Vol. 115, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1983 Pages 212-219

MULTISITE PHOSPHORYLATION OF τ PROTEINS FROM RAT BRAIN

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Received July 18, 1983

τ proteins from adult and young rat brains were phosphorylated in vitro by protein kinases present in microtubule preparations. Several phosphates were incorporated in each molecular species of this group of proteins. Cyclic AMP dependent protein kinases and casein kinase (type I) phosphorylated τ proteins on different sites. These observations indicate that τ proteins are an example of multisite phosphorylation.

Protein phosphorylation is clearly the major general mechanism by which intracellular events respond to physiological stimuli. As cyclic AMP influences both microtubule number and their organisation in living cells (1,2,3), the presence of cyclic AMP dependent protein kinases, in microtubule preparations, which phosphorylate the microtubule associated proteins (4,5,6) has generated considerable interest. This phenomenom might be a basic mechanism for regulating the behavior of microtubules in the cells. In accordance with this view a recent report (7) indicates that the phosphorylation of unidentified microtubule associated proteins affects the kinetic parameters of microtubule assembly "in vitro".

Until now, of the microtubule associated proteins involved in the polymerisation process, studies have been performed mainly on the phosphorylation of MAP_2** which appeared to be the major substrate from microtubules for the cyclic AMP dependent protein kinase. However, CLEVELAND et al. (8) have reported that τ proteins could also be phosphorylated. We present here evidence that τ proteins are examples of "multisite phosphorylation" : several phosphates were incorporated in each molecular species of this group of proteins by protein kinase(s) (5) present in

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**MAP : Microtubule Associated Proteins.

0006-291X/83 $1.50

microtubule preparations. Furthermore, cyclic AMP dependent protein kinases and casein kinase I phosphorylated τ proteins on different sites. The possibility that cyclic AMP dependent and independent protein kinase could be involved in microtubules function is discussed.

MATERIAL AND METHODS

- $[\gamma^{32}P]$-ATP was obtained from Amersham. GTP, cyclic AMP, Phenylmethyl sulfonyl fluoride, Leupeptine, cyclic AMP dependent protein kinases I and II from rabbit muscle were purchased from Sigma. Casein kinase I was prepared by two methods described previously (9,10).

- Microtubules were prepared as described by FELLOUS et al. (11). Rat brains were homogenised with 1 volume (weight/vol) of cold buffer A (0.1 M 2-(N-morpholino) ethane sulfonic acid pH 6.4 containing 0.5 mM $MgCl_2$; 1 mM EGTA ; 0.1 mM EDTA ; 1 mM 2-mercaptoethanol ; 1 mM GTP ; 0.2 mM phenylmethyl sulfonyl fluoride ; 2 µg/ml leupeptine). The supernatant obtained from centrifugation (38 000 rpm, 60 min. in a Spinco Ti 60 rotor) was diluted with an equal volume of the same buffer containing 8 M glycerol and incubated at 37° C for 30 min. Microtubules polymerised in these conditions were sedimented (38 000 rpm, 90 min.) through a cushion of 6 M glycerol in buffer A.

- MAPs were prepared according to FELLOUS et al. (11). Microtubules were resuspended in buffer A and depolymerised for 30 min. at 0° C. 0.80 M NaCl was added and 20 min. later, microtubules were treated at 90° C for 5 min.. The supernatant obtained by centrifugation (27 000 g, 30 min.) was brought to 50 % ammonium sulfate. In these conditions MAPs containing MAP_2 and TAU were sedimented by centrifugation. Then the pellet was dissolved and dialysed against buffer A.

- In all cases, phosphorylation was performed at 37° C in 0,25 ml buffer A containing 5 mM $MgCl_2$, 5 µM cyclic AMP and 50 µM ATP (0.2 µC - 1 µC/nanomole). The reaction was stopped by pipetting an aliquot on a Whatman 31ET filter. Washing was performed according to MARTELO et al. (12).

. Endogenous phosphorylation : The reaction was initiated by adding microtubules (final concentration : 0.5-5 mg/ml). When phosphorylation was followed by electrophoretical analysis, the reaction was stopped by adding 0.8 M NaCl and the reactionnal mixture was cooled to 0° C before the isolation of MAPs as indicated above.

. Exogenous phosphorylation : MAPs 0.5-1 mg/ml were incubated for the time indicated with 7.5 units cyclic AMP dependent protein kinases or casein kinase I incorporating 0.2 nmol ^{32}P in casein/mn. The reaction was initiated by adding ATP.

- Electrophoresis was performed by applying protein samples (10-50 µg) to 4-15 % polyacrylamide gel containing 0.1 % SDS (13). After staining with Coomassie blue, gels were scanned using a Vernon densitometer. Finally the gels were dried and autoradiography was performed with X-ray films (Kodak XAR5).

- Peptide "mapping" was performed according to CLEVELAND et al. (14). ^{32}P labelled MAPs were submitted to electrophoresis as indicated above. The gels were placed in a bag of non-permeable cellophane and an X-ray film was applied to localize labelled polypeptides. Individual τ bands were excised from the gel. Slices were put directly onto the stacking gel of a 15 % SDS polyacrylamide gel. Following overlaying of each slice with a given amount of V8 protease from Staphylococcus aureus, proteolysis was effected in the stacking gel during electrophoresis. Radioactive peptide patterns were obtained by applying X-ray film to the dried gels.

- Determination of phosphate contents : Electrophoresis from ^{32}P labelled adult MAPs were scanned after staining. The amount of protein

in each band was estimated by comparison with albumin used as a marker which migrated on the same plates. We have established that in our experimental conditions, Coomassie blue stains microtubule associated proteins and albumin quantitatively over the range applied. The radioactive phosphate incorporated was measured by counting each band cut after drying. The corresponding number of moles of phosphate was calculated from specific radioactivity of ATP.

- Protein determination was performed according to LOWRY et al. (15).

RESULTS AND DISCUSSION

1.- τ phosphorylation by protein kinase(s) present in microtubule(s) preparation (endogenous phosphorylation). Effect of cyclic AMP.

Figure 1 shows the autoradiography from the gel electrophoresis of MAPs from adult rat brains phosphorylated by the protein kinases present in the microtubule preparations. MAP_2, τ and an intermediary polypeptide had incorporated phosphate. The addition of cyclic AMP stimulated the incorporation of ^{32}P in all of these polypeptides. In the case of τ, the stimulation by cyclic AMP was better demonstrated by cutting out bands of the dried gel and counting them (not shown).

Figure 2 shows similar observations made for MAPs from young animals (three days old). In particular, the two polypeptides τ_f and τ_s which constitute τ in these animals (13) were also phosphorylated and this

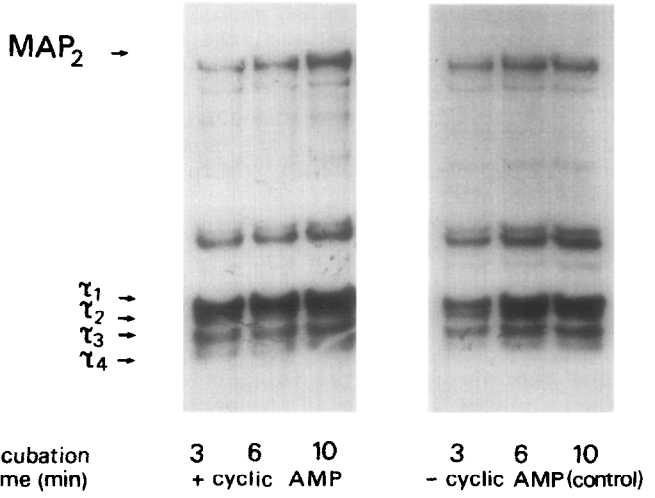

Fig. 1 : Adult τ phosphorylation by protein kinase(s) present in microtubule preparations.

After endogenous phosphorylation of MAPs from adult rat brain, ^{32}P MAPs were isolated and submitted to electrophoresis as indicated under Material and Methods. Stained gels were dried and autoradiography was performed.

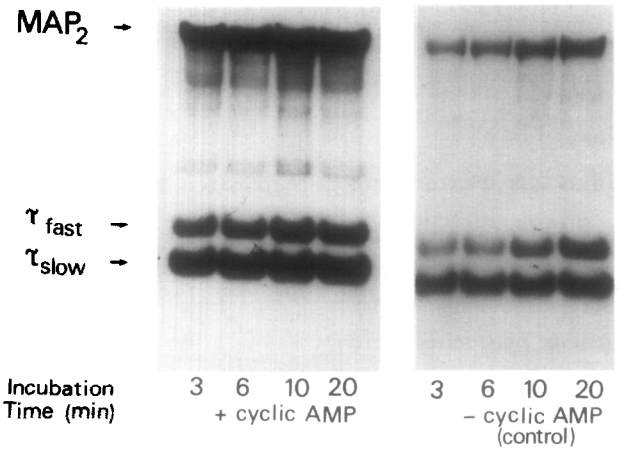

Fig. 2 : Young τ phosphorylation by protein kinase(s) present in microtubule preparations.
(see legend Fig. 1).

phosphorylation was markedly stimulated by cyclic AMP. The efficiency of the labelling (counts/mg protein) was similar for τ in both adult and young animals.

In all cases it was difficult to ascertain whether phosphorylation was catalysed only by cyclic AMP dependent protein kinases since ^{32}P incorporation in the absence of cyclic AMP was still high.

The number of phosphate groups incorporated in each molecular species of τ was measured. Table I gives the results obtained from two

TABLE I : AMOUNT OF PHOSPHATE INCORPORATED IN τ PROTEIN FROM ADULT BRAIN

	Experiment 1 (microtubules 5 mg/ml)		Experiment 2 (microtubules 0,5 mg/ml)	
	cpm/µg	mole P/ mole protein	cpm/µg	mole P/ mole protein
τ_1	4511	4,43	6227	5,22
τ_2	2655	2,50	2786	2,62
τ_3	2819	2,46	3279	2,48
τ_4	1978	1,64	1970	1,63

Determination of ratio P/protein was performed as indicated in the text.

typical experiments. It appears that several phosphates were incorporated in each molecular species of adult τ. At least 5 for τ_1, 3 for τ_2 and 3, 2 for τ_4.

Between 10-12 phosphates were incorporated into MAP_2 according to the observations of ISLAM and BURNS (23).

2.- τ phosphorylation by cyclic AMP dependent protein kinases and casein kinase I (exogenous phosphorylation).

All MAPs, and among them τ proteins, could be phosphorylated by the forms I and II of cyclic AMP dependent protein kinase(s) as shown by the example in figure 3 with form II of these enzymes. MAPs and τ did not autophosphorylate. The presence of both forms of the kinase during the reaction did not change the level of maximum incorporation (not shown).

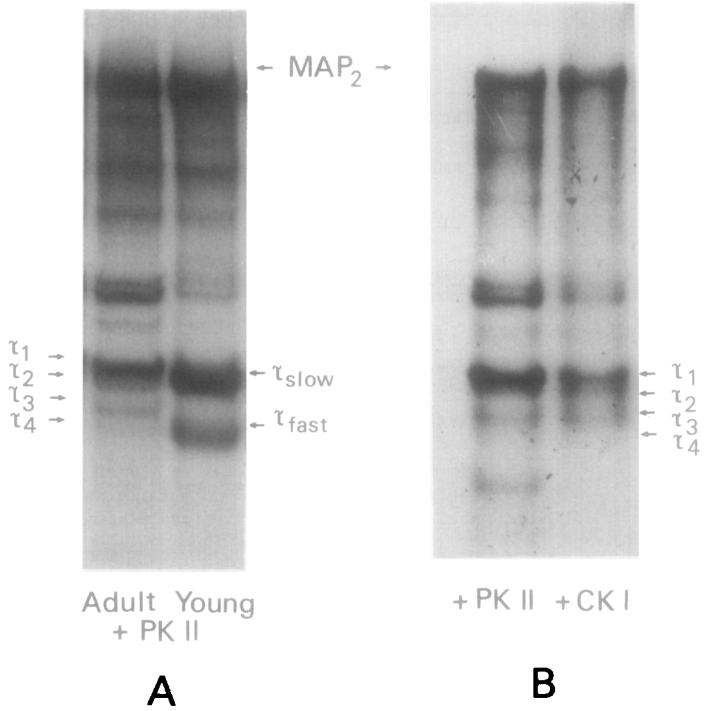

Fig. 3 : τ phosphorylation by cyclic AMP dependent protein kinase and caseine kinase I.
(A) : MAPs (1 mg/ml) from adult and young animals were phosphorylated by form II of cyclic AMP dependent protein kinase.
(B) : Phosphorylation of MAPs from adult animals by cyclic AMP dependent protein kinase (+ PK II) and casein kinase I (+ CK I).
Phosphorylation was performed as described under Material and Methods. Stained gels were dried and autoradiography was performed.

In these conditions, the four τ proteins from adult animals and the two τ proteins from young animals were phosphorylated with a similar efficiency (counts/mg protein). The number of phosphates incorporated in each molecular species of adult τ by cyclic AMP dependent protein kinases was also determined. This incorporation was lower than the one obtained in the presence of protein kinases associated with microtubules = 0.7 to 0.8 mole phosphate/mole protein for τ_1 and between 0.3 to 0.4 for the other τ species.

It is possible to imagine that all sites phosphorylated by endogenous phosphorylation are recognized by cyclic AMP dependent protein kinases in microtubule preparations. One possible explanation is that many sites were masked on τ free in solution due to a conformation change, although this is unlikely since isolated τ is a linear molecule with minimum folding (10). An other explanation is that a part of these molecules were phosphorylated in vivo on each site and that a greater incorporation was obtained in vitro by "endogenous phosphorylation" due to the presence of protein phosphatase, associated with microtubules, which acts on in vivo phosphorylated residues. In this case mapping of τ labelled by endogenous and exogenous phosphorylation would be very similar. But, this hypothesis is not verified as shown in figure 4A for τ_1.

Then, it is likely that several protein kinases present in the microtubule preparations are able to phosphorylate τ, and the presence of cyclic AMP independent protein kinases in these preparations (15) lends support to this view. This view was further supported by the observation that casein kinase I was also able to phosphorylate τ proteins on other sites than cyclic AMP dependent protein kinases (fig. 4B).

This phenomenon of "multisite phosphorylation" in which proteins are phosphorylated at two or more sites by two or more protein kinases is turning out to be common, for example glycogen synthase (18), phosphorylase kinase (19), acetyl-coA-carboxylase (20), protein I in synaptic membranes (22) and histone III (22).

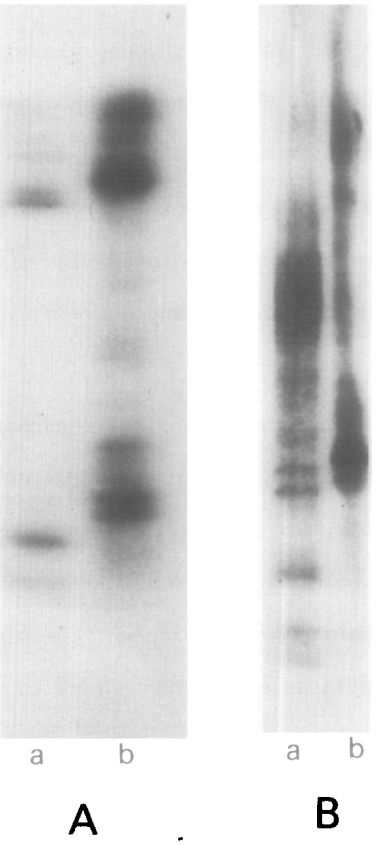

Fig. 4 : Peptide mapping from adult τ_1 labelled in various conditions.
(A) : τ_1 phosphorylated in the same experiment, by cyclic AMP dependent protein kinase (a) and by protein kinases associated to microtubules (b).
(B) : τ_1 phosphorylated in the same experiment, by casein kinase I (a) and by cyclic AMP dependent protein kinase (b).

Previous works (2,3,7) have indicated that τ was much less phosphorylated than MAP_2. This discrepancy with our results may be due to differences in methods of τ preparation ; it was easier to study τ when it was isolated by the method of FELLOUS et al. (11). The observations that several sites are phosphorylated on MAP_2 (23) and on τ (this paper) suggests that various changes in cell microtubules could be promoted by phosphorylation (number, size, organisation, interaction with other organites (24) or molecules). On other hand the indication that several protein kinases are able to phosphorylate τ suggests the possibility that the behaviour of microtubules is controlled by various signals and not only by stimuli which affect intracellular cyclic AMP concentration.

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