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Identification of the phosphorylated β-tubulin isotype in differentiated neuroblastoma cells

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The tubulin molecule consists of an α- and a β-subunit, each of which exists in several isotypic forms. It has been previously shown that one of the isotypes of neuroblastoma β-tubulin is phosphorylated at a serine residue in vivo [(1985) J. Cell Biol. 100, 764–774]. Here we identify the phosphorylated isotype as β_2 (type III). Moreover, the large size of the phosphorylated tryptic peptide and sequence comparisons of vertebrate β-tubulins suggest that one of the two serines in positions 444 and 446 is the phosphorylated residue. Our results raise the possibility that β_2-tubulin differs functionally from the other β-tubulin isotypes.

β-Tubulin; Tubulin isotype; Tubulin phosphorylation; Neuroblastoma tubulin

1. INTRODUCTION

Microtubules are cylindrical organelles involved in a variety of cellular processes, such as mitosis, transport and ciliary and flagellar motility [1]. The precise mechanisms by which microtubule assembly is regulated in vivo are unknown. One possible mechanism is phosphorylation, a widespread regulator of enzyme function. Cytoskeletal proteins, including the microtubule-associated proteins (MAPs) are known to be highly phosphorylated in vivo, but little is known about the phosphorylation of tubulin, the building block subunit of microtubules [2–4]. The tubulin molecule (100 kDa) is a heterodimer of two 50 kDa subunits, designated α and β [5]. Phosphorylation of β-tubulin in vivo was first noted by Eipper [6] using rat brain slices incubated in organ culture in the presence of [^{32}P]orthophosphate. Eipper [7] observed that the phosphorylation was in the C-terminal tryptic peptide. Later, Gard and Kirschner [8] observed that an isotype of β-tubulin in neuroblastoma cells was phosphorylated in vivo when the cells were induced to differentiate in the presence of [^{32}P]orthophosphate. The sequences of the various isotypes of β-tubulin are now known and it appears likely that there may be functionally significant differences between them [9–12]. Gard and Kirschner [8] did not, however, determine which of the isotypes of β-tubulin is phosphorylated, nor in which part of the protein it is phosphorylated. More recently, Serrano et al. [13] repeated the experiments of Gard and Kirschner [8] and located the radioactive serine in the C-terminal tryptic peptide; by hydrolyzing that peptide with CNBr and analyzing the resulting fragments, they tentatively identified the radioactive serine as that in position 423 [13]. Serrano et al. [13], however, also indicated that if the phosphorylated isotype was the neuronal specific β-tubulin isotype (type III) then the labeled residue could be much closer to the C-terminus [13]. This tubulin isotype has unique serines near the C-terminus [9] and using their approach they would not have been able to rule out the possibility of these residues being

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phosphorylated if indeed type III tubulin is the phosphorylated isotype.

We took advantage of the fact that the type III isotype, which we previously designated β_2, has a markedly lower electrophoretic mobility after carboxymethylation than any of the other vertebrate β-isotypes [14–17]; we incubated neuroblastoma cells in vivo with [^{32}P]orthophosphate and found that the β_2-isotype of tubulin is the one that becomes phosphorylated. Moreover, the site of phosphorylation is probably one of the two serines near the C-terminus of β_2. Since the C-terminal region of β-tubulin is known to be the site where the MAPs bind and since β_2 lacks the MAP-binding site [18], our results raise the possibility that tubulin isotypes in the brain may have their assembly regulated in different fashions.

2. MATERIALS AND METHODS

GTP and Na iodoacetate were from Serva (Heidelberg, FRG). Affinity purified trypsin was the kind gift of Dr K.D. Jany [19]. Sephadex G-50 and Sephadex G-10 were from Pharmacia (Uppsala, Sweden). [^{32}P]Orthophosphate (5 mCi/ml) was from Amersham (Buchler, Braunschweig, FRG). Dulbecco's minimal essential medium was from Biochrom (Berlin). Nitrocellulose sheets were from Schleicher and Schuell (Dassel, FRG) and X-omat AR X-ray film was from Eastman Kodak (Rochester, NY).

Twice-cycled pig brain microtubule protein (C_2S) was the kind gift of Dr Karl Doenges and Joachim Buchholz. It was prepared at a protein concentration of 20 mg/ml in 0.1 M Pipes, pH 6.9, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA and stored at −80°C. After being thawed, the C_2S was made 1 mM in GTP and incubated at 37°C to allow microtubules to polymerize. The microtubules were centrifuged at 20000 × g (in a 50 Ti rotor) for 80 min at 30°C. The resulting pellets, each one containing 12 mg of protein, were stored at −72°C until use. During an experiment where neuroblastoma tubulin was to be used, a pig brain microtubule pellet was resuspended in 0.7 ml of a buffer (PB) consisting of 0.1 M Mes, pH 6.55, 0.5 mM $MgCl_2$, 2 mM ethyleneglycol-bis-(β-aminoethyl ether), N,N'-tetraacetic acid, 0.1 mM EDTA, 1 mM ATP, 1 mM β-mercaptoethanol, 1 mM GTP, 10 mM NaF, 10 μg/ml pepstatin A, 10 μg/ml o-phenanthroline, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine-HCl. The suspension was then incubated at 0°C for 30–60 min and was then centrifuged at 160000 × g for 5 min at 4°C in a Beckman airfuge. The resulting supernatant, designated C_3S, was then mixed with the neuroblastoma protein as described below.

Neuroblastoma 2A cells (CCL 131) obtained from the American Type Culture Collection were maintained in DMEM with 10% fetal calf serum. For some experiments, the cells were transferred into five 75 ml flasks and were allowed to grow for three days in the presence of DMEM and 2% dimethylsulfoxide, but no fetal calf serum. They were then placed in DMEM, lacking both dimethylsulfoxide and fetal calf serum. After 4 days, the cells were placed in medium identical to the previous one, but lacking phosphate. For other experiments, the cells were grown for 9 days in DMEM containing 0.2% fetal calf serum and 2% dimethylsulfoxide and then transferred into medium lacking fetal calf serum and dimethylsulfoxide for 7 days prior to transfer to phosphate-free medium. Both protocols led to considerable neurite outgrowth, similar to that seen by Gard and Kirschner [8] (fig.1). 1 mC [^{32}P]orthophosphate was then added to each bottle. After 20 h of incubation, the cells were harvested and processed according to Gard and Kirschner [8]. The neuroblastoma cell extract (about 0.2 ml) was passed by centrifugation through two 2 ml columns of Sephadex G-10 and was then mixed with 0.3 ml of pig brain microtubule protein (C_3S, 9 mg/ml). The mixture was then incubated at 37°C for 30 min and then layered on top of PB containing 50% sucrose in an airfuge tube and centrifuged at 160000 × g for 20 min at 25°C. The pellets were then reduced and carboxymethylated [20] and subjected to electrophoresis on 5.5% polyacrylamide gels containing 1% Na dodecyl sulfate [21]. For preparative gel electrophoresis, the sample was made 5% in trichloroacetic acid at 0°C and then centrifuged to remove any residual free [^{32}P]orthophosphate. The pellet was resuspended in 5% trichloroacetic acid and centrifuged again and then resuspended in the Laemmli gel sample buffer and subjected to electrophoresis on a 5.5% polyacrylamide slab. The α, β_1, and β_2 bands were visualized in 4 M Na acetate, extracted from the gel, dialyzed extensively against water and lyophilized [22].

The β_2-tubulin was mixed with previously purified β-tubulin (0.4 mg/ml) in 0.05 M NH_4HCO_3. To this was added 20 μg of trypsin and the digestion carried out for 7 h at 37°C. The digest was then lyophilized and dissolved in 200 μl of 8 M urea, 0.1 M NH_4HCO_3 and applied to a column of Sephadex G-50. The fractions were pooled as shown in fig.3 and the individual pools were subjected to chromatography on a column of Sephadex G-10 equilibrated in 0.1 M NH_4HCO_3 to remove the urea. Samples from this column were lyophilized.

Protein concentrations were determined by the method of Lowry et al. [23] as modified by Schacterle and Pollack [24], using bovine serum albumin as a standard.

Gels were dried on a slab gel dryer. Peptides were dissolved in water and spotted on nitrocellulose.

3. RESULTS AND DISCUSSION

Neuroblastoma cells that were induced to differentiate developed an extensive network of neurites (fig.1). The cells were incubated with [^{32}P]orthophosphate and a cell extract was prepared as described above. Microtubule protein from pig brain that had been mixed and copolymerized with ^{32}P-labeled neuroblastoma cell extract was reduced and carboxymethylated and subjected to electrophoresis on polyacrylamide gels. Autoradiography of the gels (fig.2) revealed the presence of radioactivity that co-migrated with

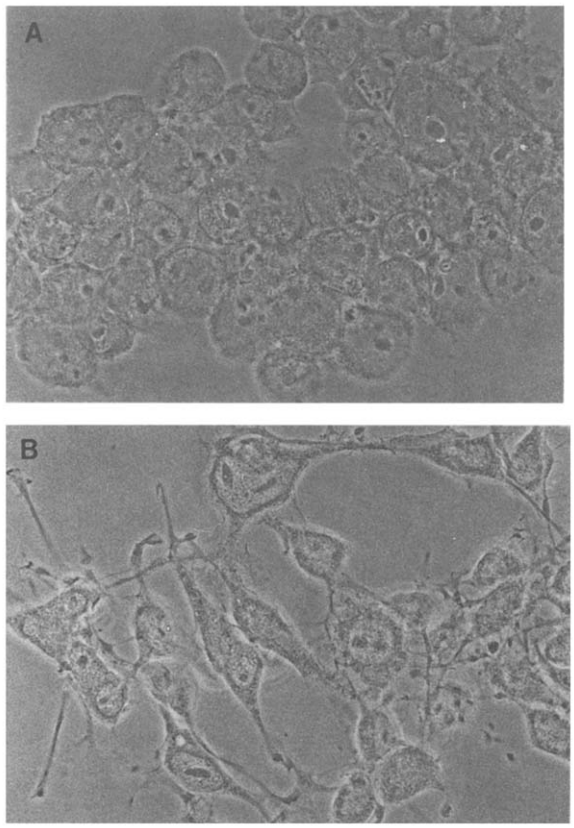

Fig.1. Micrograph of neuroblastoma cells. The figure shows two phase-contrast micrographs of neuroblastoma cells in a non-differentiated state (A) and in a differentiated state after being incubated in the absence of fetal calf serum (B). (× 700).

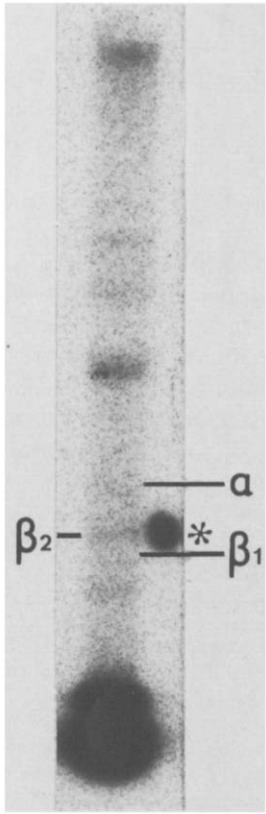

Fig.2. Incorporation of ^{32}P into neuroblastoma proteins. Neuroblastoma cells were incubated with [^{32}P]orthophosphate and the microtubule proteins prepared as described in section 2. The microtubule protein was electrophoresed on a 5.5% polyacrylamide gel slab [18]. The gel was stained with Coomassie blue and dried on filter paper. An autoradiograph of the dried gel is shown. Prior to autoradiography, spots of radioactive ink (*) were applied to the filter paper. One of the spots was next to the β_2-band. The positions of the α-, β_1- and β_2-bands are shown.

the β_2-band. There was also radioactivity at other positions on the gel, but very little label co-migrated with either the α- or the β_1-bands. The positions of the non-tubulin labeled bands roughly matched those seen by Gard and Kirschner [8].

Another portion of the radioactive material was subjected to electrophoresis and the β_2-band was visualized using 4 M Na acetate and sliced from the gel. The protein was extracted from the gel slice and digested with trypsin. The tryptic digest was chromatographed on a column of Sephadex G-50 and the elution profile is shown in fig.3. The fractions were pooled in four groups as shown on the figure. Each pool was then run on a column of Sephadex G-10 to remove the urea. Portions of each pool were spotted on nitrocellulose and subjected to autoradiography (fig.3, inset). The approximate relative amounts of each pool that were spotted on nitrocellulose and shown in fig.3 were as follows (based on protein determination of the fractions eluted from the G-10 column and expressed relative to pool I): pool I, 1.0; pool II, 2.6; pool III, 3.0; pool IV, 0.7. Only pool I was found to contain any label. An autoradiogram, developed under identical conditions, of a nitrocellulose sheet spotted with double the amounts of pools II–IV showed no radioactivity in these peaks. The only peptide to elute at the position of pool I at the time when β-tubulin from pig brain was sequenced was the particularly large C-

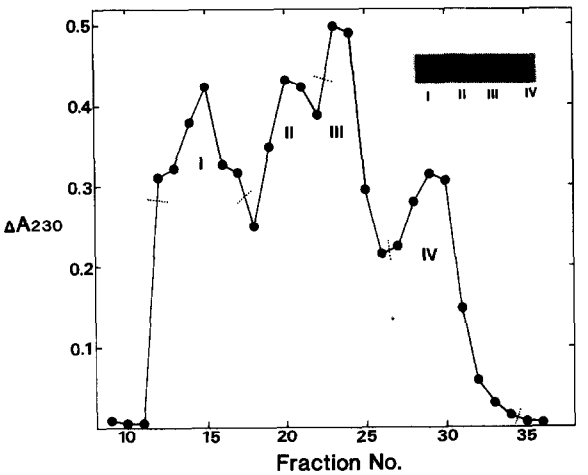

Fig.3. Profile of the tryptic digest of β_2-tubulin. The mixture of ^{32}P-labeled neuroblastoma and carrier bovine tubulin was digested with trypsin and the resulting peptides subjected to chromatography on Sephadex G-50. The figure shows the A_{230} of the fractions. The fractions were grouped, as indicated on the figure, into pools I, II, III and IV. Samples from each pool in relative amounts of (I) 1.0, (II) 2.6, (III) 3.0 and (IV) 0.7 were spotted on a nitrocellulose sheet which was then subjected to autoradiography (inset).

terminal tryptic peptide [25]. Our evidence therefore suggests that the label may be located in the C-terminal tryptic peptide.

Gard and Kirschner [8] showed that when neuroblastoma cells are incubated with [^{32}P]orthophosphate, the phosphate label is attached to a serine residue in a β-tubulin isotype. Our evidence suggests that the label is in the C-terminal tryptic peptide of the β_2-tubulin isotype. By sequencing β_2-specific peptides it was possible to identify this isotype as corresponding to the type III β-tubulin in the classification scheme of Sullivan and Cleveland [9,14].

Table 1 shows the published sequences of the vertebrate β-tubulins C-terminal to arginine 380. It is noteworthy that two serines, those at positions 413 and 420, are common to all of the β-isotypes found in the brain. Similarly, the sequences in the vicinity of these serines are highly conserved. It is therefore highly unlikely that a protein kinase that phosphorylates serines would phosphorylate these serines in the type III β-tubulin isotype and not in the others. It is much more likely that a protein kinase would phosphorylate only those serines that are unique to type III. These serines are the ones at positions 444 in chickens and humans and at

Table 1
C-terminal sequences of mammalian and avian β-tubulins

	380	390	400	410	420	430	440
Type I[a]	RISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQDATAEEEED*FGEEAEEEAX						
Type II (β_1)[b]						D QGE* E G	DEAX
Type III (β_2)[c]						GEMYEDDE	SEQGAKX
Type III (β_2)[d]						GEMYEDDE	SESQGPKX
Type IV[e]						*GE* E	VAX
Type IV[f]						GE* E	VAX
Type V[g]	S	F			E	NDG EA EDDE	INEX
Type VI[h]	V S			S G T		DV **Y	ASPEKETX
cβ3[i]						GE* E	EX
Mβ1[j]	V H S	R V S		IS G DIH	F	VR GL DS*EEDA	AEVEAEDKDHX

[a] Constitutive; sequenced in chicks, mice and humans [9,26]
[b] Major neuronal; sequenced in chicks and pigs [9]
[c] Minor neuronal; sequenced in chicks [9]
[d] Minor neuronal; sequenced in humans [9]
[e] Brain-specific; sequenced in humans and mice [9]
[f] Constituve; sequenced in humans and mice [9]
[g] Ubiquitous, except brain; sequenced in chicks [9]
[h] Thrombocytes and erythrocytes; sequenced in chicks [27]
[i] Testes; sequenced in chicks [9]. Recently classified as a form of type IV [26]
[j] Bone marrow; sequenced in mice [10]

The table gives the C-terminal sequences of the known isotypes of β-tubulin. Sequences start at position 380; except for the sequence of type I, only those residues are indicated which occupy positions where the sequence differs from that of type I. The serines (S) are underlined

position 446 in humans. If the phosphorylation of β_2-tubulin has a general physiological significance, then the likeliest of these two serine residues to be phosphorylated is serine 444 since that is common to the known β_2(type III)-tubulins (table 1). It is interesting that the sequence at positions 434–440 present in types II and IV but not in type III has been shown to be the site of interaction with the microtubule-associated proteins tau and MAP 2 [18]. The C-terminal sequence of the type III isotype differs from other β-isotypes at positions 436, 438, 439 and 440 [9]. It is possible that the type III isotype does not interact as well with tau and MAP 2 as do the other isotypes. It is conceivable, therefore, that the type III β-tubulin has its assembly and/or function regulated by phosphorylation while the other isotypes are regulated by microtubule-associated proteins.

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