Effects of microtubule-associated proteins on network formation by neurofilament-induced polymerization of tubulin

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It has been revealed that neurofilaments stimulate polymerization of tubulin and thereby cause gelation. Addition of a very small amount of MAPs to the reaction mixture of tubulin and neurofilaments resulted in promotion of gelation. This could not be ascribed to MAP-induced cross-linking between microtubules and neurofilaments because further increases in the MAP concentration (still substoichiometric amount) resulted in total suppression of gelation. It is concluded that MAPs promote microtubule assembly independently of neurofilaments, and lower the concentration of tubulin available for neurofilament-induced polymerization, then preventing network formation.

Neurofilament *Tubulin* *Tubulin polymerization* *Gelation* *Network formation* *Microtubule-associated protein*

1. INTRODUCTION

Electron microscopic observations of the squid giant axon revealed that microtubules and neurofilaments in the axon run parallel to the longitudinal axis of the axon with cross-bridges formed by thin filaments [1–3]. Mammalian neurons also contain an axoplasmic matrix composed of microtubules and neurofilaments [4–9]. Runge et al. [10] have reported that a complex between microtubules and neurofilaments is formed in vitro. We also found that the neurofilament preparation obtained from porcine brain stimulates tubulin polymerization, thereby causing gelation [11]. These findings held true with the highly purified neurofilaments and the ability to promote tubulin assembly was revealed to reside in the 200 kDa subunit of neurofilament proteins [12]. Moreover the exteriorly projecting portion of the 200 kDa subunit was supposed to be responsible for this ability [13], and dephosphorylation of the 200 kDa subunit suppressed the activity [14].

On the other hand, Leterrier et al. [15] and Aamodt and Williams [16] have described that MAPs bound to neurofilaments promote microtubule assembly and that a viscous complex of microtubules and neurofilaments is formed.

Here we further present evidence that purified neurofilaments induce polymerization of tubulin, thereby causing gel formation. Only a small amount of exogenous MAPs stimulated gel formation but an increase in the amount of MAPs (still substoichiometric amount) inhibited gelation.

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Abbreviations: MAPs, microtubule-associated proteins; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PC-MAPs, phosphocellulose-prepared MAPs; h-MAPs, heat-resistant MAPs

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2. MATERIALS AND METHODS

2.1. *Preparation of proteins*

Microtubule proteins were purified from porcine brain by 3 cycles of temperature-dependent assembly-disassembly [11] according to Shelanski et al. [17]. Tubulin was purified from these microtubule proteins by phosphocellulose column chromatography [11] following the procedure of Weingarten et al. [18]. MAPs were then eluted from the tubulin-depleted phosphocellulose with a buffer solution containing 0.8 M KCl. The peak fraction containing MAPs was concentrated in the presence of 0.1 M β-mercaptoethanol with Aquacide IIA and then dialyzed against PM solution (100 mM Pipes, 2 mM EGTA and 1 mM $MgSO_4$ at pH 6.7). h-MAPs [19] were prepared from microtubule proteins as described [11]. Neurofilaments were prepared from porcine brain as described by Runge et al. [20] with slight modifications [11]. Though these partially purified neurofilaments contained tubulin corresponding to about 30% of the total protein, very few, it any, MAPs were detected in this preparation [14]. Further purification was performed by hydroxyapatite column chromatography in the presence of 6 M urea [12] following Liem [21].

2.2. *Falling ball assay*

Low-shear viscometry was performed in a falling ball device [22] as described in our previous paper [11]. When the reaction mixture was too viscous for a ball to fall through a capillary, it was designated as 'gel'.

2.3. *Protein determination*

Protein concentration was determined by the method of Lowry et al. [23] using bovine serum albumin as a standard.

2.4. *SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was carried out on 7.5% slab gels according to Laemmli [24]. Gels were stained with Coomassie brilliant blue and then scanned.

3. RESULTS AND DISCUSSION

3.1. *Inhibition of gelation by PC-MAPs*

PC-MAPs suppressed gelation of a mixture of tubulin and partially purified neurofilaments (fig.1a and b). The ratio of PC-MAPs to tubulin was about 0.18, which was almost equal to that for the 3-cycled microtubule proteins. In contrast, once tubulin and neurofilaments had formed a gel, PC-MAPs could no longer exhibit the ability to inhibit gelation (fig.1d). The inhibitory effect of PC-MAPs on gel formation has also been reported for h-MAPs [11]. There was no possibility that MAPs cross-linked microtubules and neurofilaments. This was reinforced by the following experiments (fig.1c). Microtubules pre-assembled from tubulin and PC-MAPs were not efficient for the gelling reaction when mixed with partially purified neurofilaments (fig.1c). Even though MAPs could associate with neurofilaments the network was not as strong as a gel as measured by low-shear viscometry. The results in fig.1 indicate that mere coexistence of MAPs-associated microtubules and neurofilaments cannot account for the formation of the highly viscous gel. It could be inferred that MAPs prevent a mixture of tubulin and neurofilaments from forming a gel by causing

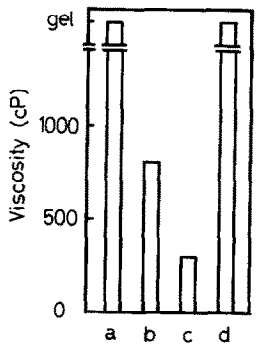

Fig.1. Inhibitory effect of PC-MAPs on gelation. Tubulin and partially purified neurofilaments were combined in the presence (b) or absence (a) of PC-MAPs, incubated at 34°C for 20 min, and then drawn up into falling ball apparatuses. Partially purified neurofilaments and a mixture of tubulin and PC-MAPs (c) or PC-MAPs and a mixture of tubulin and partially purified neurofilaments (d) were separately incubated at 34°C for 20 min, respectively, combined, and then drawn up into viscometers. Each measurement (a–d) was performed after a further 10 min incubation. The final reaction mixture contained 2.8 mg/ml tubulin, 2.4 mg/ml partially purified neurofilaments, 0.5 mg/ml PC-MAPs, 100 mM Pipes (pH 6.7), 1.7 mM EGTA, 0.6 mM GTP and 3.1 mM Mg^{2+}.

tubulin to polymerize independently of neurofilaments. Although neurofilaments appeared to be less competent to induce polymerization of tubulin than MAPs, the inhibitory effect of MAPs was cancelled by elevating the concentration of neurofilaments (cf. fig.4).

3.2. *Effects of h-MAPs on gelation*

It has already been reported that neurofilaments highly purified by hydroxyapatite column chromatography retain the ability to promote tubulin polymerization and give rise to gelation, and this ability was revealed to reside in the 200 kDa subunit of neurofilaments [12]. Fig.2 shows that the purified neurofilament preparation contained only triplet subunits. Here, it should be noted that the activity of neurofilaments to stimulate tubulin polymerization was totally suppressed when the neurofilament preparation was first treated with phosphatase to be dephosphorylated [14]. Because dephosphorylation of MAPs would enhance the nucleating ability for tubulin assembly [25,26], if MAPs contaminated the purified neurofilament preparation and promoted the gelling reaction, dephosphorylation reaction should have occurred in MAPs as well and thus caused promotion of gelation. However, this was not the case. This result suggested that it could not be anything other than neurofilaments that induced assembly of tubulin and thereby caused gelation. Although we concluded that MAPs were not required for the gelling reaction, we then further investigated the effect of varying concentration of h-MAPs on gelation of tubulin and neurofilaments (fig.3). When tubulin was present at 2.8 mg/ml, addition of 5.0 mg/ml purified neurofilaments resulted in an increase in viscosity only up to 1030 cP (closed circles). Under the same conditions except for the presence of 0.04 mg/ml h-MAPs, 4.0 mg/ml neurofilaments caused gelation (open circles). If this were due to MAP-induced cross-linking between neurofilaments and microtubules, increase in the concentration of MAPs should have further enhanced gelation. However, this did not occur. In contrast, when the amount of h-MAPs was slightly increased to 0.10 mg/ml, the critical gelling concentration of neurofilaments increased to 4.5 mg/ml (fig.3, crosses). Even this amount of h-MAPs corresponds to one-eightieth of that of tubulin on a molar basis, which is quite insufficient to occupy each binding site on microtubules and neurofilaments in contrast to the argumentation by Aamodt and Williams [16]. Data are not available at present to elucidate the role of a very small amount of h-MAPs on the stimulation of gelation. It may be possible that the small amount of MAPs serves to reinforce neurofilament-induced tubulin assembly so that it decreases the critical concentration of neurofila-

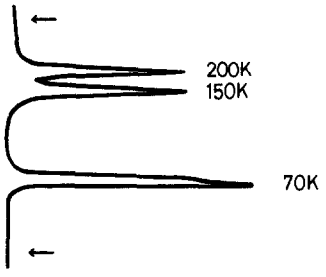

Fig.2. Densitometric scan of SDS-polyacrylamide gel (7.5%) of purified neurofilament proteins (200, 150 and 70 kDa). The upper and lower arrows indicate the top of the gel and the dye front, respectively.

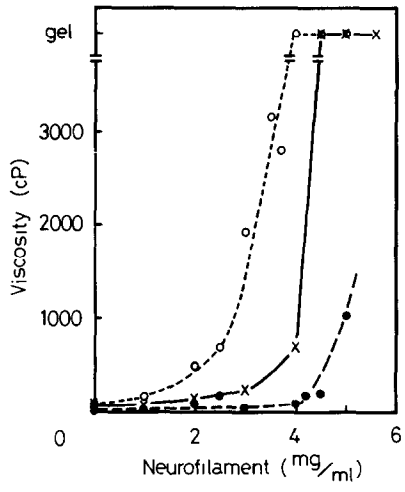

Fig.3. Effects of h-MAPs on gelation of a mixture of tubulin and neurofilaments. Varying amounts of neurofilaments were combined with mixtures of tubulin (2.8 mg/ml) and h-MAPs [(○) 0, (●) 0.04, (×) 0.1 mg/ml]. The reaction medium contained 85 mM Pipes (pH 6.7), 1.7 mM EGTA, 0.52 mM GTP and 3.2 mM Mg^{2+}. After incubation at 34°C for 40 min, low-shear viscosities were measured.

ments necessary for gelation (cf. section 3.3). The results in the next section summarize the action of MAPs on the gelling reaction.

3.3. *Biphasic effects of h-MAPs on gelation*

A mixture of 2.8 mg/ml tubulin and 2.0 mg/ml neurofilaments freshly prepared exhibited very low viscosity, but the addition of a small amount of h-MAPs (0.04–0.07 mg/ml) gave rise to gelation (fig.4, closed circles). Further addition of h-MAPs, on the contrary, decreased the viscosities as their amounts increased. The optimal concentration range of h-MAPs for stimulating gelation was extended to both the lower and higher concentrations when the amount of neurofilaments was elevated (fig.4, crosses). Eventually, 2.8 mg/ml tubulin and 4.1 mg/ml neurofilaments could cause gelation without the assistance of h-MAPs (fig.4, open circles).

A tubulin:MAP (MAP-2) stoichiometry of 12:1 was deduced first by Amos [27] on the basis of ultrastructural studies of reassembled brain microtubules. This was later confirmed by Burns and Islam [28] using 4-cycled microtubule proteins from chick brains. In the present conditions for stimulation of gelation by MAPs, 0.2 μM MAPs was sufficient for gelation of 28 μM tubulin and 2.0 mg/ml neurofilaments. This corresponded to one-tenth the stoichiometry between tubulin and MAPs. When the concentration of MAPs increased to 1 μM, which was still half the stoichiometric amount, gelation was totally inhibited.

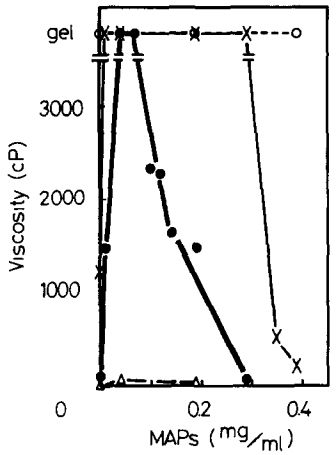

Fig.4. Biphasic effects of h-MAPs on gelation. Varying amounts of h-MAPs were added to the reaction mixture of tubulin (2.8 mg/ml) and neurofilaments [(△) 0, (●) 2.0, (×) 3.2, (○) 4.1 mg/ml]. The medium conditions were the same as shown in fig.3.

From these results, it was assumed that neurofilaments at a low concentration were insufficient for the formation of gels with tubulin below a certain concentration. In fact, the lower the concentration of neurofilaments, the larger was the amount of tubulin required for gelation. This was consistent with the result that when the concentration of neurofilaments was low, a smaller amount of h-MAPs was sufficient to inhibit gelation.

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