Vol. 192, No. 1, 1993
April 15, 1993
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 268-272

ABSENCE OF 2'-DEOXY-GTP IN ADULT BRAIN TUBULIN*

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Received February 17, 1993

Summary-- Nerve growth factor can stimulate incorporation of 2'-deoxy-GTP into the non-exchangeable nucleotide sites in tubulin and cytoskeletal microtubules of PC12 pheochromocytoma cells and embryonic chick dorsal root ganglion neurons [J. M. Angelastro and D. L. Purich (1992) *J. Biol. Chem.* 267, 25685-25689]. We replaced and hydrolyzed exchangeable-site GTP and GDP in adult bovine brain tubulin by incubation with the non-hydrolyzable nucleotide analogue 5'-guanylyl-methylenediphosphonate and alkaline phosphatase, thereby allowing us to analyze the non-exchangeable guanine nucleotides for GTP and dGTP. HPLC analysis reveals no evidence of dGTP in adult tubulin, suggesting further that the appearance of dGTP in tubulin and microtubules may be a characteristic of recently dividing neurons in response to nerve growth factor.

Tubulin is a guanine nucleotide binding protein which irreversibly hydrolyzes its exchangeable (or E-site) GTP to GDP during microtubule self-assembly (1-4). This heterodimeric protein also binds a second molecule of GTP at a nonexchangeable (or N-) site, so named for its lack of exchange with nucleotide added to the medium (5,6). While nucleotides other than GTP can bind to the tubulin E-site *in vitro* (6), until recently there had been no evidence suggesting that the E- or N-sites contain any nucleotide other than GTP or GDP. Our laboratory developed a protocol (7,8) for directly analyzing the nucleotide content of microtubules isolated intact from non-muscle cells by extraction using Triton X-100 (9). We studied PC12 pheochromocytoma cells which extend elaborate long, branching neurite processes characteristic of sympathetic neurons, but

*This investigation was supported in part by funds from the University of Florida.

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Abbreviations used are: NGF, nerve growth factor; Tb, tubulin; $Gpp(CH_2)p$, 5'-guanylyl-methylene-diphosphonate.

0006-291X/93 $4.00

only when the cells are cultured in the presence of nerve growth factor (10). Intriguingly, microtubules isolated from NGF -treated PC12 cells, but not untreated cells, contained a significant fraction (*i.e.*, 30-45%) of their N-site nucleotide as 2'-dGTP which has heretofore been thought of only as a proximal DNA precursor (8). Similar results were also obtained with chick embryo dorsal root ganglion neurons, also cultured with NGF. Using PC12 cells, metabolic labeling experiments with [^{14}C]guanine resulted in radiolabeled GDP incorporation into microtubule-bound nucleotide in a manner suggesting that any E-site dGTP would have been detectable; moreover, the absence of any dGDP (an expected product of E-site nucleotide hydrolysis during assembly) also suggests that the dGTP was lodged in the non-exchangeable site. We were also interested to ascertain whether dGTP is present in adult neuronal tubulin, and we analyzed "cycle-purified" adult bovine brain tubulin. Accordingly, we present here a method that allows analysis of N-site nucleotide composition, and we further report on the absence of dGTP in adult brain tubulin.

Experimental Procedures

Materials-- GTP and Gpp(CH_2)p were purchased from Sigma Chemicals, Inc., and calf intestinal alkaline phosphatase (catalogue number 108-138) was a Boehringer Mannheim product. Microtubule protein (composed of approximately 75% tubulin and 25% microtubule-associated proteins) were isolated from adult bovine brain as outlined by Terry and Purich (11).

Methods-- Replacement of exchangeable-site guanine nucleotide with nonhydrolyzable analogue was achieved as follows. Microtubule protein (tubulin and microtubule-associated proteins, 8 mg/ml) in 80 mM piperazine-N,N'-bis[2-thanesulfonic acid] buffer (containing 1 mM $MgCl_2$ and adjusted to pH 6.8 with KOH) was incubated with 0.5 mM Gpp(CH_2)p and 3 units of calf intestinal alkaline phosphataseper mg microtubule protein for 45 min at 37° C, followed by an additional 30 min incubation at 4°C. The protein was then rewarmed to 37°C for another 45 min with an additional 1 mM analogue and 1 mM EGTA. After assembly, the microtubule fraction was harvested by centrifugation (100,000 x g, 2 hours) through a 30% (w/v) sucrose, such that 2-3 volumes of sucrose were overlaid with one volume microtubules prior to sedimentation. Pellets were resuspended in buffer, deproteinized by addition of an equal volume of cold 50% (v/v) ethanol for 30 min at 4° C. Nucleotides in the supernatant fluid after centrifugation (12,000 *x* g for 20 min) were subsequently analyzed with a diode array detection system using a Hewlett-Packard Model 1091A HPLC instrument, equipped with a Hypersil-ODS reverse phase microbore column by isocratic elution as described by Manser and Bayley (12).

Results

Spiegelman *et al.* (2) first demonstrated that cycles of warm-induced microtubule assembly and cold-promoted tubule depolymerization (with intervening sedimentation to separate remove

contaminants) are accompanied by complete replacement of E-site nucleotide. They observed, however, that no such change occurs with respect to N-site guanine nucleotide. To analyze the identity and amount of nucleotide bound at the non-exchangeable nucleotide sites of adult brain tubulin samples, an essential operation is the complete removal of exchangeable nucleotide introduced into tubulin during *in vitro* assembly/disassembly cycling. Because E-site GTP and GDP are bound tightly (*i.e.*, with sub-micromolar dissociation constants), efficient displacement can be problematical. For this reason, the protocol shown in Fig. 1 offers the advantage that the non-hydrolyzable analogue, 5'-guanylyl-methylenediphosphonate, can displace E-site GTP and GDP for enzymatic dephosphorylation by alkaline phosphatase. This approach offers an additional attribute in that one-to-one stoichiometry of N- and E-sites permits one to gauge the extent of E-site nucleotide removal by comparing the amount of analogue present in gel filtered tubulin directly to the amount of guanine nucleotide arising from the N-site. This comparison is achieved by the integrated area of the $Gpp(CH_2)p$ peak with those of GTP and other nucleotides released from tubulin by acid extraction and then analyzed by HPLC .

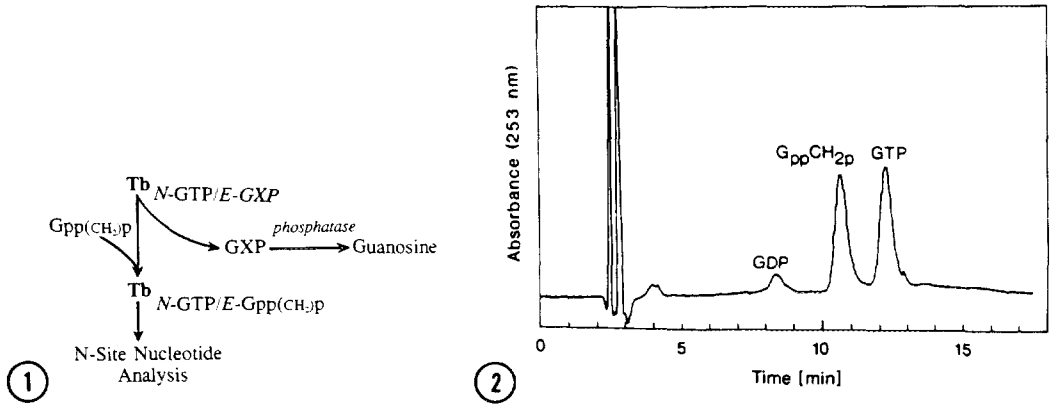

Fig. 1. Scheme for removal of exchangeable-site guanine nucleotide from tubulin.

Fig. 2. HPLC elution profile of non-exchangeable nucleotides and exchangeable-site $Gpp(CH_2)p$ from adult bovine brain tubulin. See "Experimental Procedures" for other details.

The elution profile data shown in Fig. 2 provide information on adult bovine brain tubulin N-site nucleotide composition. Three well resolved peaks were evident: first, the GDP peak; second, the Gpp(CH_2)p peak; and finally a single symmetric GTP peak. We observed no indication that any dGTP was present in the tubulin non-exchangeable nucleotide site of adult bovine brain tubulin, and the inset shows the a typical elution profile dGTP and GTP. Integration of the absorbance peaks indicates that GDP, Gpp(CH_2)p, and GTP amounted to 11, 42, and 47% of the guanine nucleotide. In view of the 1:1 stoichiometry of N- and E- sites, these values suggest that about 84% of the exchangeable-site nucleotide was replaced during analogue/phosphatase treatment used to deplete E-site nucleotide. Most significantly, however, despite our reasonably successful removal of E-site nucleotide to maximize the signal from the N-site, there was no evidence for the occurrence of any detectible dGTP in four separate experiments.

Discussion

Adult neurons are post-mitotic, and little is currently known about the capacity of post-mitotic cells to synthesize deoxy-nucleotides. Certainly, neurons have active pathways for efficient salvaging of purine bases, but again we cannot locate any literature regarding the ability of neurons to convert deoxy-nucleosides into deoxy-nucleotides. The data presented in the present communication indicate the absence of dGTP in adult brain tubulin and suggest that only recently dividing neurons may incorporate this DNA precursor into their microtubules. Interestingly, Ignatius *et al* (11) reported that PC12 cells treated with NGF for up to 15 days continued to synthesize DNA, and these investigators observed mitotic activity even in cultures of morphologically differentiated PC12 cells. Our earlier finding (8) of dGTP in microtubules from such cells suggests a hitherto unrecognized link between microtubule-bound nucleotide composition and cell division. One potential mechanism would involve trapping of cellular dGTP into microtubules such that the dCTP, dTTP, and dATP would collectively feedback inhibit ribonucleotide reductase; this could retard any restoration of dGTP and thereby block DNA synthesis. The presence of dGTP in tubulin of NGF-treated cells is also indicative of reduced availability of GTP, an essential

element in all G-regulatory signal transduction processes. The likely delicate poise between continued cellular division and initiation of morphogenesis in PC12 cells may respond to signal transduction at several different echelons, and sequestration of dGTP into tubulin may be a component of the overall regulatory mechanism underlying the commitment to neurite outgrowth and stabilization.

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