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Inhibition of Microtubule Assembly by HPC-1/Syntaxin 1A, An Exocytosis Relating Protein

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ABSTRACT. **HPC-1/syntaxin 1A (HPC-1), which has been identified as a presynaptic membrane protein, is believed to regulate the synaptic exocytosis as a component of t-SNARE. The distribution of the protein, however, is not restricted to the synaptic terminal, but it has been found to locate on the axonal membrane. When the expression of HPC-1 was suppressed, neurite sprouting was enhanced in cultured neurons. These findings suggest that HPC-1 possesses other functions than the regulation of the membrane fusion in neurotransmitter release. Rather it may also participate in the morphogenesis of neurons through membrane fusion, and possibly through cytoskeleton. HPC-1 has a sequence resemble to the assembly promoting sequence of heat stable MAPs in residues 89–106, suggesting that it can bind tubulin and be involved in microtubule system. Thus, both the tubulin binding property and the effect on microtubule assembly of HPC-1 were examined *in vitro* using a mutated HPC-1 lacking the C-terminal transmembrane region (HPC-ΔTM), which was overexpressed in *E. coli*.**

Affinity column chromatography showed that tubulin was found to bind HPC-1 directly. Synthetic peptide which corresponds to the residues 89–106 competitively inhibited the tubulin-HPC-1 binding, indicating that the sequence is responsible for the tubulin binding. In addition, chemical cross-linking with EDC revealed that one HPC-1 molecule can bind per one monomeric tubulin molecule. Light scattering measurement of microtubule polymerization showed that HPC-1 decreased the rate of the pure tubulin polymerization. Direct observation of single microtubules under dark-field microscopy showed that the growth rate of microtubule decreased by HPC-1. After shortening stopped, microtubules often spent attenuate phases, in which neither growing nor shortening was detected. When another mutant HPC-1 which is composed of residues 1–97 and lacks tubulin binding activity was used, however, the suppression of microtubule polymerization was not observed. These results suggest that HPC-1 is a potent regulator of microtubule polymerization, which directly bind tubulin subunit and decrease the polymerization activity.

Key words: tubulin/HPC-1/syntaxin 1A/cross-linking/dark-field microscopy

Introduction

Microtubule is a cylindrical polymer of α and β tubulin heterodimers, which is common to most eukaryotic cells and is involved in many cellular processes, for example, chromosome segregation and determination of cell polarity (5, 8). One of the most prominent features of cytoplasmic microtubules is their dynamic behavior (3). Various components called microtubule-associated proteins (MAPs) have been identified to be regulators of microtubule assembly (8). Those contain classical MAP1, MAP2, and tau in neurons, and MAP4 in non-neuronal cells. All of them possess a common feature that favors microtubule polymerization by promoting microtubule nucleation or by stabilizing polymers. Recently, however, a variety of factors that are unfavorable to microtubule polymerization have been discovered in various types of cell (1, 7, 11, 15, 20, 22). A member of kinesin family has also been identified as the

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Abbreviations: EDC, 1-ethyl-3-(3-dimethylamonopropyl)carbodiimide; MAPs, microtubule-associated proteins; MBP, maltose binding protein.

microtubule-destabilizing factor (4) which preferentially targets to the microtubule end. So far, regulatory systems of microtubules within cells became to resemble to the well-established actin system since either contains monomer-sequestering factor, polymer-severing factor, and so on. Moreover, several transmembrane proteins have been reported to interact microtubules directly via their cytoplasmic regions (12, 24), indicating that molecular basis of the stable interaction between membrane and microtubule now become to subject for investigation.

HPC-1/syntaxin 1A, which is a 35 kDa protein with 288 amino acids (Fig. 1a), was originally found as a neuronal plasma-membrane protein (9). It belongs to the syntaxin protein family, which mediates intercellular membrane transport in many types of cells (2). This molecule could bind many other "exocytosis-relating proteins" such as synaptobrevin, SNAP-25 (23 kDa synaptosomal-associated protein) and synaptotagmin, and is considered to be involved in the neurotransmitter release as a component of SNAREs (23). However, its precise function is still obscure since it was distributed widely throughout on the axonal plasma membrane as well as the presynaptic region (13). The exocytotic processes in various cells were suppressed by increasing its expression, and were enhanced by decreasing its amount with anti-HPC-1 injection (14, 16, 17). These results indicate that HPC-1 would play an inhibitory role in the exocytotic process. In addition, when the expression of HPC-1 was suppressed by antisense oligonucleotide against HPC-1 mRNA, neuronal sprouting was highly enhanced in cultured dorsal root ganglion neurons (26). This result suggests that HPC-1 participates in the morphogenesis of neuronal cells presumably by controlling the cytoskeletal systems as well as by suppressing membrane fusion. At the N-terminal residues 89–106, HPC-1 has a sequence which resembles to an assembly-promoting motif of heat stable MAPs (Fig. 1a), suggesting that the target of HPC-1 could be microtubules (6). In this article, we describe a direct interaction of HPC-1 and tubulin, and show its inhibitory effects on microtubule polymerization *in vitro*.

(a)

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MKDRTQELRT AKDSDDDDDV TVTVDRDRFM DEFFEQVEEI  40
RGFIDKIAEN VEEVKRKHSA ILASPNPDEK TKEELEELMS  80
DIKKTANKVR SKLKSIEQSI EQEEGLNRSS ADLRIRKTQH 120
STLSRKFVEV MSEYNATQSD YRERCKGRIQ RQLEITGRTT 160
TSEELEDMLE SGNPAIFASG IIMDSSISKQ ALSEIETRHS 200
EIIKLENSIR ELHDMFMDMA MLVESQGEMI DRIEYNVEHA 240
VDYVERAVSD TKKAVKYQSK ARRKKIMIII CCVILGIIIA 280
STIGGIFG                                    288
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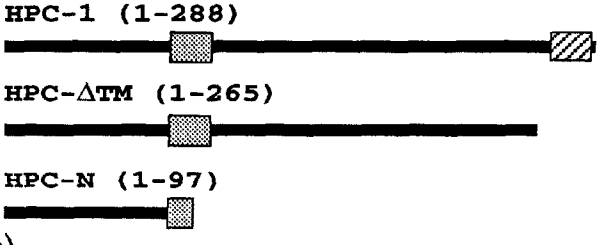
(b)



(c)

pep-1: 89**VRSKLKSIEQSIEQEEGL**106

pep-2: 69**EKTKEELEELMSDIKKTAN**87

pep-3: 44**IDKIAENVEEVKRKH**58

Fig. 1. (a) Amino acid Sequence of HPC-1/syntaxin 1A. Sequence that resembles to the assembly promoting repeat in heat stable MAPs (89–106) is underlined. (b) Schematic illustration of the primary structures of HPC-1 and its mutants used in the present study. Sequence that resembles to the assembly promoting repeat in each construct is shown by meshed box. The putative transmembrane region is shown by hatched box in HPC-1. (c) Synthetic peptides for competition assay.

Binding between tubulin and HPC-1

HPC-1 is a transmembrane protein with a putative transmembrane region at the C-terminal end (Fig. 1b), and the N-terminal bulk is located in cytoplasm. To examine the role of the cytoplasmic N-terminal region of this protein, a deletion mutant was constructed using site directed mutagenesis. This mutant lacked C-terminal transmembrane region, and was named HPC-ΔTM (Fig. 1b). The mutated cDNA was subcloned into pMAL-p2 vector (New England Biolab, Beverly, MA, USA). The recombinant was overexpressed in *E. coli* expression system as a fusion protein with maltose binding protein (MBP).

The binding between tubulin and the recombinant was analyzed by affinity column using amylose resin. When purified tubulin alone was applied to the amylose resin column without recombinant protein, no tubulin was detected in fraction obtained by 10 mM maltose. With HPC-ΔTM, tubulin was recovered in the fraction eluted by 10 mM maltose. This result clearly demonstrated that tubulin bound recombinant HPC-1 *in vitro*. To determine which region in HPC-ΔTM was responsible to the tubulin binding, three polypeptides were synthesized as in Figure 1c (6). Among them, only pep-1, which corresponds to the residues 89–106, competitively inhibited by about 60%. The other peptides (pep-2 and pep-3) did not affect the tubulin binding. In addition, the inhibition of tubulin-binding by pep-1 was dose-dependent. From these results, we can conclude that HPC-1 bound tubulin at the residues 89–106, which resemble to the assembly-promoting se-

quence in heat stable MAPs.

The mode of binding was examined in detail using a chemical cross-linker. In this case, MBP was proteolytically removed from the recombinant by Factor Xa, which selectively cleave between the MBP and the mutant protein. The apparent molecular weight of HPC-ΔTM in SDS-PAGE was 23 kDa (Fig. 2a). When HPC-ΔTM alone was treated with 5 mM of 1-ethyl-3-(3-dimethylamonopropyl)carbodiimide (EDC), a "zero-length cross-linker," at 27°C for one hour, two major bands reactive to the anti-HPC-1 antibody appeared at 37 kDa and 58 kDa instead of a 23 kDa band as shown in Figure 2 (lane 4). In the case of tubulin alone, a band corresponds to the tubulin dimer was detected by CBB staining (Fig. 2, lane 2). When the mixture of 15 μM tubulin and 2 μM HPC-ΔTM was subjected to the cross-linking, new band reactive to the anti-HPC-1 antibody appeared between tubulin monomer and tubulin dimer (Fig. 2, lane 6). In this case, the density of lowest band which appeared in lane 4 was reduced. The newly appeared band with apparent molecular weight of 72 kDa was also detected by anti-tubulin antibody. Since its apparent molecular mass is almost same to a sum of that of tubulin monomer and that of HPC-ΔTM, it is possible that the minimum ratio of the tubulin-HPC-ΔTM complex is 1:1. The bands derived from HPC-ΔTM tended to be invisible by CBB after EDC treatment. This is perhaps due to the decrease in the affinity with CBB dye by the chemical modifications.

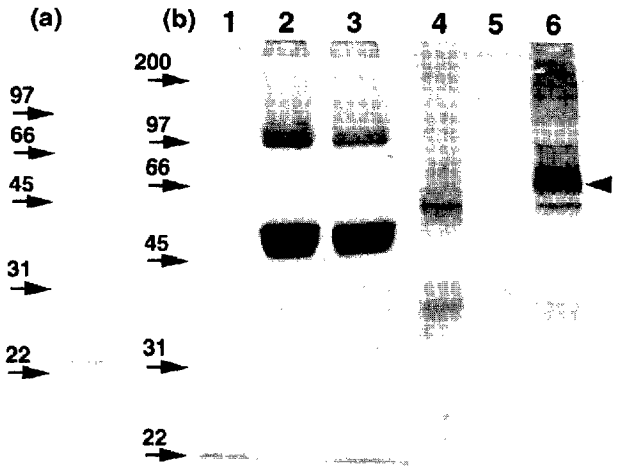


Fig. 2. (a) Purified HPC-ΔTM. The calculated molecular mass of HPC-ΔTM from the sequence is 28 kDa, but it migrates at 23 kDa in SDS-PAGE (12.5% gel). Both the antibodies against N-terminal and C-terminal of HPC-1 similarly recognized this band, so that the apparent molecular mass is not due to the proteolytic digestion during the preparation. (b) EDC-cross-linking of tubulin to HPC-ΔTM. Samples were treated with 5 mM EDC for 1 hr at 27°C. The products were separated on a 10% polyacrylamide gel and stained with CBB (lane 1–3). For the immunological analysis, samples were transferred to PVDF membrane after separating with SDS-PAGE. The membrane was then probed mono-clonal antibody against HPC-1 (lane 4–6). Lane 1 and 4, 2 μM HPC-ΔTM; lane 2 and 5, 15 μM tubulin; lane 3 and 6, 2 μM HPC-ΔTM and 15 μM tubulin. Arrowhead indicates a band derived the tubulin-HPC-ΔTM complex. Arrows in (a) and (b) indicate positions of molecular weight marker.

Inhibition of microtubule polymerization of HPC-ΔTM

The effect of HPC-1 on the microtubule polymerization was first examined by measuring the increase in light scattering at 350 nm. When 1.6 μM HPC-ΔTM was added into crude brain tubulin fraction, which contained high amount of MAPs, no change occurred in the profile of the increase in light scattering. However, when 20 μM of pure tubulin, which was purified by DEAE-sepharose column chromatography, was mixed with various amounts of HPC-ΔTM before polymerization, the initial rate of increase in light scattering decreased depending on the HPC-ΔTM concentrations as shown in Figure 3. After prolonged incubation at 37°C, the mixtures of tubulin and various concentrations of HPC-ΔTM were subjected to ultracentrifugation to measure the amount of polymer at steady state. No obvious decrease in the amount of tubulin obtained in pellet was detected by increasing HPC-ΔTM concentration. These results indicate that HPC-ΔTM suppress the rate of tubulin polymerization without affecting the final extent of polymer.

Individual microtubules were observed under dark-field microscopy to analyze the effects of HPC-ΔTM on microtubule dynamics. The mixture of 20 μM tubulin and HPC-ΔTM was incubated at 37°C for 5 min to allow the spontaneous polymerization of microtubules.

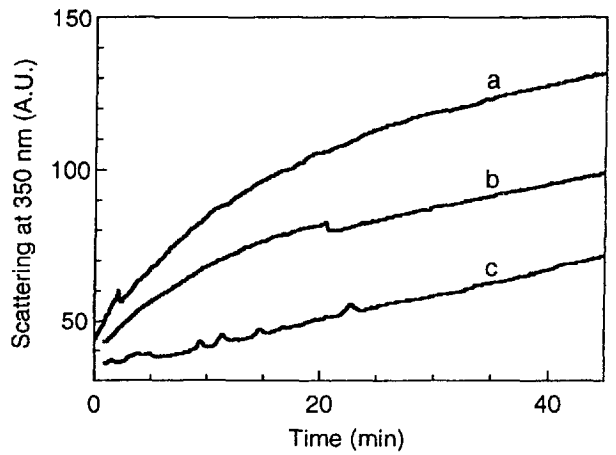


Fig. 3. Microtubule assembly in the presence of various concentrations of HPC-ΔTM. Assemblies were carried out with 20 μM tubulin at 37°C and followed by light scattering measurement at 350 nm. The concentrations of HPC-ΔTM were 0 μM (a), 0.9 μM (b), or 1.6 μM (c), respectively.

Then, 5 μl of sample was observed as described before (10). In this condition, several numbers of microtubules could be observed in the absence of HPC-ΔTM as seen in Figure 4a. In the presence of 0.7 μM HPC-ΔTM, number of microtubules decreased slightly as in Figure 4b. These observations correspond to the results obtained by the light scattering measurement. In these conditions, ends of microtubules were easily distinguished, so that the length changes in both ends of microtubules were examined. Figure 5a shows a profile of the length change of a microtubule in the absence of HPC-ΔTM. This microtubule shows a typical dynamic instability, alternating growing phases and rapidly shortening phases. At an end that grew faster than the other end, phase transitions occurred more frequently than the other one. Throughout present observation, we assumed more dynamic end, at which microtubule grow faster, was plus end. In the presence of 0.7 μM of HPC-ΔTM, however, growth rates in individual growing phase of more active end of the microtubule decreased as in Figure 5b. The growth rates in less active ends of microtubules were also slow, and as an extreme case, no obvious length change was observed as in the lower trace in Figure 5b. In addition, microtubule spent an attenuate phase, in which neither growing nor shortening was detected, between a shortening phase and the subsequent growing phase as indicated by arrow in the figure. Sometimes, the length of the attenuated phase was more than two min. In contrast, the profiles of the length change in microtubules were almost same to the control in the presence of another mutant HPC-1, HPC-N, which consisted of residues 1–97 and lacked tubulin binding capacity. In this case, no

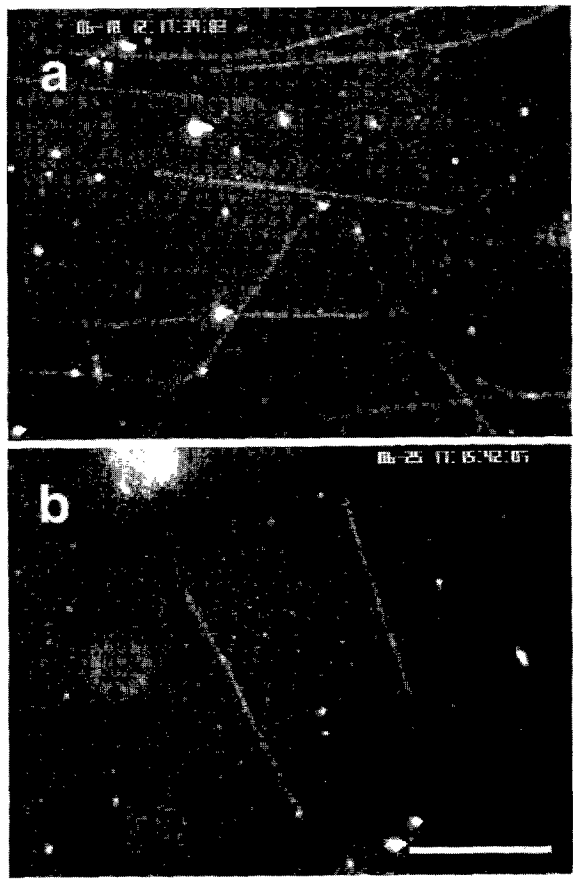


Fig. 4. Microtubules observed under dark-field microscopy. 20 μM tubulin was incubated at 37°C for five min in the absence of HPC-ΔTM (a) or in the presence of 0.7 μM HPC-ΔTM (b). Scale bar = 10 μm.

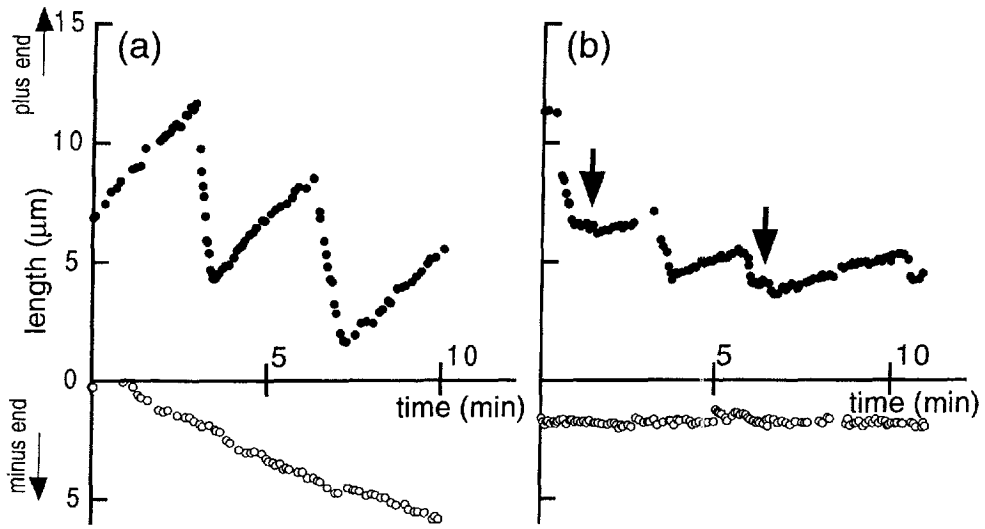


Fig. 5. Changes in length at both the plus and minus ends of single microtubules without HPC-ΔTM (a), or with 0.7 μM of HPC-ΔTM (b). The concentration of tubulin was 20 μM. Filled and open circles show the plus and minus ends of the microtubule.

suppression of microtubule growth was detected up to 7.7 μM (data not shown). These results suggest that binding of HPC-ΔTM decreased polymerization activity of tubulin molecules.

There are two possibilities to explain the molecular basis of HPC-1 dependent suppression of microtubule growth. i) HPC-ΔTM may sequester available tubulin subunit as stathmin (11). ii) Tubulin-HPC-ΔTM complex may inhibit the addition of tubulin competitively at growing microtubule end. In the former case, tubulin could lose polymerizing activity upon HPC-ΔTM binding, resulting in the decrease in the total amount of polymerizable subunits. Supposing that all HPC-ΔTM formed the complex in the present condition, the decrease of tubulin pool was only 0.7 μM out of 20 μM. Simple decrease in tubulin concentration does not result in the irregular profile of microtubule growth as observed in the presence of HPC-ΔTM since only the growth rate of microtubule slightly decreased at lower tubulin concentrations (data not shown). In addition, cross-linking data suggests that all HPC-ΔTM were not available to form the complex, so that the practical amount of the tubulin-HPC-ΔTM complex could be much lower than 0.7 μM. Taken together, tubulin-sequestering is not likely to explain the HPC-ΔTM dependent suppression of microtubule growth. Rather, we believe the second possibility is more conceivable. When small amount of complex of tubulin and mitotic drug such as colchicine were added into tubulin solution, similar attention phases were often observed (18, 19). Although there is difference in the frequency of catastrophe between colchicine and HPC-ΔTM, attenuation in dynamic microtubule may be similarly resulted by the disturbance of the end dependent addition of subunits.

Present data suggest that HPC-1 is a novel membrane protein that inhibits microtubule polymerization. Because HPC-1 is established as the negative regulator of membrane fusion at the plasma membrane, it is likely that this protein is multi-functional for the regulation of cell morphology. If HPC-1 also suppresses the microtubule growth *in vivo*, it would be a candidate of a regulator which restricts the presence of microtubules near the plama membrane of neuronal processes. Indeed, density of microtubules is high around the central axis, but gradually decreased toward the periphery in neuronal processes (21, 25). When the expression of HPC-1 is suppressed by means of antisense oligodeoxynucleotide (26), it could be possible that the restriction of assembly of microtubules near the plasma membrane is disrupted. The enhancement of sprouting might be caused by a combined effect of the enhanced membrane fusion at plasma membrane and the uncontrolled microtubule assembly.

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