Biochem. J. (1984) **224**, 623–627
Printed in Great Britain

Phosphorylation of the microtubule-associated protein MAP2 by GTP

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(*Received 25 June 1984/Accepted 22 August 1984*)

The chick brain microtubule-associated protein MAP2 can be phosphorylated *in vitro* to the extent of 12mol/mol with GTP at the same sites as can be labelled by the cyclic AMP-independent protein kinase utilizing [γ-^{32}P]ATP as the phosphoryl donor. Consequently, the microtubule protein is chemically modified by the conditions usually employed for studies of microtubule assembly, so that the derived kinetic parameters may not relate to steady-state conditions.

Microtubule assembly requires, under most conditions, GTP and the presence of microtubule associated proteins, of which the best characterized is the high-M_r (280000) MAP2 (see, e.g., Burns & Islam, 1982). MAP2 is a phosphorylated protein, and 12 sites can be labelled *in vitro* with ATP as the phosphoryl donor (the 'labile' sites) (Islam & Burns, 1981), and a similar number of residues cannot yet be labelled *in vitro* (the 'stable sites') (Theurkauf & Vallee, 1983; Selden & Pollard, 1983). We have shown that 10 of the 12 labile sites lie within the known tubulin-binding domain at one end of the MAP2 primary sequence (Burns & Islam, 1984), that the number of sites labelled is determined by the ATP concentration (Islam & Burns, 1981), and that ATP-induced phosphorylation specifically affects the dissociation rate constant without affecting the association rate constant (Burns *et al.*, 1984).

Since GTP is normally used to promote microtubule assembly *in vitro*, the assembly properties of the microtubule protein might change during polymerization if GTP could also be utilized as a phosphoryl donor. Indeed, the thermodynamic analysis of microtubule assembly assumed that the protein is not chemically modified during polymerization (Johnson & Borisy, 1977).

GTP has though been shown to phosphorylate microtubule protein (Jameson *et al.*, 1980), although the maximal extent was significantly lower with [γ-^{32}P]GTP than with [γ-^{32}P]ATP, and a protein kinase activity associated with vinblastine-precipitated protein has been described that utilizes both ATP and GTP (Piras & Piras, 1974). In view of the possible effect of phosphorylation by GTP on the assembly kinetics, the identification of labile and stable phosphorylation sites and the reported presence of a protein kinase activity utilizing GTP, the phosphorylations of MAP2 with ATP and GTP as the phosphoryl donor have been compared.

Abbreviations used: MAP1 and MAP2, microtubule-associated proteins 1 and 2; SDS, sodium dodecyl sulphate; 2 × microtubule protein, microtubule protein that has been purified through two cycles of assembly and disassembly.

Methods

Preparation of microtubule protein

Microtubule protein was purified from 1-day-old chick brains through two cycles of assembly/disassembly as previously described (Burns & Islam, 1981), with a 2h cold dissociation step in the absence of added nucleotides after the second cycle to permit the complete dephosphorylation of the labile sites (Burns & Islam, 1984). The protein was then stored in liquid N_2 until use and was not further purified.

Phosphorylation of microtubule protein

This was done *in vitro* by using either [γ-^{32}P]ATP or [γ-^{32}P]GTP as the phosphoryl donor and the co-purifying endogenous cyclic AMP-independent protein kinase as previously described (Islam & Burns, 1981; Burns & Islam, 1984). The incorporation into MAP2 was determined either from the radioactivity co-migrating with MAP2 on SDS/polyacrylamide-gel electrophoresis (Islam & Burns, 1981) or by precipitation of unfractionated microtubule protein with 10% (w/v) trichloroacetic acid (Islam & Burns, 1981). SDS/polyacrylamide-gel electrophoresis was performed by the method

of Laemmli (1970), and the gels were stained and scanned as previously described (Burns & Islam, 1984).

Nucleotide analysis

The concentration of free nucleotide after incubation was determined by quenching the reaction mixture with 1% (w/v) SDS, precipitating the detergent with 0.5M-KCl and spotting a sample of the supernatant on to polyethyleneimine–cellulose plates (Camlab, Cambridge, U.K.; CEL 300 PEI, pre-washed with 10mM-Tris/HCl buffer, pH8.0), and chromatographing in methanol (2min), followed by 2M-LiCl/10mM-Tris/HCl buffer, pH8.0 (2h). After location of the γ-^{32}P-labelled nucleotide and [^{32}P]P_i by autoradiography, the appropriate portions of the plate were cut out and the radioactivity was counted in a scintillation counter.

Protein determination

This was done with bovine serum albumin as the calibration standard (Hartree, 1972).

Materials

All biochemical reagents were purchased from Sigma Chemical Co. Other reagents were of Fisons A.R. grade. [γ-^{32}P]ATP (24.2Ci/mmol) and [γ-^{32}P]GTP (21.1Ci/mmol) were purchased from Amersham International.

Results

Chick brain microtubule protein can be phosphorylated *in vitro* by the co-purifying cyclic AMP-independent protein kinase activity with either [γ-^{32}P]ATP or [γ-^{32}P]GTP as the phosphoryl donor (Fig. 1). Incubation of the 2× microtubule protein with either 0.5mM-[γ-^{32}P]ATP or 0.5mM-[γ-^{32}P]-GTP shows increasing incorporation into trichloroacetic acid-precipitable protein with time, and the kinetics of incorporation with GTP are, as previously reported (Jameson *et al.*, 1980), significantly slower than with ATP.

Fractionation of the pre-labelled microtubule protein by SDS/polyacrylamide-gel electrophoresis shows that [γ-^{32}P]GTP and [γ-^{32}P]ATP label the same proteins (Figs. 2*a*–2*c*). The pattern of incorporation is very similar for the two donors, with MAP2 and the τ-like proteins (M_r 50000–70000) being the predominant substrates. We consider below whether the two donors phosphorylate different residues on the same proteins.

The incorporation by [γ-^{32}P]GTP is directly proportional to the donor GTP concentration over a considerable range (3–300μM), and only approaches saturation at 500μM-GTP (Fig. 3*a*). Measurement of the free GTP and phosphate concentrations at the end of the incubation period (60min) indicated substantial GTP hydrolysis (Fig. 3*b*), and that free GTP only remained when the initial concentration exceeded 100μM-GTP. No attempt has been made to identify the GTPase(s) involved, but the formation of free phosphate is not due to the turnover of the labile phosphorylated residues, as the contaminating phosphoprotein phosphatases are totally inhibited by the 20mM-NaF present in the assay mixture. The GTPase activity may include hydrolysis of the GTP bound to the exchangeable site of tubulin during microtubule assembly (David-Pfeuty *et al.*, 1977).

An effect of the GTP concentration on MAP2 phosphorylation could either reflect the kinetic properties of protein kinase or describe the competency of MAP2 to function as a substrate. These two alternatives can be distinguished by plotting

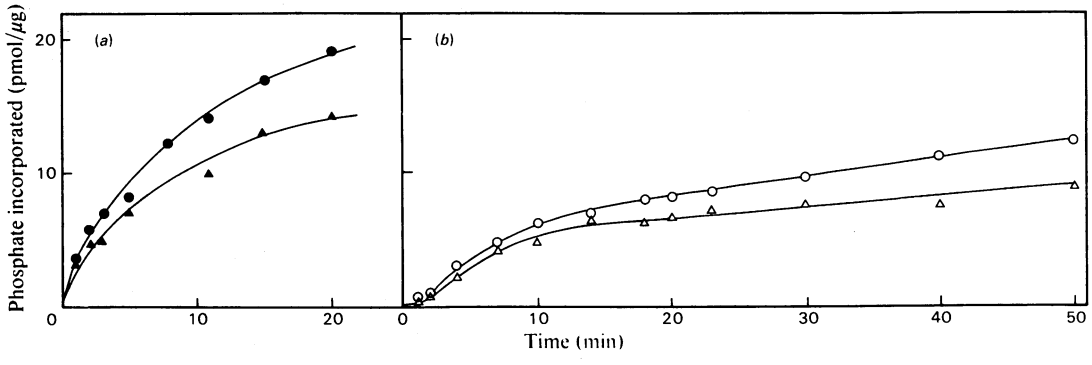

Fig. 1. *Incorporation of [^{32}P]phosphate into trichloroacetic acid-precipitable protein at 30°C as a function of time with ATP and GTP as the phosphoryl donors*

The 2× microtubule protein (1.18mg/ml) was incubated with (*a*) (●) 0.5mM-[γ-^{32}P]ATP or (▲) 0.5mM-[γ-^{32}P]ATP+0.5mM unlabelled GTP, or (*b*) (○) 0.5mM-[γ-^{32}P]GTP or (△) 0.5mM-[γ-^{32}P]GTP+0.5mM unlabelled ATP, for increasing times, and then precipitated with 10% trichloroacetic acid and the radioactivity counted.

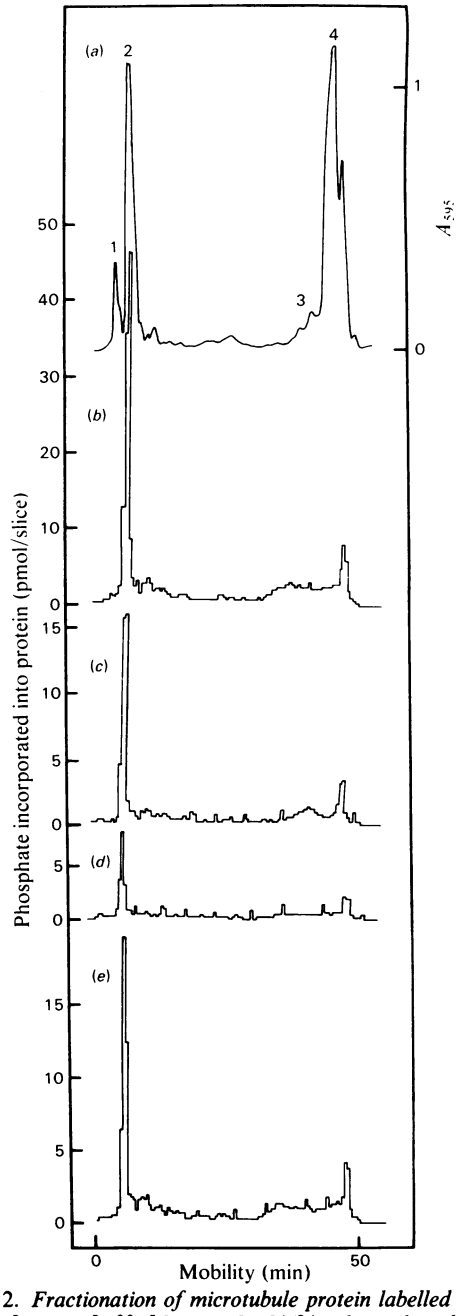

Fig. 2. *Fractionation of microtubule protein labelled with [γ-^{32}P]ATP or [γ-^{32}P]GTP on SDS/6%-polyacrylamide-gel electrophoresis*

(*a*) Profile of a typical gel stained with Coomassie Blue G-250 and scanned at 595nm, showing MAP1, MAP2 and tubulin as the principal components. (*b*)–(*e*) Distribution of the ^{32}P-labelled protein after labelling at 30°C with: (*b*) 0.5mM-[γ-^{32}P]ATP for 60min; (*c*) 0.5mM-[γ-^{32}P]GTP for 120min; (*d*) 0.5mM unlabelled ATP for 60min, followed by 0.5mM-[γ-^{32}P]GTP for 120min; (*e*) 0.5mM unlabelled GTP for 120min, followed by 0.5mM-[γ-^{32}P]ATP for 60min. The peaks refer to: 1, MAP1; 2, MAP2; 3, the τ-like components; 4, tubulin.

1/plateau value versus 1/[GTP], as an effect mediated by the protein kinase will only be apparent from initial-rate measurements. Such an analysis will only be valid if free GTP remains when phosphorylation is complete, and substantial GTP hydrolysis is observed at low initial concentrations (Fig. 3*b*). A plot of 1/plateau value versus 1/[GTP] for the initial GTP concentrations above 200μM (Fig. 3 inset) shows that the maximal extent of phosphorylation by GTP is equivalent to 17.4pmol/μg of microtubule protein. This yields, on correction for the amount of MAP2, its M_r and the fraction of label co-migrating with MAP2 [see Islam & Burns (1981) for details], a stoichiometry of 12mol/mol of MAP2. Adopting a revised M_r of 280000 for MAP 2 (Burns & Islam, 1982), compared with the earlier value of 300000, yields corrected stoichiometries of 12mol/mol for the GTP-induced phosphorylation and 10.7–12.8mol/mol for ATP.

As this strongly suggests that ATP and GTP phosphorylate the same residues, microtubule protein was pre-incubated with 0.5mM-ATP or -GTP for increasing times and then challenged with the alternative nucleoside triphosphate (0.5mM-[γ-^{32}P]GTP for 120min or 0.5mM-[γ-^{32}P]ATP for 60min). The incorporation by [γ-^{32}P]GTP without prior pre-incubation with the unlabelled nucleotide was one-third of that for [γ-^{32}P]ATP, and the axes scales reflect this difference (Fig. 4). Pre-incubation with the alternative nucleotide inhibits the subsequent phosphorylation, clearly indicating that the same residues are phosphorylated by the two donors.

Furthermore, SDS/polyacrylamide-gel electrophoresis of microtubule protein pre-incubated with 0.5mM-ATP (60min) or 0.5mM-GTP (120min) and challenged with 0.5mM-[γ-^{32}P]GTP (120min) or 0.5mM-[γ-^{32}P]ATP (60min) respectively show that no component is preferentially labelled for pre-incubation with the alternative donor (Figs. 2*d* and 2*e*).

Discussion

MAP2 can be phosphorylated *in vitro* with either ATP or GTP as the phosphoryl donor to the same maximum stoichiometry (Fig. 2), and pre-incubation experiments demonstrate that the two nucleotides label the same residues on the same proteins (Figs. 2 and 4). The protein kinase activity present in these preparations of microtubule protein is cyclic AMP-independent (Burns & Islam, 1984) and utilizes both ATP and GTP.

The final plateau value of phosphorylation is directly proportional to the nucleotide concentration for both GTP (0–300μM; Fig. 3) and ATP (0–400μM; Islam & Burns, 1981). A simple explana-

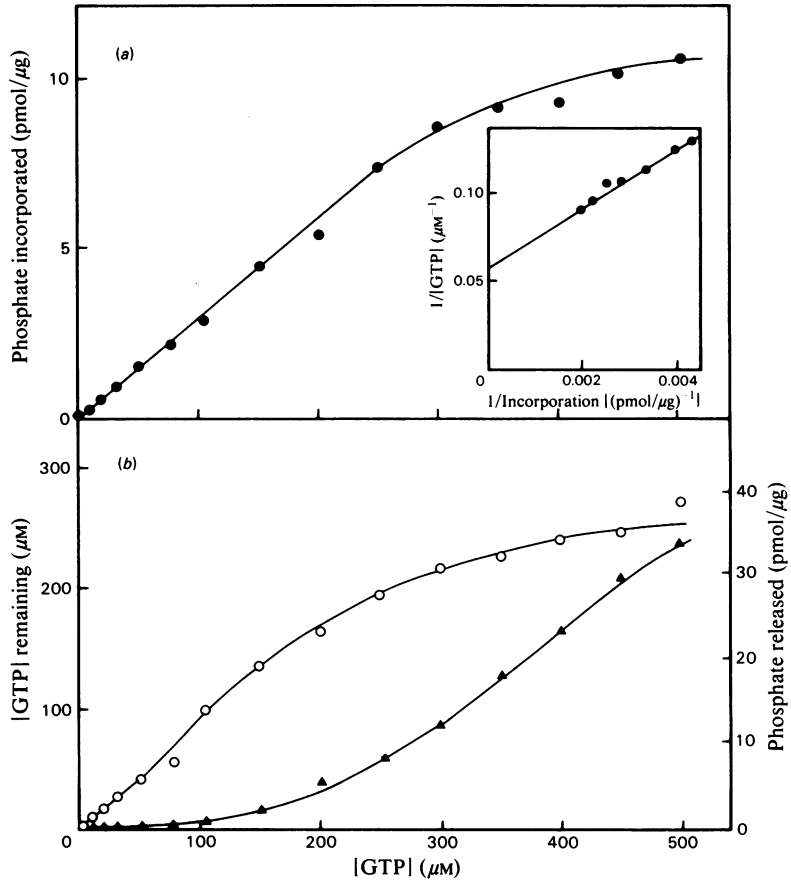

Fig. 3. *Endogenous phosphorylation of microtubule protein as a function of the GTP concentration*
(*a*) Incorporation into trichloroacetic acid-precipitable protein after incubation of 2× microtubule protein (2.78 mg/ml) for 60 min at 30°C with 3–500 μM-[γ-^{32}P]GTP. Inset: double-reciprocal plot of 1/incorporation (pmol/μg of protein) versus 1/[GTP]. (*b*) Concentrations of (▲) GTP remaining and (○) phosphate formed at the end of the 60 min incubation period.

tion would be that the phosphoryl-donor concentration is depleted by contaminating GTPases or ATPases before the phosphorylation of MAP2 is complete. Although considerable GTPase activity is observed (Fig. 3), significant concentrations remain when the phosphorylation is complete, provided that the initial GTP concentration exceeds 200 μM. This GTP concentration phosphorylates MAP2 to only approximately half the maximal extent (Fig. 3), so that the termination of phosphorylation cannot simply be due to the complete exhaustion of GTP. The sub-maximal phosphorylation is not due to the promotion of microtubule assembly by GTP, as the same effect is observed with ATP under non-assembly conditions (Islam & Burns, 1981) and as the kinetics of ATP-induced phosphorylation are unaffected by the extent of microtubule assembly (Islam & Burns, 1984). Consequently, the nucleotide concentration appears to determine the number of potential phosphorylation sites.

Finally, the observation that GTP phosphorylates the same sites as ATP indicates that the conditions commonly used to induce microtubule assembly also cause the phosphorylation of MAP2. This may account for why a high critical concentration for assembly (1.5–3.0 μM-tubulin dimer) is generally observed for microtubule assembly with 1 mM-GTP (see, e.g., Johnson & Borisy, 1977). More recent studies measuring the critical concentration from both the initial rates and final plateau values at 100 μM-GTP and with protein that had been extensively dephosphorylated yielded a critical concentration below 0.1 μM-tubulin dimer, but higher values were observed after rephosphorylation with ATP as the phosphoryl donor (Islam & Burns, 1981). Consequently, although the kinetics of MAP2 phosphorylation by GTP are compara-

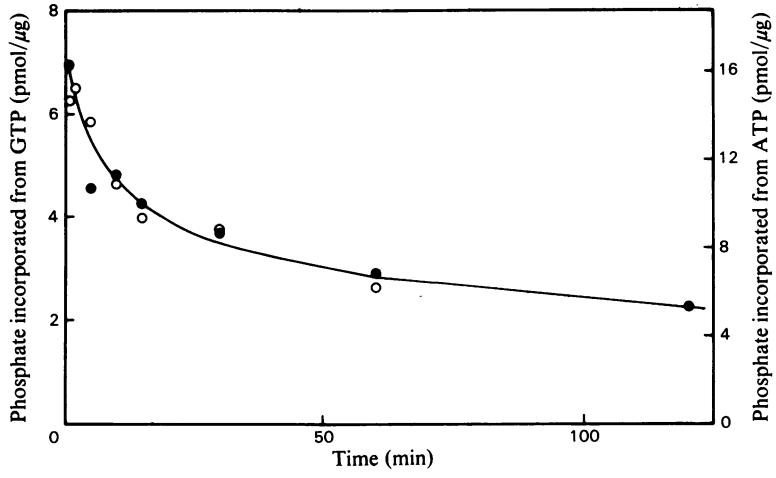

Fig. 4. *Effect of pre-incubating microtubule protein for increasing times with unlabelled GTP or ATP on the subsequent incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP or [γ-^{32}P]GTP into trichloroacetic acid-precipitable protein*
The 2× microtubule protein (0.83mg/ml) was incubated at 30°C for increasing times either (●) with 0.5mM unlabelled ATP and then challenged with 0.5mM-[γ-^{32}P]GTP for 120min, or (○) with 0.5mM unlabelled GTP and then challenged with 0.5mM-[γ-^{32}P]ATP for 60min.

tively slow (Fig. 1), there may be a significant effect on the assembly properties unless care is taken to dephosphorylate the microtubule protein extensively, and the kinetic parameters may change during the course of assembly as the MAP2 becomes phosphorylated.

We thank the Medical Research Council and the University of London for financial support, and Mr. A Luttriano of Ross Breeders Ltd. for generously donating the chicks.

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