubules in an effort to understand the potential regulation of microtubule function by protein phosphorylation. In this study we describe some of the properties of a unique cofactor-independent protein kinase which co-purifies with pig brain microtubules.

METHODS

Microtubule preparation

Twice-cycled microtubules were prepared from pig and rat brain by the temperature-dependent assembly-disassembly method (Murphy, 1982). The protease inhibitors leupeptin (5 μ M), benzamidine (1 mM) and phenylmethanesulphonyl fluoride (0.1 mM) were added to the homogenization and polymerization buffers. After two cycles of purification, the microtubule pellets (H_2P) were stored at -70°C . When needed, the H_2P pellets were homogenized in polymerization buffer, incubated for 30 min at 4°C , and the cold-stable microtubules were removed by centrifugation at 40000 *g* for 30 min at 4°C . The supernatant material (H_3S) containing kinase activity was further analysed as described below.

Phosphorylation assay

In the standard assay, 15–30 μ g of H_3S was incubated at 30°C for 10 min in a 30 μ l reaction mixture containing 20 mM-Tris/HCl, pH 7.5, 2 mM-EGTA, 5 mM-MgCl₂ and 200 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (600–1000 c.p.m./pmol), in the absence or presence of 2 mg of histone H1/ml. Unless specifically noted, all assays contained 1 μ M-PKI5–24, a peptide corresponding to amino acids 5–24 of the heat-stable protein inhibitor of cAMP-kinase. Samples (10 μ l) of the reaction mixture were spotted on to Whatman P81 paper, and the paper strips washed for 3×15 min in 75 mM- H_3PO_4 to remove unincorporated $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The strips were washed in 95% (v/v) ethanol for 5 min, dried and quantified for radioactivity in scintillation fluid (ReadySolv HP; Beckman). When protein substrates other than histone H1 were used, the reaction was terminated by adding 50 μ g of bovine serum albumin followed by precipitation with 12% (w/v) trichloroacetic acid. The precipitate was washed twice with 12% trichloroacetic acid, dissolved in 1 M-NaOH, mixed with scintillation fluid and quantified for protein-bound ^{32}P . When synthetic peptides were used as substrates, the phosphorylation reaction was terminated with bovine serum albumin and trichloroacetic acid as described above. The supernatant, containing the acid-soluble peptide, was passed over a column containing AG1X8 ion-exchange resin to remove $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Kemp *et al.*, 1976a). The eluent was mixed with ReadySolv HP and quantified for radioactivity.

Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was per-

formed in 4–20% gradient polyacrylamide gels by the method of Laemmli (1970). After electrophoresis, gels were dried and autoradiographs prepared by exposing Kodak XRP-5 film to the gels.

Analytical methods

Phosphoamino acid analyses were performed on phosphorylation reaction mixtures and on ^{32}P -labelled proteins excised from SDS/polyacrylamide gels in accordance with published procedures (Haring *et al.*, 1984). Protein was measured by the Bio-Rad Protein Assay with γ -globulin as protein standard.

Materials

Pig brains were obtained from a local abattoir. Brain tissue was collected immediately after killing the animal, and was kept on ice during transport. Tubulin was purified from pig brain as described by Murphy (1982), neurofilaments were prepared from Sprague-Dawley rat brain (Toru-Delbauffe & Pierre, 1983) and tau proteins from pig brain by method 3 of Grundke-Iqbal *et al.* (1986). Synapsin I was generously provided by Dr. Teresa McGuinness and Dr. Paul Greengard. Peptides GRG-LSLSR and RRKSGP were purchased from Bachem Inc., and peptide DRVQSKI was provided by Dr. C. Thorber, ICI Pharmaceuticals. All other peptide substrates were purchased from Peninsula Laboratories. Characterization of these peptides for use as substrates for the various protein kinases is described in the following references: LRRASLG (Kemp *et al.*, 1976b); RRKASGP (Pomerantz *et al.*, 1977); GRGLSLSR (Kishimoto *et al.*, 1985); PLSRTLSSVSS-NH₂ (Pearson *et al.*, 1985); RRKASGPPV (O'Brian *et al.*, 1984); LRRASLG-NH₂ (C. W. Scott, unpublished work); RRREEETEE (Kuenzel & Krebs, 1985); RKRSRKE (Glass & Krebs, 1979); RKRSRAE (Glass & Krebs, 1982); SSEGESPDFPEELEK and DRVQSKI (Caputo *et al.*, 1989). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from New England Nuclear, AG 1×8 resin was from Bio-Rad, and dioleoylglycerol and myelin basic protein were from Calbiochem. The protease inhibitors, phosphoamino acid standards, various cofactors and inhibitor compounds and remaining proteins were purchased from Sigma Chemical Co. Precast 4–20% gradient polyacrylamide gels were purchased from Integrated Separation Systems.

RESULTS

Identification of a microtubule-associated cofactor-independent protein kinase

Microtubules were prepared from rat and pig brain by two cycles of temperature-dependent assembly-disassembly. When these microtubules were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, then run on an SDS/polyacrylamide gel and analysed by autoradiography, a number of ^{32}P -labelled proteins were observed (Fig. 1, lanes 1, 2). Both preparations contained an active protein kinase which phosphorylated various microtubule proteins. In addition to the endogenous microtubule proteins, the microtubule-associated protein kinase (MTAK) phosphorylated histone H1 added to the microtubule preparation (lanes 3, 4). Phosphorylation of histone H1 by MTAK was quantified by the P81 filter-paper method described in the Methods section and was used as an assay to characterize this kinase activity further.

MTAK activity is defined here by its phosphorylation

Fig. 1. Phosphorylation of microtubule proteins and histone H1 by a microtubule-associated protein kinase (MTAK)

A 30 μ g portion of twice-cycled rat brain microtubules (lanes 2, 4) or pig brain microtubules (lanes 1, 3) was incubated with 200 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 5 mM- MgCl_2 and 1 μ M-PKI5-24 in the absence (lanes 1, 2) or presence (lanes 3, 4) of 2 mg of histone H1/ml. The reaction mixtures were boiled in SDS sample buffer, run on a 6–14% acrylamide slab gel and analysed by autoradiography. M_r values ($\times 10^{-3}$) of protein standards are shown in the margin.

of histone H1. This activity is detected in the absence of added cofactors and in the presence of PKI5-24, a peptide inhibitor of cAMP-kinase. Since histone H1 is also a substrate for cAMP-kinase (Kishimoto *et al.*, 1985), the possibility was raised that MTAK was actually the free catalytic subunit of cAMP-kinase. However, MTAK activity was detected in the presence of 1 μ M-PKI5-24, a concentration of inhibitor that completely abolishes cAMP-stimulated cAMP-kinase activity in the microtubule preparation with synthetic peptides, endogenous microtubule proteins or exogenous proteins as substrates (see below).

To determine whether MTAK was truly a microtubule-associated enzyme or a simple contaminant of the microtubule preparation, pig brain microtubules were purified through four cycles of assembly–disassembly, and samples from each disassembly step were analysed for MTAK activity. MTAK specific activity was found to be relatively constant during the repeated cycling procedure (Table 1). This suggests that the kinase is an integral component of microtubules and is capable of associating with microtubule proteins during microtubule polymerization.

When microtubules were cycled through repeated steps of assembly and disassembly in the presence of 10 μ M-cAMP, a continual loss of microtubule-associated cAMP-kinase activity was observed (Table 1). cAMP promotes the dissociation of the catalytic subunits of cAMP-kinase from the regulatory dimer (which is bound to MAP2) and therefore the release of catalytic subunits from the microtubule structures (Theurkauf & Vallee, 1982). The dissociated catalytic subunits are separated from microtubules during centrifugation, creating a loss

Table 1. MTAK activity co-purifies with microtubules through repeated cycles of assembly–disassembly

Pig brain microtubules were purified through one cycle of polymerization, then were separated into two groups; one group was cycled three more times in the presence of 10 μ M-cAMP, whereas the other group was cycled in the absence of cAMP. The preparations were assayed for MTAK activity and cAMP-dependent protein kinase activity as described in the Methods section. Data represent means $\pm$ S.E.M. of triplicate measurements of a typical preparation.

Microtubule sample	MTAK activity (pmol/min per mg)	cAMP-dependent protein kinase activity (nmol/min per mg)
Cycled in the absence of cAMP		
1 cycle	638 $\pm$ 23	4.24 $\pm$ 0.06
2 cycles	810 $\pm$ 30	4.06 $\pm$ 0.22
3 cycles	660 $\pm$ 17	3.80 $\pm$ 0.14
4 cycles	786 $\pm$ 29	3.13 $\pm$ 0.27
Cycled in the presence of cAMP		
2 cycles	770 $\pm$ 08	2.76 $\pm$ 0.08
3 cycles	804 $\pm$ 13	1.37 $\pm$ 0.04
4 cycles	774 $\pm$ 11	0.58 $\pm$ 0.03

of microtubule-associated cAMP-kinase activity. There was no change in MTAK activity with cAMP treatment, however. MTAK specific activity was the same in microtubule samples prepared without cAMP as in those continuously cycled in the presence of cAMP. These data provide further evidence that MTAK is a unique protein kinase and not cAMP-kinase.

Effects of various compounds on MTAK activity with endogenous proteins and histone H1 as substrates

Several protein kinases can be selectively activated or inhibited by specific compounds. These compounds were evaluated for their effects on MTAK activity in the microtubule preparation. In the presence of 1 μ M-PKI5-24, cAMP did not stimulate the phosphorylation of either endogenous proteins or histone H1, demonstrating that PKI5-24 completely inhibited the microtubule-associated cAMP-kinase (Table 2). cGMP stimulates cGMP-dependent protein kinase (cGMP-kinase), but did not enhance the phosphorylation of microtubule proteins or histone H1. At 200 μ M, CaCl_2 inhibited MTAK activity by 40% with histone H1 as substrate, but showed no inhibitory effect with endogenous proteins alone as substrates. It is possible that this Ca^{2+} inhibition is a substrate-directed effect rather than an enzyme-inhibitory effect. A second possibility is that MTAK is a Ca^{2+} -sensitive enzyme and the phosphorylation of endogenous proteins (which is Ca^{2+} -insensitive) is due to a second protein kinase. This dilemma will be resolved when MTAK is purified and its Ca^{2+} -sensitivity re-assessed by using multiple substrates.

Pig brain microtubules were assayed for Ca^{2+} -calmodulin-stimulated protein kinase activity, but none was observed (Table 2). No increase in ^{32}P incorporation by endogenous proteins or histone H1 was detected

Table 2. Effect of various cofactors and inhibitors on MTAK activity

Samples of twice-cycled microtubule preparation were incubated with the indicated compounds in the absence or presence of 2 mg of histone H1/ml. Samples of the reaction mixture were spotted on to P81 paper and ^{32}P incorporation into protein was measured. Data represent means $\pm$ S.E.M. of three experiments. Abbreviations: DAG, diacylglycerol; PS, phosphatidylserine.

Additive	Concn.	MTAK activity (%)	
		No histone H1	Histone H1
None	—	100 $\pm$ 4*	100 $\pm$ 2*
cAMP	10 μM	104 $\pm$ 7	103 $\pm$ 4
cGMP	10 μM	95 $\pm$ 5	110 $\pm$ 4
CaCl_2	200 μM	97 $\pm$ 2	61 $\pm$ 10
+ calmodulin	20 $\mu\text{g/ml}$	93 $\pm$ 8	54 $\pm$ 7
+ PS, DAG	20 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$	112 $\pm$ 8	60 $\pm$ 1
Spermine	3 mM	55 $\pm$ 7	70 $\pm$ 4
Spermidine	3 mM	63 $\pm$ 2	92 $\pm$ 9
Heparin	1 $\mu\text{g/ml}$	114 $\pm$ 7	106 $\pm$ 4
Trifluoperazine	1 mM	11 $\pm$ 9	18 $\pm$ 7

* 100% = 26 pmol of ^{32}P incorporated in the absence of histone H1, 218 pmol of ^{32}P in its presence.

when microtubules were incubated with 200 μM - CaCl_2 and 20 μg of calmodulin/ml, compared with CaCl_2 alone or an EGTA-treated control. Calmodulin showed no stimulatory effect when it was used with lower concentrations of CaCl_2 , concentrations which alone had no inhibitory effect on MTAK (results not shown). MTAK activity was not stimulated in the presence of Ca^{2+} , phosphatidylserine and diacylglycerol (Table 2). Spermine and spermidine, polyamines which stimulate casein kinase II, did not stimulate the phosphorylation of microtubule proteins or histone H1. Heparin is a potent inhibitor of casein kinase II, but had no inhibitory effect on MTAK. Trifluoperazine inhibited the phosphorylation of both endogenous proteins and histone H1. Trifluoperazine and related phenothiazines bind to calmodulin in a Ca^{2+} -dependent manner and block calmodulin activation of various enzymes. Phenothiazines have also been reported to inhibit calmodulin-independent kinases (Abdel-Ghany *et al.*, 1988; Pelech *et al.*, 1987), possibly by interacting with hydrophobic regions on the enzymes (Prozialeck & Weiss, 1982). Trifluoperazine is probably directly inhibiting MTAK rather than inhibiting calmodulin activation of MTAK, because MTAK activity is measured in the absence of added Ca^{2+} (and the presence of 2 mM-EGTA), and because Ca^{2+} -calmodulin showed no stimulation of MTAK activity.

Proteolytically activated forms of CaM-kinase II and protein kinase C have been identified (Levine & Sahyoun, 1987; Takai *et al.*, 1979). These proteolytic fragments are active in the absence of their physiological cofactors, therefore it was necessary to determine whether MTAK represented one of these active fragments. Synthetic peptides which are known substrates for protein kinase C, CaM-kinase II and other protein kinases were tested

Table 3. Phosphorylation of peptide substrates by microtubule-associated kinases

Samples of twice-cycled microtubule preparation were incubated with the various peptides in the absence or presence of the relevant cofactors. PKI5-24 was present in all assays except those measuring cAMP-stimulated activity. ^{32}P incorporation into peptide substrates was measured as described in the Methods section. Data represent the means $\pm$ S.E.M. of triplicate measurements. When histone H1 was used as substrate under the same assay conditions, 203 $\pm$ 11 pmol of ^{32}P was incorporated. The cofactors used with the various peptide substrates were: cAMP-kinase substrates, 10 μM -cAMP; Ca^{2+} -calmodulin-dependent kinase substrates, 200 μM - CaCl_2 plus 20 μg of calmodulin/ml; protein kinase C substrates, 200 μM - CaCl_2 , 20 μg of phosphatidylserine/ml and 5 μg of diacylglycerol/ml; cGMP-dependent protein kinase substrate, 10 μM -cGMP.

Peptide	Concn. (μM)	^{32}P incorporation into peptide (pmol)	
		No cofactors	+ Cofactors
cAMP-dependent pro- tein kinase substrates			
LRRASLG-NH ₂	50	7 $\pm$ 0.8	1016 $\pm$ 14
LRRASLG	50	13 $\pm$ 2.4	990 $\pm$ 10
RRKASGP	2000	2 $\pm$ 0.1	1544 $\pm$ 64
GRGLSLSR	300	6 $\pm$ 0.3	1052 $\pm$ 25
Ca/calmodulin- dependent kinase substrates			
PLSRTLVS-S-NH ₂	60	27 $\pm$ 3.6	18 $\pm$ 2.2
LLRPQRATSNVFS-NH ₂	50	3 $\pm$ 0.1	4 $\pm$ 0.3
Protein kinase C substrates			
RRKASGPPV	300	3 $\pm$ 0.3	3 $\pm$ 0.1
LRRASLG-NH ₂	100	13 $\pm$ 0.6	8 $\pm$ 0.1
GRGLSLSR	100	5 $\pm$ 0.2	6 $\pm$ 0.3
Casein kinase II substrate			
RRREEETEEE	500	< 1	< 1
cGMP-dependent protein kinase substrates			
RKRSRKE	100	5 $\pm$ 0.1	7 $\pm$ 0.4
RKRSRAE	100	2 $\pm$ 0.1	< 1
Neurofilament- associated kinase substrates			
SSEGSPDFPEELEK	300	< 1	—
DRVQSKI	500	< 1	—

as substrates for MTAK (Table 3). Each of the peptides was used at a concentration which was 1–10 times its reported K_m for the appropriate kinase. None of the peptides was substantially phosphorylated in the absence of cofactors, indicating that (1) the pig microtubule preparation does not contain cofactor-independent forms of CaM-kinase II, cGMP-dependent protein kinase or protein kinase C, nor does it contain casein kinase II, and (2) these peptides are not substrates for MTAK. Adding cAMP to the reaction mixture stimulated the phosphorylation of cAMP-kinase substrate peptides. This was expected, since cAMP-kinase is known to be

Fig. 2. pH-dependence of MTAK activity

Equal samples of microtubule preparation were incubated with 2 mg of histone H1/ml under standard conditions at different pH values. Each data point represents the mean of three measurements. Buffers, used at a final concentration of 50 mM, were: $\blacklozenge$, succinate; $\triangle$, Mes; $\blacksquare$, Pipes; $\circ$, Hepes; $\blacktriangle$, Tris; $\square$, glycylglycine.

Fig. 3. Bivalent-cation-dependence of MTAK activity

The requirement of MTAK activity for Mg^{2+} or Mn^{2+} was measured at various concentrations of $MgCl_2$ ($\triangle$) or $MnCl_2$ ($\blacktriangle$) in the standard reaction mixture as described in the Methods section. Each value is the mean $\pm$ S.E.M. of three measurements.

present in microtubule preparations and since PK15-24 was omitted from the assay. No other cofactors produced an increase in ^{32}P incorporation into the various peptides. These data, together with the results from Table 2, demonstrate that MTAK is a distinct microtubule-associated protein kinase with a unique substrate specificity.

Characterization of MTAK

Various properties of MTAK in twice-cycled microtubule preparations were assessed by the histone-

H1 phosphorylation assay. MTAK activity was detected over a range of pH values (Fig. 2). A broad optimum was observed between pH 7.5 and 8.5. Mg^{2+} was required for the phosphorylation reaction, and maximal activity was obtained with 5 mM- Mg^{2+} (Fig. 3). Concentrations above this were inhibitory. Mn^{2+} was a poor substitute for Mg^{2+} .

The phosphorylation reaction catalysed by MTAK was measured at various concentrations of ATP (Fig. 4). Kinetic constants were determined from the double-reciprocal plot (Fig. 4, inset). The apparent K_m for ATP was 45 μM , and the V_{max} was 1.5 nmol/min per mg of

Fig. 4. MTAK activity as a function of ATP concentration

Samples of twice-cycled microtubule preparation were incubated with various concentrations of ATP and assayed for MTAK activity under standard conditions with 2 mg of histone H1/ml as substrate. Data represent the means of triplicate measurements. Inset: double-reciprocal plot of initial velocity (v ; nmol of ^{32}P incorporated/min per mg of microtubule preparation) versus ATP concentration (μM).

protein. Microtubules contain an intrinsic GTPase activity (Dustin, 1984), therefore the effectiveness of GTP as a phosphate donor for MTAK could not be assessed.

MTAK activity is sensitive to both trifluoperazine and CaCl_2 (Table 1). The dose-response curves for these two inhibitors are depicted in Fig. 5. The IC_{50} values (median inhibitory concn.) for trifluoperazine and CaCl_2 were 0.6 mM and 4 mM respectively. Since Ca^{2+} causes microtubule disassembly (Dustin, 1984) and inhibited MTAK activity, the influence of the microtubule polymerization state on MTAK activity was examined. At 0.1 mM, colchicine effectively blocks microtubule formation (Borisy *et al.*, 1972), but had no effect on MTAK activity (results not shown). This suggests that MTAK activity is unaffected by the polymerization state of microtubules, and that the Ca^{2+} inhibitory effect on MTAK is not a consequence of microtubule disassembly.

A sample of twice-cycled microtubule preparation was incubated with histone H1 and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and the reaction mixture analysed for phosphoamino acid content. Both serine and threonine residues on histone H1 were labelled by MTAK (Fig. 6).

Various proteins were tested as substrates for MTAK. The different proteins were incubated with portions of the microtubule preparation under the standard phosphorylation conditions. The reaction mixtures were electrophoresed on SDS/polyacrylamide gels and analysed by autoradiography (Fig. 7). Tubulin, myosin light chains, histones VIS and VIIS and casein were not phosphorylated by the microtubule preparation. The M_r -200 000, -150 000 and -68 000 subunits of neurofilaments were poorly phosphorylated, whereas two low- M_r proteins in the neurofilament preparation showed prominent ^{32}P incorporation. The relationship between these low- M_r proteins and the neurofilament triplet is not known. Histone IIA, myelin basic protein, tau protein and synapsin showed intense ^{32}P incorporation and are possible substrates for MTAK. Because the presence of

Fig. 6. Phosphoamino acid analysis of histone H1 phosphorylated by MTAK

Samples of microtubule preparation were incubated with $30\ \mu\text{M}$ - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5000 c.p.m./pmol) and 2 mg of histone H1/ml under standard conditions as described in the Methods section. Phosphorylated histone H1 was isolated from SDS/polyacrylamide gels and processed for phosphoamino acid analysis. Circled areas on the autoradiogram represent migration of phosphoamino acid standards.

Fig. 5. Inhibition of MTAK activity by trifluoperazine or CaCl_2

Samples of the standard microtubule preparation were incubated with various concentrations of trifluoperazine ($\circ$) or CaCl_2 ($\blacktriangle$) and assayed for MTAK activity with histone H1 as substrate. All assays were run in the presence of 2 mM-EGTA; the abscissa for the CaCl_2 curve represents the amount of free Ca^{2+} . Data are plotted as percentages of activity in the absence of inhibitors and represent means $\pm$ range from two separate experiments.

Fig. 7. Phosphorylation of exogenous proteins by MTAK

A $25\ \mu\text{g}$ sample of twice-cycled microtubule protein was incubated with various exogenous proteins for 15 min at 30°C in a standard phosphorylation reaction mixture. The samples were boiled in SDS sample buffer and run on a 4-16% acrylamide gradient gel. An autoradiogram of the dried gel shows the ^{32}P -labelling of the various proteins. The M_r values ($\times 10^{-3}$) of protein standards are shown in the margin. The exogenous proteins used in the assays and their M_r values are: (1) histone IIA, 12000-30000; (2) histone VIS, 12000-30000; (3) histone VIIS, 12000-30000; (4) casein, 24000; (5) myosin light chains, 20000; (6) tubulin, 50000; (7) neurofilaments, 200000, 150000 and 68000; (8) myelin basic protein, 19000; (9) tau protein, 50000-65000; (10) synapsin, 80000 and 86000. The exogenous proteins were present at 2 mg/ml, except for neurofilaments (1 mg/ml), tau protein and synapsin (0.5 mg/ml).

another protein kinase in the microtubule preparation cannot be ruled out, an unequivocal study of the substrate specificity of MTAK can only be achieved with the purified enzyme. However, those proteins which were not phosphorylated by the microtubule preparation are probably not substrates for MTAK. The lack of phosphorylation of casein supports the argument that MTAK is not cAMP-kinase, casein kinase I or casein kinase II.

The ^{32}P -labelled high- M_r band observed in several lanes in Fig. 7 probably represents the endogenous microtubule-associated protein, MAP2. Purified bovine MAP2 is a substrate for MTAK and migrates identically with the endogenously phosphorylated protein (C. W. Scott, unpublished work). As shown in Figs. 1 and 7, phosphorylation of this endogenous substrate is markedly decreased in the presence of various exogenous proteins.

DISCUSSION

This paper describes a unique protein kinase in pig brain that localizes to microtubules. This microtubule-associated protein kinase, termed MTAK, was identified and characterized with histone H1 as substrate. Histone H1 can be phosphorylated by a number of protein kinases, including cAMP-kinase and protein kinase C (Kishimoto *et al.*, 1985). Because histone H1 is a non-selective substrate, several approaches were taken to determine whether MTAK was a unique protein kinase. Initial experiments examined the effects of various protein kinase cofactors on MTAK activity. Polyamines, cyclic nucleotides, Ca^{2+} -calmodulin and phospholipids did not stimulate MTAK activity (Table 2), suggesting that MTAK was not casein kinase II or a second-messenger-dependent protein kinase.

Although previous reports have detected CaM-kinase II in microtubule preparations from rat brain (Vallano *et al.*, 1985), no Ca^{2+} -calmodulin-stimulated kinase activity was observed in pig brain microtubule preparations. CaM-kinase II is an extremely labile enzyme and requires rapid isolation techniques to retain activity (Goldenring *et al.*, 1983). Since 30 min lapse between isolating the pig brain at the abattoir and processing it at the laboratory, it is likely that all CaM-kinase II is inactivated in our tissue samples. A second possibility is that CaM-kinase II was present in the pig microtubule preparation, but in a cofactor-independent form. Both autophosphorylation (Miller & Kennedy, 1986) and limited proteolysis (Levine & Sahyoun, 1987) will convert CaM-kinase II into an activated Ca^{2+} -independent state. Protein kinase C can also undergo proteolytic activation (Takai *et al.*, 1979). Since cofactor-independent states exist for various cofactor-dependent enzymes, experiments were conducted to determine whether MTAK was a unique protein kinase or a cofactor-independent form of a known cofactor-dependent kinase.

The cofactor-independent forms of CaM-kinase II and protein kinase C have substrate specificities identical with those of their cofactor-dependent counterparts (Levine & Sahyoun, 1987; Sakanoue *et al.*, 1987). Therefore, synthetic peptide substrates can be used to identify both forms of each of these kinases. By this approach it was shown that the pig microtubule preparation was devoid of cofactor-dependent and -independent CaM-kinase II, protein kinase C and cGMP-kinase activities as well as casein kinase II activity (Table 3). These data

support the hypothesis that MTAK is a unique protein kinase. Furthermore, since the different peptide substrates were not phosphorylated by MTAK, these data imply that MTAK has a substrate specificity distinct from the cofactor-dependent kinases and casein kinase II.

Several points concerning the substrate specificity of MTAK can be extracted from Table 3 and Fig. 7. Histone H1 was a substrate for MTAK, yet a peptide corresponding to amino acids 30–40 of histone H1 (peptide RRKASGP) was not phosphorylated by MTAK. Serine-38 on histone H1 is a phosphorylation site for cAMP-kinase (Pomerantz *et al.*, 1977), and peptide RRKASGP was a substrate for the microtubule-associated cAMP-kinase (Table 3). These results suggest that MTAK has a substrate specificity distinct from cAMP-kinase, and that MTAK phosphorylates histone H1 at a site other than serine-38.

The microtubule preparation phosphorylated myelin basic protein, yet a peptide corresponding to amino acids 108–114 of myelin basic protein (GRGLSLSR) was not phosphorylated under similar assay conditions. Serine-110 of myelin basic protein is a phosphorylation site for both cAMP-kinase and protein kinase C (Kishimoto *et al.*, 1985), but apparently not for MTAK.

Cofactor-independent protein kinase activities have been previously identified in microtubule preparations (Piras & Piras, 1974; Sheterline, 1977; Burns & Islam, 1984). Two of these reports (Sheterline, 1977; Burns & Islam, 1984) contain minimal characterizations of the microtubule-associated kinase, and comparisons with MTAK are not possible. The third report (Piras & Piras, 1974) described a microtubule-associated kinase with a substrate specificity and bivalent-cation requirement different from MTAK, suggesting that the two kinase activities are disparate. Although various cytosolic cofactor-independent protein kinases have been described (Pant *et al.*, 1986; Cobb, 1986; Pelech *et al.*, 1987; Palczewski *et al.*, 1988; Ray & Sturgill, 1988), their properties are not similar to those of MTAK. MTAK appears to be a unique cofactor-independent protein kinase.

In summary, several properties of MTAK are described in the present paper, yet many questions remain unanswered. MTAK activity is unaffected by the polymerization state of microtubules, but it is not known if both active and inactive forms of the enzyme can bind microtubules. Indeed, it is not known how MTAK activity is regulated *in vivo*. The role of MTAK in regulating microtubule stability has not been defined, nor is it known whether MTAK is a brain-specific enzyme or if it is present on microtubules in non-neuronal tissues. These and other questions await further characterization of this microtubule-associated enzyme.

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