Dephosphorylation suppresses the activity of neurofilament to promote tubulin polymerization

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The phosphate content of neurofilament was diminished by half, from 49 4 to 22 9 nmol/mg, by treatment with alkaline phosphatase Dephosphorylation decreased the activity of neurofilament to promote tubulin assembly This suppression was supposed to be mainly due to dephosphorylation of the 200 kDa subunit of neurofilament Dephosphorylation of the isolated 200 kDa subunit caused suppression of its activity to promote tubulin polymerization These results suggest that changes in the phosphate content modulate interaction of neurofilament with tubulin

Neurofilament *Tubulin* *Tubulin polymerization* *Alkaline phosphatase* *Dephosphorylation* *Phosphate content*

1. INTRODUCTION

It has been revealed that neurofilament stimulates tubulin assembly and then causes gelation in vitro [1,2] This ability of neurofilament was found to reside in the protruding portion of the 200 kDa subunit [2,3] Therefore, it is speculated that the projection of the 200 kDa subunit cross-bridges between neurofilament and microtubule. The 200 kDa subunit contains a number of phosphorylation sites [4–6] and these phosphate groups are located on the projecting domain of the 200 kDa subunit [7]. It is very intriguing whether phosphorylation/dephosphorylation alters the activity of the 200 kDa subunit to stimulate polymerization of tubulin. This paper describes that the activity of neurofilament to promote tubulin assembly is reduced by dephosphorylation. The suppression appears to be mainly due to dephosphorylation of the 200 kDa subunit.

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Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride

2 MATERIALS AND METHODS

2.1 *Preparation of proteins*

Microtubule proteins were purified from porcine brain [1] according to Shelanski et al. [8]. Tubulin was separated from microtubule proteins [1] by the procedure of Weingarten et al. [9]. Neurofilament was prepared from porcine brain [10] and then purified by hydroxyapatite column chromatography [11] as described [1–3]. The 200 kDa subunit was separated from two other subunits [2] by the method of Geisler and Weber [12]

2.2. *Dephosphorylation*

Neurofilament or the 200 kDa subunit was incubated with alkaline phosphatase (grade I, Boehringer) in the reaction medium [120 mM Tris·HCl (pH 8.5), 1 mM $MgSO_4$, 1 mM PMSF, 10 μg/ml leupeptin] at 30°C for 15 h. For the control experiment, 5 mM sodium phosphate, 1 mM 1,10-phenanthroline and 2 mM EDTA were added in order to prevent the dephosphorylation reaction.

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After this treatment, alkaline phosphatase was removed from neurofilament by washing it with centrifugation or from the 200 kDa subunit through gel filtration (Sepharose CL-4B, Pharmacia).

2.3. *Phosphate determination*

Neurofilament or the 200 kDa subunit was precipitated with and washed in trichloroacetic acid by the method of Stull and Buss [13]. The precipitate was ashed after addition of 10% $Mg(NO_3)_2 \cdot 6H_2O$ in ethanol, solubilized in 0.1 N HCl and boiled for 15 min [14]. This solution was mixed with the ascorbate-molybdate mixture, incubated at 20°C for 45 min and then measured at 820 nm [14,15].

2.4. *Protein determination*

Protein concentration was determined by the method of Lowry et al. [16] using bovine serum albumin as a standard

2.5. *SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was performed on 7.5% slab gels according to the method of Laemmli [17]. Gels were stained with Coomassie brilliant blue.

3. RESULTS AND DISCUSSION

Alkaline phosphatase was efficient in removing phosphate groups from neurofilament. The phosphate content was reduced by half (table 1) The dephosphorylation treatment affected the electrophoretic mobility of the 200 and 150 kDa subunits of neurofilament. However, the electrophoretic mobility of the 70 kDa subunit did not change after this treatment (fig.1a and b) The same phenomenon has been reported by Julien and Mushynski [5]. It appeared that these alterations of the electrophoretic mobility were not due to proteolysis but to conformational changes of the 200 and 150 kDa subunit molecules by removal of phosphate groups. This is supported by the finding that the molecular masses of the 200 and 150 kDa subunits are overestimated in SDS-polyacrylamide gel electrophoresis [18] In other words, dephosphorylation may cause a highly extended form of the 200 and 150 kDa subunit molecules to be folded to some extent. This may alter the configuration of the protein in SDS, giving rise to an apparent reduction of each molecular mass.

Table 1

Phosphate content of neurofilament and the 200 kDa subunit

Preparation	Phosphate content (nmol/mg)
Neurofilament	
Non-dephosphorylated	49 4
Dephosphorylated	22 9
200 kDa subunit	
Non-dephosphorylated	44 5
Dephosphorylated	3 3

The dephosphorylation treatment explicitly in-

Fig 1 SDS-polyacrylamide gel electrophoresis of neurofilament (a,b) and the 200 kDa subunit (c,d) a and c, non-dephosphorylated; b and d, dephosphorylated

fluenced the activity of neurofilament to promote tubulin polymerization, causing network formation (fig.2). The dephosphorylated neurofilament lost potency to a great extent in comparison with the non-dephosphorylated one. This suppression of promoting activity was ascribed to dephosphorylation of the 200 kDa subunit, since the 200 kDa subunit was previously shown to be responsible for stimulation of tubulin assembly and since it was effectively dephosphorylated as shown in fig.1b

Therefore, we examined dephosphorylation of the isolated 200 kDa subunit to verify this assumption. The treatment with alkaline phosphatase removed almost all of the phosphate groups from the 200 kDa subunit (table 1). The apparent molecular mass of the 200 kDa subunit was also lowered by dephosphorylation (fig.1c and d). The non-dephosphorylated 200 kDa subunit showed a

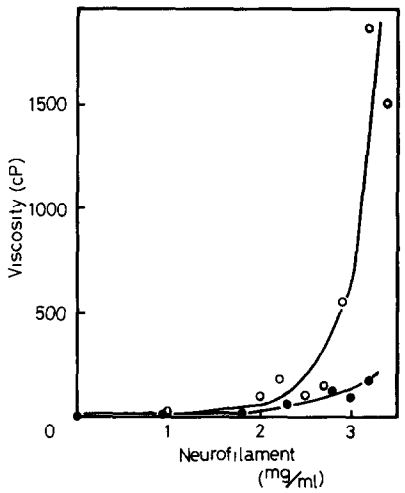

Fig 2 Inhibition of cross-linking between neurofilaments and microtubules by dephosphorylation of neurofilament measured by low-shear viscometry Varying amount of non-dephosphorylated (○) or dephosphorylated neurofilament (●) was combined with tubulin (3 3 mg/ml) and incubated at 34°C for 30 min Then low-shear viscosity was measured by a falling ball apparatus The reaction medium contained 84 mM Pipes (pH 6 7), 1 4 mM EGTA, 0 99 mM GTP and 3.1 mM $MgSO_4$ GTP-regenerating reagents (1 4 mM phospho*enol*pyruvate and 71 μg/ml pyruvate kinase) were further added to the reaction mixture to avoid exhaustion of GTP by alkaline phosphate that slightly contaminates the neurofilament preparation

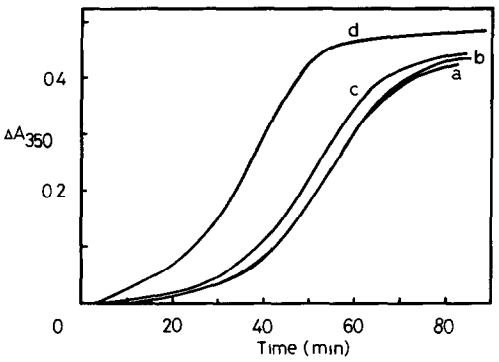

Fig 3 Inhibition of the 200 kDa subunit-induced promotion of tubulin polymerization by dephosphorylation of the 200 kDa subunit a, tubulin (3 2 mg/ml) alone, b and c, dephosphorylated 200 kDa subunit (0 38 and 0 75 mg/ml, respectively) was added; d, non-dephosphorylated 200 kDa subunit (0 34 mg/ml) was added The reaction medium contained 85 mM Pipes (pH 6 7), 1 6 mM EGTA, 1 0 mM GTP and 3 1 mM $MgSO_4$ GTP-regenerating reagents (1 1 mM phospho*enol*pyruvate and 53 μg/ml pyruvate kinase) were further added to the reaction mixture

significant acceleration of the initial rate of tubulin polymerization and a slight enhancement of assembly at steady state (fig.3, cf. a and d). On the other hand, the dephosphorylated subunit scarcely exhibited either acceleration or enhancement of assembly (fig.3, cf a with b and c). Hence, we conclude that the dephosphorylation of neurofilament causes neurofilament-dependent assembly of microtubules to sequester and that this is mainly due to the removal of phosphate groups from the 200 kDa subunit. Moreover, the speculation may be made that the conformation of the projecting domain of the 200 kDa subunit is swayed by phosphorylation/dephosphorylation, thereby causing modulation of the activity to promote tubulin assembly. Sternberger and Sternberger [19] have also documented the possibility that phosphorylation may hold the key to the function of neurofilament in vivo

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