Septin 6 localizes to microtubules in neuronal dendrites

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Abstract In neuronal dendrites, septins localize to the base of the spine, a unique position which is sandwiched between the microtubule (MT)-rich dendritic shaft and the actin filament-rich spine. Here, we provide evidence for the association of SEPT6 with MTs in cultured rat hippocampal neurons. In normal cultures, SEPT6 clusters localized to MTs, but not to actin clusters. Only MT-disrupting agents (vincristine and nocodazole), but not microfilament-disrupting one (latrunculin A), induced the redistribution of SEPT6 to the disrupted MTs. The nascent MT fibers that were recovered from vincristine or nocodazole treatments also accompanied SEPT6. Blocking MT disruption by Taxol prevented such phenomena, proving that the redistribution of SEPT6 was due to the MT disruption. Our results indicate that SEPT6 complexes at the base of the dendritic spine are associated with MTs.

Keywords Microtubule · Neuron · Septin 6 · Spine · Vincristine

Abbreviations

DIV	Days in vitro
IR	Immunoreactivity
MT	Microtubule
PBS	Phosphate-buffered saline
RT	Room temperature
SEPT	Septin

Introduction

Septins are conserved GTP-binding proteins that assemble into hetero-oligomeric higher-order structures such as filaments and rings. Originally characterized as regulators of cytokinesis in the budding yeast *Saccharomyces cerevisiae* (Hartwell 1971), septins were then found in all fungal and animal cells, but not in plants or slime *Dictyostelium* (Field and Kellogg 1999; Longtine et al. 1996). Septins are usually localized underneath a discrete region of the plasma membrane and function as a cell scaffold or diffusion barrier to effect cytokinesis, cell polarity, and many other functions (recently reviewed in Oh and Bi 2011). In yeast, septins polymerize to form a highly-ordered ring (septin ring) at the incipient site of bud growth (Byers and Goetsch 1976; Longtine et al. 1996; Oh and Bi 2011). The septin ring at the mother-bud neck serves as (1) a scaffold to recruit selective proteins at the site (Douglas et al. 2005), and (2) as a diffusion barrier across the plane of the mother-bud neck (Caudron and Barral, 2009). As protein scaffolds

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and diffusion barriers, septins have conserved their key roles in cell polarity and septation (Gladfelter et al. 2001; Kinoshita 2006; Longtine and Bi 2003).

Developing neuronal dendrites are highly decorated with filopodia, long, thin, headless protrusions. As a neuron matures, the filopodia develop into either new branches attaining a highly complex dendritic tree or spines, which serves as excitatory input sites (Fiala et al. 1998; Tada and Sheng 2006; Ziv and Smith 1996). Despite the continuity of the membrane, the spines are independent subcellular compartments that are insulated from the rest of the cell (Ashby et al. 2006). This in turn suggests the presence of a diffusion barrier at the neck of spines, reminiscent of the yeast bud neck. Indeed, recent reports have shown that septin complexes are positioned at the base of dendritic branch points and filopodia of developing neurons and at the spines of mature neurons [SEPT6 (Cho et al. 2011); SEPT7 (Li et al. 2009; Tada et al. 2007; Xie et al. 2007)]. Furthermore, high-resolution confocal microscopy revealed ring-like structures of SEPT6 (Cho et al. 2011) and SEPT7 (Tada et al. 2007) complexes at the spine neck. Since septins are required for spine maturation and proper spine morphogenesis (Tada et al. 2007; Xie et al. 2007), they may play a critical role in spinogenesis, and dendritic branching as well.

Microtubules (MTs) are dynamic polar structures with different polymerization rates at their two ends; the faster growing end is referred to as the plus (+) end and the slower growing end as the minus (−) end (Allen and Borisy 1974). In the dendrites of mature neurons, MTs are predominantly present as bundles in dendritic shafts, whereas actin filaments are predominantly concentrated in spines (Landis and Reese 1983; Matus et al. 1982). Recent reports, however, have shown that MT (+) ends grow into spines transiently (Gu et al. 2008; Hu et al. 2008; Jaworski et al. 2008). Therefore, localization of septins to the base of the spine is unique in that its position is sandwiched between the MT-rich dendritic shaft and the actin-rich spine. It is an interesting question to ask whether the septins at the spine neck is associated with MTs or actin cytoskeletons, and the answer to this question would shed light on their functions. To answer this question we performed disruption assays where MT or actin filaments were specifically dismantled. Here, we show that SEPT6 redistributes following MTs, but not actin filaments, during their

dismantling and recovery, indicating that the SEPT6 complexes at the base of spines interacts with MT.

Materials and methods

Antibodies

The following antibodies were used at the indicated dilutions: MAb pan-actin (1:1,000; Sigma); MAb tubulin α -subunit (1:2,000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA); and rabbit polyclonal affinity-pure SEPT6 (SEPT6; 1:1,000; Cho et al. 2011).

Cell culture

Hippocampi from Sprague–Dawley rat pups at embryonic day 18 (E18) or E19 were dissected, dissociated by trypsin treatment and mechanical trituration. Cells were plated onto 12-mm diameter polylysine/laminin-coated glass coverslips in neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA), as described (Brewer et al. 1993; Cho et al. 2011; Goslin et al. 1998). Latrunculin A (5 μ M; Calbiochem Company, La Jolla, CA, USA; now Merck Chemicals International, Darmstadt, Germany), vincristine (5 μ M; Sigma, St. Louis, MO, USA), Taxol (10 μ M, Sigma), and nocodazole (30 μ M, Sigma) were added directly to the culture medium from concentrated DMSO (for Latrunculin, nocodazole, Taxol) or methanol (for vincristine) stocks.

Immunocytochemistry

On 21 days in vitro (DIV), coverslips were rinsed briefly in Dulbecco's phosphate-buffered saline (D–PBS; Invitrogen) and fixed by a sequential paraformaldehyde/methanol fixation procedure [incubation in 4 % paraformaldehyde in PBS (20 mM sodium phosphate buffer, pH 7.4, 0.9 % NaCl) at room temperature (RT) for 10 min followed by incubation in methanol at −20 °C for 20 min] (Moon et al. 2007). Immunocytochemistry was performed with indicated primary and secondary antibodies [Alexa Fluor 488-conjugated goat anti-mouse and Alexa Fluor 568-conjugated goat anti-rabbit IgG (each diluted 1:1,000; Molecular Probes, Inc., Eugene, OR, USA)] as described (Moon et al. 2007).

Laser-scanning confocal microscopy

Confocal images ($1,024 \times 1,024$ pixels) were acquired using a Leica TCS SP2 confocal system with laser lines at 488 and 543 nm. Digital images were processed with the use of Adobe Systems Photoshop 5.0 software.

Analysis

A singlet SEPT6 puncta or a rod-shaped cluster (probably by stacking of singles) was considered to be one cluster. Co-localization was defined as the contact of each cluster regardless of their extent of overlap. Co-localized SEPT6 clusters in certain areas, typically in $10 \mu\text{m} \times 10 \mu\text{m}$, were counted, and expressed in the % of total SEPT6 clusters (mean $\pm$ SD). Data were analyzed using a one-way analysis of variance (ANOVA) with post hoc Duncan multiple comparison, with significant difference at $p < 0.05$ (SPSS software, version 16.0).

Results and discussion

Disruption of actin filaments does not change the distribution profile of SEPT6

Septins can associate with either actomyosin or MT fibers. Since F-actin cytoskeletons are highly enriched in the dendritic spine (Landis and Reese 1983; Matus et al. 1982), there is a possibility that SEPT6 is associated with actin filaments in the spine neck. To test this possibility, we first investigated the co-localization of SEPT6 with actin filaments. For this purpose, rat hippocampal dissociated cultures (DIV 21) grown in normal conditions were double-labeled with anti-SEPT6 and anti-pan-actin antibodies. Both SEPT6- and actin-immunoreactive (IR) signals formed clusters along neuronal dendrites (Fig. 1a). As we previously reported (Cho et al. 2011), the SEPT6 clusters were frequently ($\sim 80\%$) positioned adjacent to those of actin (arrowheads in Fig. 1a, merge inset). However, the two kinds of clusters usually did not co-localize with each other. Statistical analysis showed that only $22.6 \pm 9.9\%$ ($n = 143$) of total SEPT6 clusters co-localized with actin clusters (Fig. 1d, control).

Fig. 1 Disruption of actin filaments does not change the distribution profile of SEPT6. Confocal microscopic images showing dissociated rat hippocampal cultures (DIV 21), which were double-labeled with anti-SEPT6 and anti-pan actin antibodies. **a** Control culture. **b** Latrunculin A-treated ($5 \mu\text{M}$, 24 h) culture. **c** Recovered culture. Cultures were treated with Latrunculin A and recovered for 24 h in the control medium. The boxed area of a dendrite (box) was enlarged and shown at the bottom of each panel. Actin clusters and SEPT6 punctae are marked with arrowheads to show their relative positions neighboring each other. Note that there is no apparent change in the distribution profile of SEPT6 among different conditions. Scale bar $15 \mu\text{m}$. **d** Statistics. The percentage of SEPT6 punctae that co-localize with MT was analyzed. The numbers of SEPT6 punctae counted [$n = 143$ (control), 112 (latrunculin), 196 (lat/recovered)]. $**p < 0.01$; $*p < 0.05$

Next, we conducted disruption experiments to examine whether or not actin filament disruption would affect the SEPT6 distribution. We reasoned that if one protein is associated with a certain filament, the distribution profile of the protein would be altered when the fiber is disrupted. The F-actin cytoskeleton in mature primary cultures from embryonic rat hippocampus can be disrupted by latrunculin A reversibly and separately from MT (Allison et al. 2000; Spector

et al. 1983). Treatment of latrunculin A (5 μ M, 24 h) significantly reduced the actin-IR signal intensity in the dendritic shafts (Fig. 1b, actin). However, there was still a significant amount of actin-IR clusters which did not disappear even after 48 h of treatment. These remaining actin clusters were located at the periphery of dendritic shafts and frequently neighbored SEPT6 clusters (arrowheads in the merge inset of Fig. 1b). This change in the actin-IR profile indicates that the actin microfilaments in the dendritic shaft were dismantled, although those actin clusters at the spines still survived the latrunculin treatment. Despite the significant disruption of actin microfilaments, the general distribution profile of SEPT6 was not changed (Fig. 1b, SEPT6). The co-localization rate of SEPT6 and actin was very significantly ($p < 0.01$) decreased to $10.4 \pm 4.6 \%$ ($n = 112$) (Fig. 1d, latrunculin). This effect was mainly due to the loss of actin clusters, especially those in the dendritic shafts, by the latrunculin A treatment. When the latrunculin A-treated cells were incubated in the normal medium for 24 h, the actin-IR signal intensity in general was increased (Fig. 1c, actin), indicating the partial recovery of actin filaments. Despite this recovery, there was no apparent change in the general distribution profile of SEPT6 (Fig. 1c, SEPT6). The co-localization rate of SEPT6 and actin was significantly ($p < 0.05$), increased back to $17.8 \pm 10.4 \%$ ($n = 196$) (Fig. 1d, lat/recovered), which was statistically similar to the control cultures. This increase was mainly due to the portions of increase at the dendritic shafts. These results show that the distribution of SEPT6 is not affected by the disruption of actin filaments and strongly suggest that SEPT6 is not associated with actin filaments in neuronal dendrites.

SEPT6 locates on MT fibers and follows them after their disruption

As we recently reported (Cho et al. 2011) and showed in Fig. 2a, SEPT6 clusters are located on MT fibers of neuronal dendrites. This positional relationship suggests the association of SEPT6 with MTs. To prove this assumption we conducted disruption and recovery experiments for MT fibers. First, we employed MT disruption using vincristine that binds to tubulin dimmers. When bound to vincristine, microtubule subunits are unable to assemble into normal microtubules but may associate to form intracytoplasmic

paracrystalline aggregates of microtubular protein (Weber et al. 1975). Since vincristine successfully depolymerizes MTs without associated toxicity in cultured hippocampal neurons (Allison et al. 2000), we treated dissociated rat hippocampal cultures (DIV 21) with vincristine (5 μ M, 4 h). As reported before (Allison et al. 2000; Weber et al. 1975), treatment of vincristine disrupted MT fibers and resulted in the formation of paracrystals (Fig. 2b, Tub). In contrast to the disruption of actin cytoskeletons, the disruption of MTs induced changes in the profile of SEPT6 distribution: as shown in Fig. 2b (SEPT6), SEPT6-IR signals formed short and occasionally extended rods (Fig. 2b, SEPT6; arrows). This profile is a change from the round punctate clusters in control cultures. Interestingly, the SEPT6 rods were frequently located in close apposition to MT paracrystals (arrowheads in Fig. 2b and its merge inset) or overlapping each other (Fig. 2b, arrowhead in the merge inset). Statistical analysis showed that $53.2 \pm 18.7 \%$ ($n = 119$) of the SEPT6-IR rods co-localized with MT paracrystals (Fig. 2d, vincristine), indicating that SEPT6 follows MTs. This decrease in the colocalization rate was very significant ($p < 0.01$) from the control culture. The reasons might be either the disruption of MT leaving the SEPT6 punctae alone or the paracrystallization of MT interrupting its association with SEPT6 or both.

Next, we used another MT disrupting agent, called nocodazole (30 μ M, 2 h). Nocodazole has been shown to reversibly inhibit the assembly of tubulin from rat brain (De Brabander et al. 1977). As reported previously (Allison et al. 2000), nocodazole did not efficiently disrupt neuronal MTs (Fig. 2c, Tub). However, partial disruption was evident by the decrease in the density of MT fibers, which was manifested by spaces between fibers (Fig. 2c, Tub, inset). Disruption of MTs by nocodazole also induced a change in the profile of SEPT6 distribution: there were often SEPT6 clusters that are considerably larger than control clusters (Fig. 2c, SEPT6). Interestingly, these large SEPT6 clusters, and the small ones as well, were positioned on the MT fibers (Fig. 2c, merge). Statistical analysis showed that most (>99 %) SEPT6 clusters were co-localized with MT fibers (Fig. 2d, nocodazole), indicating that the redistributed SEPT6 followed MTs. This colocalization rate was very significantly ($p < 0.01$) different from the vincristine-treated culture but comparable to the control culture. This

Fig. 2 Disruption of MT fibers changes the distribution profile of SEPT6. Confocal microscopic images showing rat hippocampal dissociated cultures (DIV 21), which were double-labeled with anti-SEPT6 and anti-tubulin (Tub). **a** Control culture. **b** Vincristine treated (5 μ M, 4 h) culture. **c** Nocodazole treated (30 μ M, 2 h) culture. The boxed area of a dendrite was enlarged and shown at the bottom of each panel. Localization of SEPT6 punctae on MT fibers (**a**, **c**) or on tubulin paracrystals (**b**) are marked with arrowheads. Note that SEPT6 was changed into rod-shaped (arrows in **b**) or big clusters (arrowheads in **c**) in the presence of vincristine and nocodazole, respectively, but that they are still co-localized with MT fibers or paracrystals (merge images in **b** and **c**). Scale bar 15 μ m. **d** Statistics. The percentage of SEPT6 punctae that co-localize with MT was analyzed. The numbers of SEPT6 punctae counted [$n = 153$ (control), 119 (vincristine), 146 (nocodazole)]. $**p < 0.01$

may be due to the less disruption of MT by nocodazole than by the vincristine.

Blocking of MT disruption by Taxol prevents changes of SEPT6 distribution profiles

So far, we have shown that SEPT6 co-localizes with MTs in normal conditions or after their disruption by vincristine or nocodazole. We interpreted this

phenomenon as SEPT6's accompany with MTs due to their physical associations. However, there is a possibility that vincristine and nocodazole themselves affected the distribution of SEPT6 and that co-localization with MTs could be fortuitous. To eliminate this possibility we conducted blocking experiments where MT fibers were blocked by Taxol and therefore they could not be disrupted. We reasoned that, if redistribution of SEPT6 clusters was induced by the chemicals themselves, the same redistribution would occur even if MTs are intact. Pretreatment with Taxol (10 μ M, 15 min) efficiently blocked the disruption of MT by vincristine and nocodazole (Fig. 3a, b, Tub). The blocking, however, was not perfect as was indicated by the decrease in the density of MT fibers (Fig. 3a, b, Tub), being compared to control cells (Fig. 2a, Tub). Treatment of vincristine

Fig. 3 Blocking of MT disruption prevents changes in the SEPT6 distribution profile. Confocal microscopic images showing dissociated rat hippocampal cultures (DIV 21), which were double-labeled with anti-SEPT6 and anti-tubulin (Tub). The cultures were pretreated with Taxol (10 μ M, 15 min) to block MT disruption, followed by treatment with vincristine (**a**) or nocodazole (**b**). The boxed area was enlarged at the bottom of each panel. Note that SEPT6 punctae were lined up on MT fibers in preTax/vin (**a**). Due to less disruption of MT fibers in preTax/noc (**b**), SEPT6 punctae look randomly scattered. However, note that they locate to MT fibers. Statistical analysis is shown in **d**. The percentage of SEPT6 punctae that co-localize with MT was analyzed. Counted SEPT6 punctae: $n = 78$ (preTax/vin) and 142 (preTax/noc)

after the Taxol pretreatment (preTax/vin) still induced rod-shaped SEPT6 clusters (Fig. 3a, arrow). However, the frequency of the rod-shape was rarer being compared to the vincristine treatment only (compare SEPT6 panels of Figs. 2b, 3a). A more evident apparent change was the spatial distribution profile: SEPT6 clusters were linearly arranged (Fig. 3a, SEPT6). This distribution profile was very contrasting to the control cultures, where SEPT6 clusters look randomly scattered. The reason for this apparent difference was obvious: most (>99 %) SEPT6 clusters were positioned on the MT fibers (inset of Fig. 3b, merge; Fig. 3c, preTax/vinc). The sparse MT fibers, on which SEPT6 clusters are localized, thus give them a linear appearance.

Blocking of MT disruption by pretreatment with Taxol was more efficient in subsequent nocodazole treatments (preTax/noc) as was indicated by the higher density in MT fibers (Fig. 3b, Tub) than preTax/vin (Fig. 3a, Tub). The preTax/noc resulted in virtually no changes in the profile of SEPT6 distribution, although there were some clusters which showed a slightly-elongated shape (inset of Fig. 3b, SEPT6; arrow). Statistical analysis showed that virtually all SEPT6 co-localized with MTs (Fig. 3c, preTax/noc). These results strongly indicate that the redistribution of SEPT6 was not due to the chemicals themselves but to the rearrangement of MT fibers.

Recovery of MTs re-localizes SEPT6 to the nascent fibers

Next, we investigated whether or not SEPT6 would re-localize to the newly formed MT fibers after disruption and recovery processes. For this purpose, we used vincristine, which is very efficient in disrupting MT fibers. We confirmed that the treatment of vincristine (5 μ M, 4 h) resulted in complete disruption of MT fibers, inducing them to crystallize. To allow the recovery of MT fibers, the vincristine-treated cells were washed and further incubated in the normal medium. After a 24 h recovery time (vin/rec'd), cells were fixed and double-labeled with anti-Tub and anti-SEPT6. Confocal microscopic images showed that MT-IR signals were restored between crystals in dendritic shafts (arrow in Fig. 4a, Tub), and some fibers were evident (arrowheads in Fig. 4a, Tub), which were not present in unrecovered cells (Fig. 2b, Tub). The presence of MT-IR signals and fibers in

Fig. 4 Recovery from disruption re-localizes SEPT6 to the nascent MT fibers. **a** Confocal microscopic images showing dissociated rat hippocampal cultures (DIV 21), which were double-labeled with anti-SEPT6 and anti-tubulin (Tub). The cultures were treated with vincristine, washed and further incubated in the control medium for 48 h to recover MT fibers. The boxed area was enlarged at the bottom of each panel. Recovered MT-IR signals in the dendritic shaft was marked with an arrow (**a** Tub; inset), and nascent MT fibers with arrowheads (**a** Tub; inset). Note that SEPT6 punctae localize to the recovered MT fibers (merge). Scale bar 15 μ m. **b** Statistics. The percentage of SEPT6 punctae ($n = 104$) that co-localize with MT was analyzed. Data for control and vincristine (vinc) of Fig. 2 were included for comparison. $^{**}p < 0.01$

dendritic shafts indicate the partial recovery of MTs. The merge of MT- and SEPT6-IR signals showed that the nascent MT fibers accompanied SEPT6 clusters (arrowheads in Fig. 4a, merge). The co-localization rate was very significantly ($p < 0.01$) higher (84.3 ± 14.3 %, $n = 123$) (Fig. 4b, vinc/rec'd) than that of unrecovered cells (53.2 ± 18.7 %; Fig. 2b), which was, in turn, very significantly ($p < 0.01$) lower than that of control cells. The increased value was due to the fact that virtually all recovered MT fibers accompanied SEPT6. This phenomenon proves that MT and SEPT6 physically interact with each other, although it is not known whether the interaction is direct or indirect.

In this work we have shown that SEPT6 locates and follows MTs during disruption and recovery, indicating that the SEPT6 rings are associated with MTs at the base of dendritic spines. Why does SEPT6 interact with MTs in neuronal dendrites? Spines are very dynamic structures. Until recently, it is generally accepted that the remarkably dynamic actin filaments

underlie spine motility, growth, and shape (Tada and Sheng 2006), and that MTs are not considered to enter spines, nor do they play a role in spine development and dynamics. However, it is found that MTs do invade spines transiently and dynamically (Gu et al. 2008; Hu et al. 2008; Jaworski et al. 2008). Evidence suggests that this invasion plays an important role in spine development. Gu et al. (2008) reported that the MT-stabilizing agent Taxol further increased spine formation, which is elicited by the presence of brain-derived neurotrophic factor. In contrast, nocodazole, which impedes the dynamic properties of the MT (+) ends, blocked the effect of BDNF. Therefore, stabilization of the invading MT (+) ends can promote spine dynamics. The septins at the bases of dendritic filopodia and spines are ideally positioned to interact with the invading MTs and may stabilize them. Since filopodia can develop into nascent dendrite branches as well as dendritic spines (Fiala et al. 1998; Tada and Sheng 2006; Ziv and Smith 1996), it was hypothesized that septins act as a diffusion barrier at the base of the protrusion that prevents the leakage of spine and branch constituents back into the dendritic shaft (Tada et al. 2007). In addition to the role as a diffusion barrier, our results support another hypothesis that the septins at the base of dendritic filopodia may interact and stabilize the invading MT (+) ends to promote the development of dendrite branches or spines.

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