Posttranslational Glutamylation of α-Tubulin

BERNARD EDDÉ, JEAN ROSSIER, JEAN-PIERRE LE CAER, ELIZABETH DESBRUYÈRES, FRANÇOIS GROS, PHILIPPE DENOULET

The high degree of tubulin heterogeneity in neurons is controlled mainly at the posttranslational level. Several variants of α-tubulin can be posttranslationally labeled after incubation of cells with [^{3}H]acetate or [^{3}H]glutamate. Peptides carrying the radioactive moiety were purified by high-performance liquid chromatography. Amino acid analysis, Edman degradation sequencing, and mass spectrometric analysis of these peptides led to the characterization of a posttranslational modification consisting of the successive addition of glutamyl units on the γ-carboxyl group of a glutamate residue (Glu^{445}). This modification, localized within a region of α-tubulin that is important in the interactions of tubulin with microtubule-associated proteins and calcium, could play a role in regulating microtubule dynamics.

TUBULIN HETEROGENEITY IS PARticularly high in the brain where up to 21 variants can be resolved by isoelectric focusing (IEF) techniques (*1, 2*). In the mouse, five α-tubulin and six β-tubulin isogenes have been found (*3, 4*). However, the number of functional isogenes identified is substantially lower than the number of polypeptides separated by IEF, suggesting the existence of several posttranslational modifications (*5*). Some of these modifications have been characterized (*6*), including tyrosylation-detyrosylation (*7*) and acetylation of α-tubulin (*8, 9*), and phosphorylation of a specific β-tubulin isotype (*10, 11*). We now describe a modification consisting of the progressive addition of glutamyl units on a Glu residue of the COOH-terminal domain of α-tubulin.

We have previously shown that incubation of mouse brain neurons with [^{3}H]acetate, in the presence of cycloheximide, resulted in a posttranslational labeling of several acidic variants of α-tubulin (*12*). To characterize the corresponding modification, we purified the labeled tubulin, mixed the purified preparation with unlabeled mouse brain tubulin, and incubated the mixture with the proteolytic enzyme thermolysin. Tubulin peptides bearing the radioactive moiety were purified by high-performance liquid chromatography (HPLC). The whole digests were first applied on a C_8-RP300 reversed-phase column. The profile revealed three major radioactive fractions eluting between 16 and 20 min and several minor ones eluting later (Fig. 1A). The former fractions were purified further by chromatography on a C_{18} column, and three radioactive peaks, denoted TL.A, TL.B, and TL.C (Fig. 1, B to D), were thus obtained. The amino acid sequences of TL.A, TL.B, and TL.C were determined by automatic Edman degradation (*13*). The three fractions gave the same sequence, Val-Glu-Gly-Glu-Gly-X-Glu-Glu-Gly, which matches the sequence of Mα1- and Mα2-tubulin isogene products (*3*) from amino acids 440 to 448. In view of the cleavage specificity of thermolysin (*14*), these peptides probably extended to the COOH-terminus of α-tubulin: Tyr^{451} or Glu^{450} for the tyrosylated and detyrosylated forms, respectively. No other amino acid sequence was observed. This sequence differs from that deduced from the nucleotide sequence at position 445; instead of obtaining phenylthioidantoine (PTH)-Glu, no PTH–amino acid (PTH-aa) could be detected at the corresponding cycle. This gap (denoted **X** in the sequence indicated above) was observed in four out of four independent experiments and strongly supported the presence of a modified Glu residue, the corresponding PTH-derivative being either retained on the filter of the sequencer or excluded from the separating range of the PTH-aa analyzer. Fractions of the PTH-aa's stored after each cycle of the Edman degradation were processed for liquid scintillation counting. Radioactivity was not found in any of these fractions but was substantially retained on the filter of the sequencer, indicating that the modified PTH-Glu^{445} was not extracted from the filter of the sequencer.

To further characterize the modification carried by TL.A, TL.B, and TL.C, we determined the accurate masses of these peptides by mass spectrometry (MS). The underivatized forms of the peptides did not show any characteristic molecular ion. This could be explained by a difficulty of desorbtion due to the weak hydrophobicity of the peptides. However, after methylation of free carboxyl groups, abundant protonated molecular ions ($M + H^+$) with mass/charge ratios (m/z) of 1447.6, 1590.7, 1733.7, 1876.7, and 2019.8 were observed (Fig. 2, A to C).

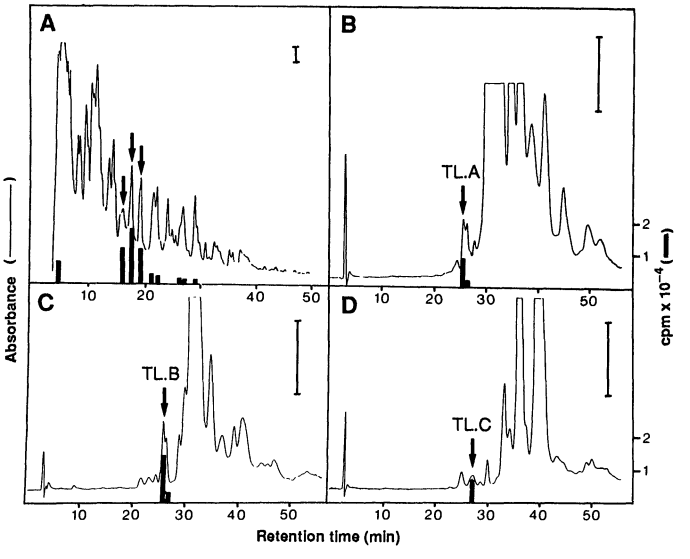

Fig. 1. HPLC purification of acetate-labeled tubulin peptides. Neurons were isolated from 15-day-old embryonic mouse brains and allowed to develop in culture for 1 week as described (*21*). They were transferred to phosphate-buffered saline containing cycloheximide (100 μg/ml), and [^{3}H]acetate (Amersham; 2 to 5 Ci/mmol) was added 30 min later to a radioactive concentration of 0.5 mCi/ml. The cells were incubated for 3 hours in the continuous presence of cycloheximide. ^{3}H-labeled tubulin (50 μg; 80,000 cpm) was purified by taxol-induced microtubule assembly (*22*) and mixed with unlabeled tubulin (1.2 mg) similarly purified from 1-month-old mice. The tubulin mixture was incubated for 24 hours at 37°C with thermolysin in 50 m*M* tris-HCl buffer (*p*H 8.0) containing 0.1 m*M* $CaCl_2$. Digestion was stopped by adding 1% (v/v) trifluoroacetic acid (TFA) and 5% (v/v) acetonitrile, and the sample was centrifuged for 2 min at 10,000*g*. The pellet, which usually contained 30 to 50% of the radioactivity, was discarded. (**A**) The supernatant was analyzed by reversed-phase HPLC on a C8-RP300 column (Brownlee; 4.6 by 220 mm). Three major radioactive fractions (arrows) were detected. (**B** to **D**) The three radioactive fractions were further purified on a C_{18} (5 μm) column (Brownlee; 2.1 by 220 mm). Samples were injected at 5% B, the elution gradient was linearly increased from 5% B to 35% B in 50 min and then from 35% B to 100% B in 10 min, and finally a 10-min wash in 100% B was applied (solution A was 0.1% TFA and solution B was 0.09% TFA and 70% acetonitrile). Flow rates were 0.9 ml/min (A) and 0.2 ml/min (B to D). The peptides were detected by their absorbance at 214 nm (A_{214}) and fractions were collected manually. The vertical bars represent a difference in A_{214} of 0.1. Samples of the collected fractions were processed for liquid scintillation counting.

B. Eddé, E. Desbruyères, F. Gros, P. Denoulet, Laboratoire de Biochimie Cellulaire, Collège de France, 75005 Paris, France.
J. Rossier and J.-P. Le Caer, Laboratoire de Physiologie Nerveuse, Centre National de la Recherche Scientifique, 91198 Gif s/Yvette, France.

These values differ by increments of 143. Moreover, the lowest mass signal at *m/z* of 1447.6 differs also by 143 from the calculated mass of the undecapeptide Val^{440}-Glu^{450} [1303.5 daltons = 1191.4 + 112.1 (from the eight methyl groups)]. Therefore, the observed mass signals must correspond to the undecapeptide Val^{440}-Glu^{450} bearing one to five posttranslationally added units of 143 daltons. This mass value corresponds to a carboxyl-methylated glutamyl group. This interpretation was further supported by the analysis of larger peptides obtained after partial digestion with Glu-C endoproteinase. These peptides all started at Val^{435} and showed again a gap at position 445. Mass signals of the underivatized peptides revealed the posttranslational addition of chemical units of 129 daltons (*15*), which is the mass of a glutamyl group. Glutamate can be metabolically labeled during cell incubation with [^{3}H]acetate. This compound enters the tricarboxylic acid cycle as [^{3}H]acetyl coenzyme A and the label can then be transferred to glutamate through α-ketoglutarate. Because incubation is always performed in the presence of cycloheximide, labeled amino acids cannot be incorporated during protein synthesis but can only be used in posttranslational events. We verified that the posttranslational modification can also be labeled after incubation of neurons with [^{3}H]glutamate. Compared to that obtained with [^{3}H]acetate, the results showed (*15*) an increased posttranslational labeling of the same α-tubulin isoforms and of the same peptides (TL.A, TL.B, and TL.C).

The identification of the added units as glutamyl groups was confirmed by amino acid analysis. Acid hydrolysis of TL.A, TL.B, and TL.C peptides, which had been labeled by incubating cells with [^{14}C]glucose (*16*), was performed. The amino acid composition of the peptides confirmed the presence of Val, Gly, and Glu (*17*). The molar ratio of Val to Gly (1:3) was similar in the three fractions, but a variation in the molar ratio of Glu was observed: 8.67 ± 0.20, 9.68 ± 0.23, and 11.41 ± 0.27 (n = 3) in TL.A, TL.B, and TL.C, respectively. These values are significantly higher than the value of 7 expected for the unmodified undecapeptide Val^{440}-Glu^{450} and are consistent with the interpretation of the MS data; that is, the addition of one to five glutamyl units. Moreover, we found that for peptides TL.A, TL.B, and TL.C, 89%, 87%, and 92%, respectively, of the input radioactivity was eluted in the Glu peak, indicating that the radioactivity in these peptides was attributable to the posttranslational addition of labeled glutamyl units.

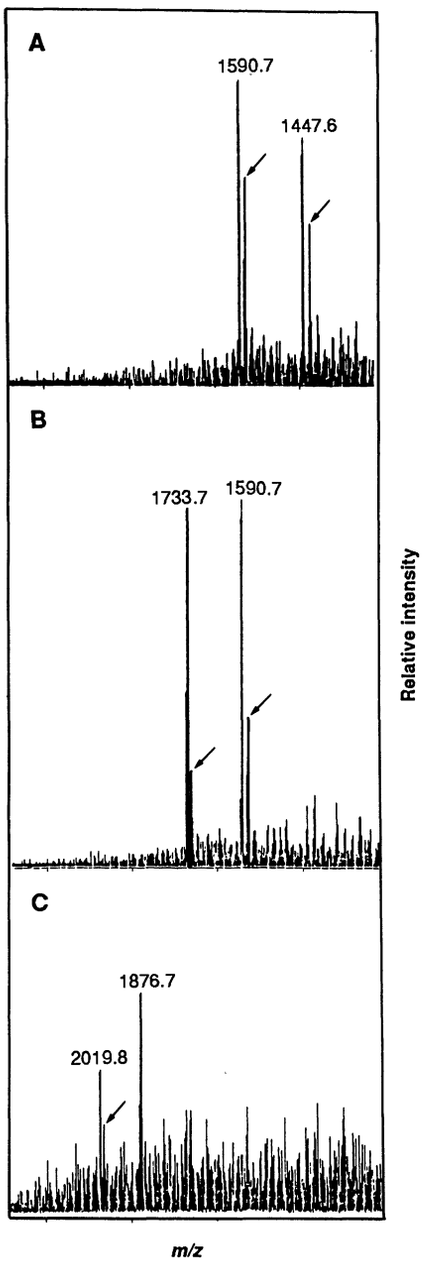

Fig. 2. Mass spectra of carboxyl-methylated TL.A (**A**), TL.B (**B**), and TL.C (**C**). Masses of the main protonated molecular ions (M + H^+) are indicated. The signals indicated by arrows, 14 mass units below the main signals, are due to the uncompleted methylation reaction. A ZAB-HS double focusing mass spectrometer (VG Analytical, Manchester, U.K.) equipped with a fast atom bombardment gun (Iontech) was used. The spectra were generated by a xenon atom beam of 8 keV. The matrix was a 1:1 mixture of glycerol and thioglycerol (1 μl) acidified with 1 μl of 10% (w/v) trichloroacetic acid. Cesium iodide clusters were used for mass calibration. Precise mass determinations were obtained with the use of linear voltage scans while maintaining the magnet field at a constant value. The spectra resulted from the averaging of 20 to 30 scans at 10 s per decade. High voltage range was 8 to 4 keV (mass range 1300 to 2400 daltons). Methylation of free carboxylic groups was performed by incubating the peptides with 50 μl of a 78:22 (v/v) mixture of methanol and acetyl chloride at room temperature for 1 hour, with constant sonication. About 50 to 100 pmol of each peptide was used in each experiment.

Previous results obtained after pulse-chase experiments (*5*) favor a mechanism of progressive glutamylation in which glutamyl groups are added unit by unit to α-tubulin. It is thus likely that the first glutamyl unit is linked by an amide bond to the γ-carboxyl group of Glu^{445}. The additional glutamyl units would be linked together by amide bonds between either the α- or the γ-carboxyl groups. It is not absolutely certain that Glu^{445} is the unique site carrying all the glutamyl units. Indeed, Glu^{449} and Glu^{450} were not released during Edman degradation, probably because of the very low recovery of the last amino acids with gas-liquid sequencing (*13*). Thus, because these two residues were out of the range of our analysis, we cannot totally exclude that they are also glutamylated. However, MS data revealed that TL.A, TL.B, and TL.C did not carry the optional residue Tyr^{451}. Other experiments (*15*) indicated that some of the minor radioactive fractions eluted from the first RP300 column (Fig. 1A) correspond to the tyrosinated counterparts of the three peptides. Thus, the modification described here could be independent from the COOH-terminal tyrosination-detyrosination process.

Glutamylation represents a major modification of neuronal α-tubulin. As estimated by the yield obtained during Edman degradation and amino acid analysis, the different glutamylated forms represent about 40 to 50% of the total α-tubulin present in brain. We also obtained evidence that neuronal α-tubulin was acetylated at Lys^{40}, but this modification appeared to be relatively minor (<5%) compared to glutamylation (*15*).

The COOH-terminal domains of α- and β-tubulin are exposed on the outer surface of the microtubule lattice and participate directly in the binding of several microtubule-associated proteins and calcium ions (*18–20*). Thus, progressive glutamylation of α-tubulin near the COOH-terminus could affect these interactions and regulate microtubule dynamics and function.

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16. Because amino acid analysis of ^{3}H-labeled peptides showed a high exchange of the radioactivity with the acidic phase, we analyzed ^{14}C-labeled peptides. [^{14}C]glutamate can be metabolically formed by conversion of [^{14}C]glucose through glycolysis and the tricarboxylic acid cycle.
17. Neurons were cultured for 1 week and were then incubated for 3 hours with D-[U-^{14}C]glucose (100 μCi/ml) (Amersham; >230 mCi/mmol) and cycloheximide. Extraction of tubulin, digestion with thermolysin, and purification of the ^{14}C-labeled peptides TL.A, TL.B, and TL.C were performed as described (Fig. 1). HPLC profiles and distribution of the radioactive peaks were similar to those observed after cell incubation with [^{3}H]acetate (Fig. 1). Hydrolysis was carried out in vapor phase of *7M* HCl and 10% (v/v) TFA for 22 min at 160°C. Precolumn derivatization with phenylisothiocyanate and reversed-phase HPLC analysis of amino acid derivatives were performed essentially as described [B. A. Bidlingmeyer, S. A. Cohen, T. L. Tarvin, *J. Chromatogr.* **336**, 93 (1984)]. The eluted fractions were processed for liquid scintillation counting. In the three cases, a single radioactive fraction, associated with the Glu peak, was detected.
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23. We thank C. Gruszczynski, A. Koulakoff, and Y. Berwald-Netter for providing cultures of mouse brain neurons; P. Gavard, F. Pratbernou, and J. C. Promé for MS analysis; S. Delay, J. Landry, and B. Ribadeau-Dumas for amino acid analysis; and R. Guénard for providing taxol. Supported by grants from Centre National de la Recherche Scientifique, Institute National de la Santé et de la Recherche Médicale (CRE N° 896005), Association Française contre les Myopathies, and Fondation pour la Recherche Médicale.

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An Identified Neuron (CPR) Evokes Neuronal Responses Reflecting Food Arousal in *Aplysia*

THOMAS TEYKE, KLAUDIUSZ R. WEISS, IRVING KUPFERMANN

Feeding behavior of *Aplysia* is associated with an arousal state characterized by a constellation of maintained behaviors and by a potentiation or depression of responses to specific stimuli. A neuron (the cerebral-pedal regulator or CPR) that has widespread actions on various systems connected with feeding has been identified. CPR excites neurons that modulate or drive (i) body posture, (ii) biting, and (iii) cardiovascular behaviors. CPR also inhibits neurons concerned with defensive responses. Food stimuli, which elicit food arousal in the animal, produce prolonged excitation of the CPR. The results suggest that the CPR may evoke a central motive state representing the neuronal correlate of feeding motivation.

MORE THAN 40 YEARS AGO IT WAS recognized that variations in behavioral responsiveness could not be adequately explained exclusively by reference to external stimuli, and it was therefore proposed that motivated behaviors are controlled by central motive states (*1*). The central motive states were postulated to evoke and maintain specific behaviors and also to modify the responses of the organism to external stimuli. The neuronal basis of central motive states has been studied in vertebrates (*2*), as well as several invertebrates (*3, 4*), including the mollusk *Aplysia*.

In *Aplysia*, food elicits behavioral changes that can be interpreted as being due to an underlying central motive state, which we have referred to as food-induced arousal (*5*). The changes include the induction of appetitive behaviors such as a characteristic feeding posture in which the head of the animal is lifted from the substratum. Furthermore, biting responses are more readily elicited by food, and once the biting responses begin there is a progressive increase in the rate and strength of the responses. Finally, defensive reflexes are inhibited, and there are modifications of cardiovascular responses (*6*). Food arousal in *Aplysia*, at least in part, is mediated by modulatory systems [for example, the serotonergic modulatory metacerebral cell (MCC) (*7*)] that are dedicated to one or another aspect of feeding behavior. In this study we sought to determine whether there are higher order neurons that modulate lower level subsystems.

We first studied isolated pedal-pleural and cerebral ganglia (*8*). We identified a single pair of bilaterally symmetrical cerebral neurons (*9*), cerebral-pedal regulators (CPRs), whose firing had excitatory or inhibitory effects on a large number of cells in the ipsilateral and contralateral pedal ganglia (Fig. 1A). A subsample of neurons studied in a solution that blocks polysynaptic transmission (*10*) indicated that most of the effects of CPR were polysynaptic (86%; $n = 32$). Of 150 pedal neurons examined (five preparations), 36% of the cells were excited and 18% were inhibited when a CPR was fired (Fig. 2). We estimate that CPR may affect the activity of as many as 2000 pedal neurons. The synaptic activity evoked by CPR in the pedal ganglion was not rhythmic, suggesting that it was not evoking a locomotor program (*11*).

Most excitatory effects exerted by the CPR were on neurons in the region where neck motor neurons have been identified [sector II (*12*) in Fig. 2]. In a reduced preparation that includes muscles that move the neck, stimulation of CPR or of pedal neurons that are excited by the CPR evoked contractions of the neck muscles. Unlike contractions evoked by presumed motor neurons, the CPR-evoked contractions were bilateral and widespread and were abolished when the ganglion was perfused with a solution that blocks polysynaptic transmission (*13*), suggesting that the CPR has no direct actions on the muscle.

To determine whether the CPR is associated with feeding circuitry we examined its actions on various neurons concerned with the consummatory aspects of feeding. Firing of the CPR evoked activity of cerebral-buccal interneuron CBI-2 (Fig. 1B), a putative command element that drives biting-like movements of the buccal mass (*14*). The resulting firing of CBI-2 was not of high enough frequency to evoke biting, but the depolarization might function to prime the neuron and enhance its responses to food stimuli. Activity of CPR also resulted in depolarization of other cerebral-buccal interneurons (*15*), including the MCC (Fig. 1B), a neuron that modulates the rate and intensity of biting (*16*).

CPR also polysynaptically modulated neurons in the abdominal ganglion and affected the cardiovascular system. Firing of CPR increased heart rate by 10 to 20%; it excited the cardiovascular command neuron L10 (*17*) and the modulatory heart motor neuron RB_{HE} (Fig. 1C). Finally, CPR firing produced a brief inhibition followed by excitation of vasoconstrictor neuron LB_{VC},

Center for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, New York, NY 10032.