POST-TRANSLATIONAL ADDITION OF TYROSINE TO ALPHA TUBULIN IN VIVO IN INTACT BRAIN AND IN MYOGENIC CELLS IN CULTURE*

William C. THOMPSON

A. Ribicoff Research Center, Norwich Hospital, Box 508, Norwich, CT 06360 and *Dept. Biobehavioral Sciences, Univ. Connecticut, Storrs, CT 06268*

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1. Introduction

Tubulin:tyrosine ligase (TTL) catalyzes the reversible addition of tyrosine to the carboxyl terminus of alpha tubulin in cell-free systems containing brain tubulin as the tyrosylable substrate [1–3]. The use of purified brain tubulin to accept tyrosine has allowed the accurate determination of TTL activity levels in extracts from many tissues of the rat and chick [4,5]. The pattern of changes in TTL activity during development in the chick have also been determined for brain, skeletal muscle and liver [6]. The present study demonstrates that tritiated tyrosine is incorporated in vivo through the action of TTL at the carboxyl terminus of alpha tubulin in brain, that some of this terminal tyrosine is released from the tubulin during subsequent warm incubations of brain supernatants in vitro, and that tubulin present in fusing myogenic cultures contains some tyrosine which is added post-translationally.

2. Materials and methods

2.1. *Tyrosine labeling of chick brain proteins in vivo*

Cycloheximide (150 μg) and chloramphenicol (150 μg) in 0.15 M NaCl was injected into each cerebral hemisphere of White Leghorn chicks one or two days after hatching. Five minutes later 10 μCi of tritiated tyrosine (54 Ci/mmol, New England Nuclear), in 0.15 M NaCl was injected into each cerebral hemisphere. Twenty minutes later the chicks were decapitated, the brains were removed to ice-cold 0.15 M NaCl and then homogenized in 1.5 ml/brain of cold buffer A (100 mM MES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.6) by twenty strokes of a Dounce homogenizer (0.125 mm clearance). The supernatant fraction following one hour centrifugation at 100 000 × *g* at 0°C. was passed through Sephadex G-25, pre-equilibrated with buffer A or buffer B (a 1:1 dilution of buffer A with 8 M glycerol). The excluded volume effluent from this column is the labeled soluble brain protein fraction.

2.2. *Carboxypeptidase A digestion and electrophoresis*

Carboxypeptidase A (EC 3.4.3.1) (Worthington Biochemical Corp.) was added to an aliquot of the labeled brain supernatant proteins at an approximate protein concentration ratio of 1 : 50, and incubated with agitation for 15 min at 35°C. A second aliquot of the supernatant protein fraction was incubated without added enzyme. The two reaction mixtures were subjected to electrophoresis according to the method of Laemmli [7]. The 9 mm diameter gels were stained with Coomassie blue, destained by diffusion, frozen and sliced into 1 mm thick discs by a regular array of razor blades. Two discs per vial were dissolved in 50 μl of H_2O_2 at 50°C, then mixed with 4 ml Aquasol.

2.3. *Polymerization of tubulin*

Microtubule assembly in vitro by the method of Shelanski et al. [8] was accomplished by incubating labeled brain supernatant proteins at 37°C in the presence of 1 mM GTP in either buffer A or buffer B

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as noted. Assembled microtubules were collected by centrifugation at 100 000 × *g* for 1 h at 25°C. Vinblastine sulfate (1 mM) was added to some aliquots of labeled chick brain supernatant proteins prior to incubation as above.

2.4. *DEAE cellulose chromatography of tubulin*

Samples in buffer A or buffer B were applied to DEAE columns equilibrated with pyrophosphate buffer (50 mM $Na_4P_2O_7$, 2.5 mM $MgCl_2$, adjusted to pH 7.0 with HCl). Elution of the protein was either by a continuous gradient of NaCl generated in the pyrophosphate buffer, or by step gradients. Tubulin and labeled protein eluted from the column between 0.1 and 0.3 M NaCl.

2.5. *Tyrosine labeling of muscle proteins by myogenic cultures*

Primary cultures of chick leg myoblasts were plated at a density of 4.5×10^6 cells per 100 mm gelatin-coated Falcon culture plate in F-10 medium containing 10% horse serum and 3% embryo extract. Cultures were fed fresh complete F-10 medium at 42 h of culture. At 46 h, when the bulk of the myoblasts had fused, puromycin (4×10^{-5} M) was added and after 5 min incubation at 37°C chloramphenicol (3×10^{-5} M) and cycloheximide (4×10^{-5} M) were added. After a further ten minute incubation the culture medium was removed and the cells were rinsed and then covered with Puck's saline G containing 3×10^{-5} M chloramphenicol, 4×10^{-5} M cycloheximide and 2.5×10^{-5} M [^{3}H]tyrosine (770 Ci/mol). After 30 min incubation at 37°C, the cultures were rinsed well with saline G, fixed in methanol, extracted extensively with 10% Cl_3CCOOH, then methanol and finally dried. Some of the cultures were then subjected to autoradiography and some were dissolved in SDS buffer for electrophoresis.

3. Results

3.1. *Tyrosylation of chick brain tubulin in vivo*

Tyrosine is incorporated in vivo in the presence of inhibitors of translation into a single soluble brain protein which co-migrates on SDS gel electrophoresis with purified rat brain tubulin (fig.1). Release of the labeled tyrosine by carboxypeptidase A digestion

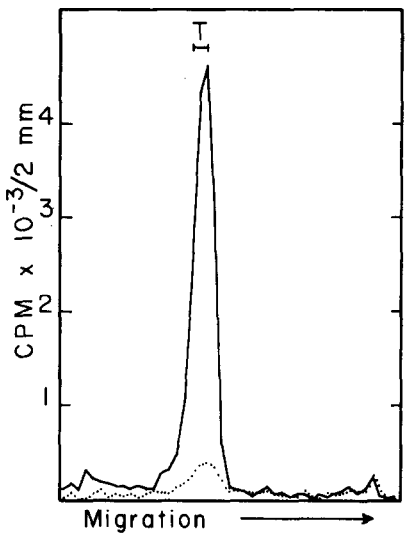

Fig.1. Electrophoretogram of heavily overloaded gels of soluble brain proteins labeled by [^{3}H]tyrosine in vivo in the presence of translation inhibitors. The position of a purified tubulin marker on a separate 10% polyacrylamide (SDS) gel is indicated. Brain proteins were incubated for 15 min in the absence (————) or presence of carboxypeptidase A (· · · · · · · ·) prior to electrophoresis.

(fig.1) shows this incorporation to be carboxyl-terminal addition to preexisting protein molecules.

When the tritiated supernatant protein mixture was subjected to chromatography on DEAE cellulose, a peak of labeled material eluted from the column at the same ionic conditions reported by Eipper [9] for the elution of tubulin (fig.2). For several experiments the labeled protein was purified from small DEAE columns by a step elution between 0.1 and 0.3 M NaCl. SDS polyacrylamide gel electrophoresis of a small aliquot of such DEAE purified material shows most of the protein to be tubulin, with the labeled material migrating with the alpha subunit (fig.3).

Purification of the tubulin protein from the crude brain homogenates by vinblastine sulfate or by in vitro assembly of microtubules by warm incubation in the presence of GTP was also accomplished. In both cases, labeled material was precipitated which co-migrated with the alpha subunit of purified tubulin on SDS electrophoresis (data not shown).

3.2. *Tyrosylation of muscle tubulin by myogenic cultures*

High levels of TTL activity have been demonstrated

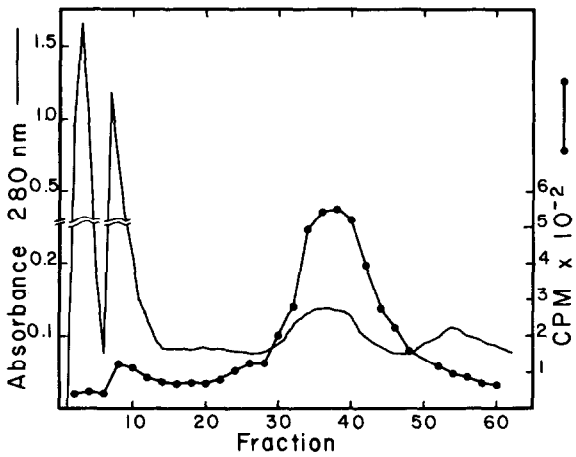

Fig.2. Effluent profile of labeled brain supernatant proteins from DEAE cellulose. The sample was added in buffer A, eluted with pyrophosphate buffer beginning with fraction 6, and with a 0–0.5 M NaCl linear gradient in that buffer beginning with fraction 11.

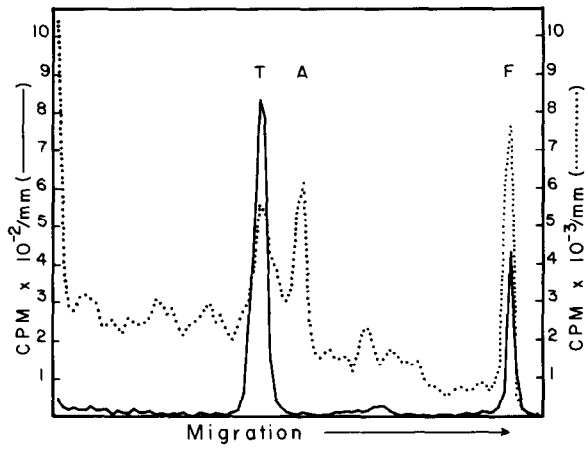

Fig.4. Electrophoretogram of total protein of cultured myogenic cells after posttranslational incorporation of [^{3}H]tyrosine (————). The pattern of [^{3}H]tyrosine incorporation by a control culture during 2 h growth in normal complete F 10 medium (· · · · · ·) is shown for comparison. The migration on these 10% polyacrylamide (SDS) gels of tubulin (T), actin (A) and the dye front (F) are indicated.

previously in extracts from 13 day embryonic chick muscle using purified brain tubulin as tyrosine acceptor [6]. Incorporation of [^{3}H]tyrosine by cultured myogenic cells in the presence of translation inhibitors demonstrates that tubulin in non-nervous cell types is capable of accepting tyrosine in vivo (fig.4). Autoradiographic analysis of such cultures following extraction of aminoacyl-tRNAs shows silver grain development over all of the cell types, including fibroblasts.

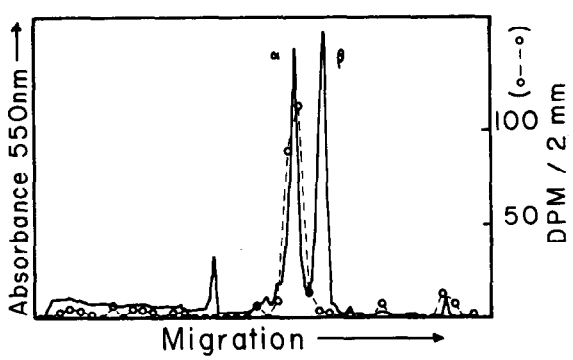

Fig.3. Electrophoretogram of DEAE cellulose – purified labeled brain protein on a 5% polyacrylamide (SDS) gel. Tubulin subunits are indicated (α,β).

3.3. *Release of terminal tyrosine during warm incubations*

During the course of purifications of tubulin involving warm incubations it was demonstrated that much of the labeled tyrosine residue had been released from the protein. Approximately one half of the labeled tyrosine was lost from total tubulin during the first 45 min incubation (table 1) and more was lost during subsequent warm centrifugation. In an attempt to determine whether tubulin competent to polymerize preferentially released its terminal tyrosine at 37°C, the specific activity of the formed microtubule tubulin and of the soluble tubulin remaining in the supernatant was determined following repurification of the tubulin from each source on DEAE cellulose columns. Both tubulin sources have a much lower specific activity at the end of the incubation and centrifugation than the original supernatant, but the specific activity of the nonpolymerizing tubulin is higher than the pelleted tubulin (table 1). Since the DEAE tubulin fraction isolated from the supernatant is less pure than that repurified from microtubules, a comparison of the specific activities based on the alpha tubulin content was made. Small samples of the repurified tubulin fractions from each source

Table 1
[^{3}H]tyrosine release during warm incubations

Expt. #	37°C. min	25°C. min	Total tubulin (DEAE) cpm/mg	Pellet tubulin (DEAE) cpm/mg	Supernatant tubulin (DEAE) cpm/mg	Ratio sup/pel sp. act. (Lowry)	Ratio sup/pel sp. act. α-tubulin (gel scan)
10	0	0	4593				
10	45	0	2263				
11	0	0	3118				
11	15	0	2715				
11	30	0	1837				
11	45	0	1566				
10-A	45	60	–	1332	2033	1.5	–
10-B	45	60	–	1233	2359	1.9	–
10 (VBS)	45	60	–	1834	1930	1.05	–
11-A	45	60	–	658	977	1.6	2.0
11-B	45	60	–	617	704	1.2	1.8

Brain supernatant fraction labeled in vivo was incubated at 37°C for the indicated times with 0.5 mM GTP in buffer A (Expt. 11) or with 0.5 mM GTP in buffer B (Expt. 10). Polymerized tubulin was collected from some samples by centrifugation at 25°C. Following a 0°C incubation to insure microtubule depolymerization, tubulin was isolated from all samples by DEAE cellulose chromatography. Specific activity of this DEAE purified tubulin is based upon hot TCA insoluble tritium and protein estimations according to Lowry et al. [11] or estimates of alpha tubulin concentration from stained gels.

were electrophoresed on 5 mm polyacrylamide gels and the stained alpha tubulin content was determined by densitometry. Although the Coomassie stain is not necessarily linear with respect to protein concentration, the alpha tubulin concentrations of the samples of interest were nearly identical. The remaining specific activity of the supernatant alpha tubulin band was approximately twice that of the microtubule pellet alpha tubulin

4. Discussion

Posttranslational addition of tyrosine at the carboxyl terminus of alpha tubulin by the action of a tubulin:tyrosine ligase in the intact brain indicates that this enzyme influences the ratio of two different alpha tubulin populations within the cell. The obvious exciting possibility is that such rapid changes in the primary structure of alpha tubulin may play an important role in controlling the assembly, maintenance and/or function of microtubules in vivo. Raybin and Flavin have recently reported the tyrosylation of a protein in neuroblastoma cells which migrates, like tubulin, on SDS electrophoresis [5]. The post-translational incorporation of tyrosine by myogenic cells in culture reported here is the first unequivocal demonstration of the ability of tubulin from non-nervous tissues to serve as a substrate in the TTL reaction in vivo. Experiments to be reported elsewhere have demonstrated the ability of fresh extracts of embryonic muscle to incorporate tyrosine into endogenous muscle tubulin in vitro.

The release of some terminal tyrosine incorporated in vivo during subsequent in vitro assembly of microtubules would indicate that the terminal tyrosine content of tubulin prepared by the method of Shelanski, et al. [8] might not reflect the terminal tyrosine content of tubulin in vivo. Barra, et al., have also noted a partial release of tyrosine from alpha tubulin during warm incubations of brain proteins previously labeled in vitro, suggesting that the loss of tyrosine was linked

to microtubule assembly [10]. The major tyrosine release reported here affects both the tubulin which forms microtubules and that which remains unpolymerized, although the unpolymerized material does retain a higher level of [^{3}H]tyrosine per alpha tubulin subunit. This may be due to a preferential labeling in vivo of tubulin incompetent to polymerize, a slight preferential release of tyrosine from assembled microtubules, or a slow inactivation of tubulin which might preclude both polymerization and enzymatic tyrosine release.

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References

[1] Arce, C. A., Rodriguez, J. A., Barra, H. S. and Caputto, R. (1975) Eur. J. Biochem. 59, 145–149.
[2] Rodriguez, R. A., Arce, C. A., Barra, H. S. and Caputto, R. (1973) Biochem. Biophys. Res. Commun. 54, 335–340.
[3] Raybin, D. and Flavin, M. (1975) Biochem. Biophys. Res. Commun. 65, 1088–1095.
[4] Deanin, G. G. and Gordon, M. W. (1976) Biochem. Biophys. Res. Commun. 71, 676–683.
[5] Raybin, D. and Flavin, M. (1977) J. Cell Biol. 73, 492–504.
[6] Deanin, G. G., Thompson, W. C. and Gordon, M. W. (1977) Develop. Biol. 57, 230–233.
[7] Laemmli, U. K. (1970) Nature 227, 680–685.
[8] Shelanski, M. L., Gaskin, F. and Cantor, C. R. (1973) Proc. Natl. Acad. Sci. USA 70, 765–768.
[9] Eipper, B. A. (1974) J. Biol. Chem. 249, 1398–1406.
[10] Hallak, M. E., Rodriguez, J. A., Barra, H. S. and Caputto, R. (1977) FEBS Lett. 73, 147–150.
[11] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.