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Biochemical and cell biological characterization of a mammalian septin, Sept11

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Abstract Septins are a family of conserved cytoskeletal GTPases implicated in a variety of cellular functions such as cytokinesis and vesicle trafficking. Here, we report identification of an yet uncharacterized septin, Sept11, in septin complexes purified from porcine brain. The transcripts were detected in all tested tissues except leukocytes. A Sept11 mutant with apparently reduced GTPase activity did not form filaments in the transient expression system using COS7 cells. By Western blot analysis using a specific antibody, Sept11 was detected in various cell lines as well as brain tissues. Septin complexes immunoprecipitated from porcine brain with anti-Sept9 and anti-Sept11 antibodies were found to contain different Sept9 isoforms based on SDS-PAGE analyses followed by silver-staining and Western blotting. Immunofluorescent study revealed cell type-dependent intracellular localization of the protein; Sept11 was colocalized dominantly with microtubules and actin stress fibers in HMEC cells and REF52 cells, respectively, and their filamentous distribution was dependent on the cytoskeleton structures with which the protein is colocalized. Sept11 partially colocalized with stress fibers and microtubules in HeLa cells.

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Keywords: Septin; Sept11; Cytoskeleton

1. Introduction

Septins, a family of heteropolymeric filament-forming proteins, were originally discovered in yeast to be essential for cytokinesis and have since been identified in most eukaryotic organisms, with the exception of plants (for review see [1–5]). Septins have a 25% or closer identity over their entire length and sequence similarity is greatest in the central domain which contains guanine nucleotide (nt) interactive motifs homolo-

gous to those of ras-related small GTPases. In addition to the conserved central domain, most septins have divergent N- and C-terminal domains, many of which contain a predicted coiled-coil region possibly involved in protein–protein interaction. Although the ras-related small GTPases function as signal transducing molecular switches through GTP/GDP-exchange and GTP-hydrolysis, physiological significance of mammalian septins is largely unknown. In addition to the important roles of septins involved in cytokinesis, the accumulating biochemical and cell biological observations suggest various physiological roles of septins such as cytoskeletal polymers and scaffolds for assembly of signaling complexes (for review see [1–5]). As for mammalian cells, 12 septin genes have so far been identified mainly based on random sequencing projects and some septins represent alternative splicing forms (reviewed in [5,6]). By using mass spectrometry analyses, we here identified a septin, Sept11, in septin complexes purified from porcine brain, and carried out biochemical and cell biological characterization.

2. Materials and methods

2.1. Materials

Fresh porcine brains were purchased from local slaughterhouse. SP-Sepharose fast flow resin, MonoS (8 ml) and MonoQ (1 ml) were from Pharmacia Biotech Inc. Prepacked hydroxyapatite column (1-ml Econo-Pac CHT-II cartridge) was from Bio-Rad Inc.

2.2. Plasmid construction

Human Sept11 was produced by PCR with Marathon-Ready cDNA (human brain) (Clontech Inc.). A48Sept11, a Sept11 mutant with a point mutation (Gly48 to Ala) in the G1 box of the GTPase region, was prepared using QuickChange Site-directed Mutagenesis Kits (Stratagene Inc.). The constructs were verified by DNA sequencing. Sept11 cDNAs were subcloned into pMal, pGEX4T3, or pRK5 vector containing Myc- or Flag-tag.

2.3. Identification of proteins by mass spectrometry

Protein identification was performed by mass spectrometry combined with sequence database searches. Protein bands excised from a Coomassie blue-stained polyacrylamide gel were rinsed, reduced, S-alkylated and then incubated with trypsin. The resulting tryptic peptide mixtures were directly analyzed by LC-MS/MS.

2.4. Northern blot analysis

A human multiple tissue Northern blot (Clontech) was hybridized in accordance with the manufacturer's instructions using a [α -³²P]dCTP-labeled cDNA probe encompassing nt 2045–3795 of Sept11 sequence.

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Abbreviations: nt, nucleotide; aa, amino acid; MBP, maltose-binding protein

2.5. Preparation and characterization of antibodies and immunoaffinity isolation

Using the glutathione *S*-transferase-fused Sept11 fragment (amino acid, aa, 366–429) expressed in *E. coli* as the antigen, a rabbit polyclonal antibody specific for Sept11 (anti-Sept11-C) was produced and affinity-purified. An antibody for Sept9 (anti-Sept9, previously referred to as MSF, anti-mixed lineage leukemia septin-like fusion) was also produced and affinity-purified as described [7]. Western blot analysis was done and immunoreactive bands were visualized as described [7]. For immunoaffinity isolation, the high speed supernatant fraction of porcine brain was prepared as described [8]. Septin complexes were isolated with CNBr-activated Sepharose 4B beads coupled with anti-Sept11-C or anti-Sept9 antibodies. Proteins bound to the beads were eluted with 0.2 M glycine-HCl (pH 2.5) and analyzed by SDS-PAGE (10%) followed by silver-staining or Western blotting.

2.6. Cell culture, transfection and immunofluorescence

A variety of cell lines were cultured essentially as described [7]. In some experiments, cells were treated with 5 μ M cytochalasin B for 10 min or 10 ng/ml demecolcine for 100 min. For immunofluorescence analyses, cells were fixed in 3.7% formaldehyde, then treated with 0.2% Triton X-100. To detect Sept11, anti-Sept11-C was used as the primary antibody. To visualize Myc-tag, Flag-tag, actin or tubulin, cells were reacted with 9E10, M2 (Sigma Inc.), rhodamine-phalloidin or anti-tubulin monoclonal antibody (Sigma), respectively. Alexa 488-labeled or Cy3-labeled antibody (Molecular Probes Inc.) was used as a secondary one. When analyzing the cells, we used an Olympus LSM-GB200 confocal microscope.

2.7. Expression and purification of recombinant proteins

Sept11 and A48Sept11 were expressed in *E. coli* as maltose-binding protein (MBP)-fusion proteins and purified in accordance with the manufacturer's instructions. The recombinant proteins were released from the beads by cleavage with Factor Xa. Purity of the protein preparations was confirmed on Coomassie blue-stained SDS-polyacrylamide gels.

2.8. GTPase activity and [α - 32 P]GTP-binding assays

The GTPase activity was estimated by the liberation of 32 P_i from [γ - 32 P]GTP in the presence of 10 mM MgCl₂ and 100 μ M GTP as described [9]. For [α - 32 P]GTP-binding analysis, 1 μ M (50 pmol) recombinant Sept11 or the A48 mutant was incubated with 10 μ M [α - 32 P]GTP in the presence of 10 μ M MgCl₂ for 15 min at 30 °C as described [9]. After terminating the reaction by adding MgCl₂ to a final concentration of 10 mM, Sept11 and the A48 mutants were precipitated with anti-Sept11-C-coupled protein G agarose. After washing the beads and denaturation of the bound proteins, eluted nt were analyzed by thin layer chromatography as described [10].

3. Results and discussion

3.1. Identification of Sept11 in porcine brain septin complexes

In the course of study to elucidate the physiological significance of Sept9, which we referred to as MSF previously [7], we wanted to identify the components of septin complexes containing Sept9. For this purpose, we purified Sept9-containing septin complexes in brain tissues. The septin complexes were purified from porcine brain by successive column chromatographies using SP-Sepharose fast flow, MonoS, MonoQ and a hydroxyapatite CHT-II, as described [8]. Sept9 in eluted column fractions was monitored by Western blotting and Sept9-enriched fractions were collected at each column chromatography step. The resultant Sept9-enriched fractions are most likely to contain some physiological septin complexes even though the purified complexes may not cover all existing brain septin complexes. To identify components in the purified septin complexes, mass spectrometry of tryptic digests (LC-MS/MS analyses) was

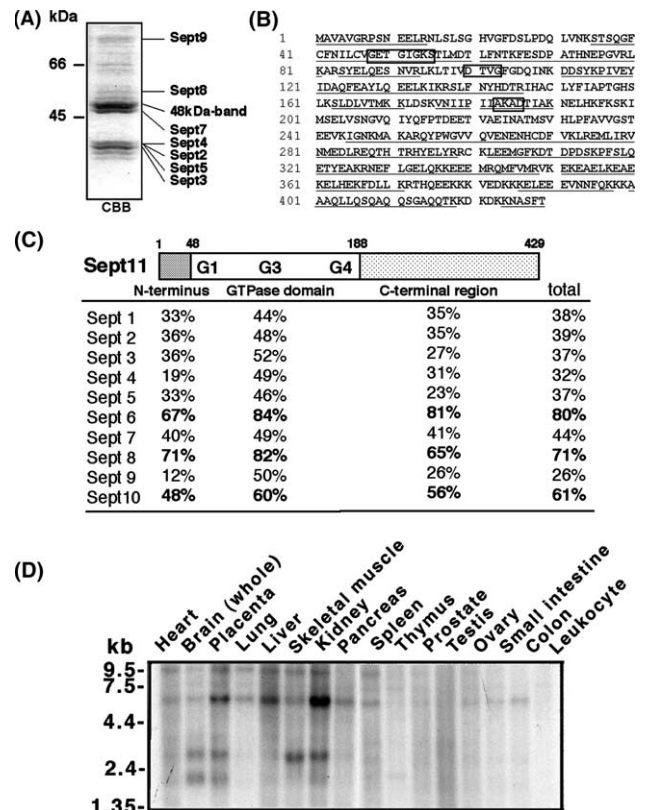


Fig. 1. Identification of Sept11 in septin complexes purified from porcine brain. (A) Coomassie blue staining of the septin complexes purified from porcine brain after SDS-PAGE (10% gel). A ~48-kDa band and septins identified by mass spectrometry analyses are marked. Molecular size markers were shown on the left. (B) Alignment of the aa sequence of Sept11. The aa sequences underlined are those identified in mass spectrometry. Three conserved regions (G1, G3, and G4) in the GTPase domain were boxed. (C) Diagram of Sept11 showing N- and C-terminal variable regions as well as a central GTPase domain. Total homologies as well as regional ones between Sept11 and 10 mammalian septins so far identified are shown. (D) Northern blot analyses. A nylon membrane containing human mRNAs (2 μ g each) from different tissues was used. Molecular sizes of RNA standards are indicated on the left.

performed, and the complexes were found to contain a variety of septins (Fig. 1A). In the course of the analyses, a protein band with molecular mass of 48 kDa was subjected to LC-MS/MS analyses. Consequently, the analyzed peptides consisted of those from a mixture of at least two proteins (data not shown). The aa sequences of a group of the digested peptides were found to be derived from Sept6 (data not shown). When database analyses of the aa sequences of remaining peptides were done, all of their sequences completely matched with FLJ10849 (GenBank Accession No. AK001711) (Fig. 1B). The deduced aa sequence of FLJ10849 possessed clear sequence similarity to septins in the central GTPase domain, and was highly homologous to Sept6 and Sept8 over the entire length (Fig. 1C). FLJ10849 was recently referred to as Sept11 [5], although its biochemical and cell biological characterization has not been carried out. As shown in Fig. 1D, Northern blot analysis revealed that Sept11 is widely expressed in all tested tissues except leukocytes, and transcribed as various combinations of ~6.5, ~3.0 and ~2.0 kb mRNAs in tissue-dependent manner.

3.2. GTPase activity is required for filament formation of Sept11 in COS cells

GTPase activity is possible to play a role for the filamentous structure of Sept2, while it is not required for polymer formation of Sept9 [7]. To test if the GTPase activity is essential for the filament-forming ability of Sept11, we introduced a mutation (Gly48 to Ala) in the G1 box of the GTPase region. MBP-fused Sept11 and the mutant, A48Sept11, were expressed in *E. coli* and purified (Fig. 2A). The GTPase activity of Sept11 and A48Sept11 was determined by measuring production of free phosphate released from [γ - 32 P]GTP as described [9]. It is to be noted that the GTPase assay used here was composed of two steps: [γ - 32 P]GTP/GDP exchange and hydrolysis of bound [γ - 32 P]GTP to GDP. As depicted in Fig. 2B, A48Sept11 showed a highly reduced in vitro GTPase activity under conditions where Sept11 has the activity, although the possibility that the reduced GTPase activity is due to the denaturation of the protein cannot be ruled out. The specific activities were calcu-

lated to be 0.115 ± 0.01 and 0.0164 ± 0.004 mmol Pi/mol of protein/min for Sept11 and A48Sept11, respectively. There are two possibilities for the apparent inhibition of the GTPase activity of A48Sept11; one is due to the reduced GTP-binding to the protein and another is reduced hydrolysis of bound GTP to GDP. When [α - 32 P]GTP-binding activity of A48Sept11 was compared to that of Sept11 by thin layer chromatography, both proteins were found to bind almost equal amount of [α - 32 P]GTP: $\sim 5.2\%$ of wild-type and $\sim 4.7\%$ of the mutant bound [α - 32 P]GTP (Fig. 2C). Although only limited portion of the used proteins interacted with guanine nt, these results suggest that A48Sept11 possesses GTP-binding ability indistinguishable from that of Sept11 but lost the GTP-hydrolysis activity.

When Sept11 and A48Sept11 were individually overexpressed in COS7 cells, the former formed filament structure while the latter had significantly abolished filament-forming ability (Fig. 2D). Endogenous Sept11 was localized as a short filamentous pattern in COS7 cells similar to that observed in HeLa cells (see Fig. 5A), although it should be noted that the staining pattern of Sept11 was not overlapping with stress fibers, microtubules and vimentin intermediate filaments.² It should be noted that the filament structure observed in COS7 cells expressing Myc-Sept11 is likely to be homopolymers which are different from the native heteropolymeric septin filaments. Despite the above possibility, expressed Sept11 was likely to have filament-forming and/or lateral association capability. It is notable that A48Sept11 was not incorporated into the filament composed of Sept11, and the filament structure by Sept11 and aggregates composed of A48Sept11 are independently present in cells expressing them (Fig. 2E). These phenotypes were very similar to those observed in the case of Sept2 as described [7]. Taken together, these results suggest that GTP-hydrolysis activity is required for polymer formation of Sept11, although the possibility that A48Sept11 was aggregated due to the mutation-mediated abnormal folding cannot be ruled out. Since it has been recently reported that the majority of septin complexes in *S. cerevisiae* do not exchange guanine nt [12], the physiological importance of GTPase activity of Sept11 in hetero-septin complexes remains to be elucidated.

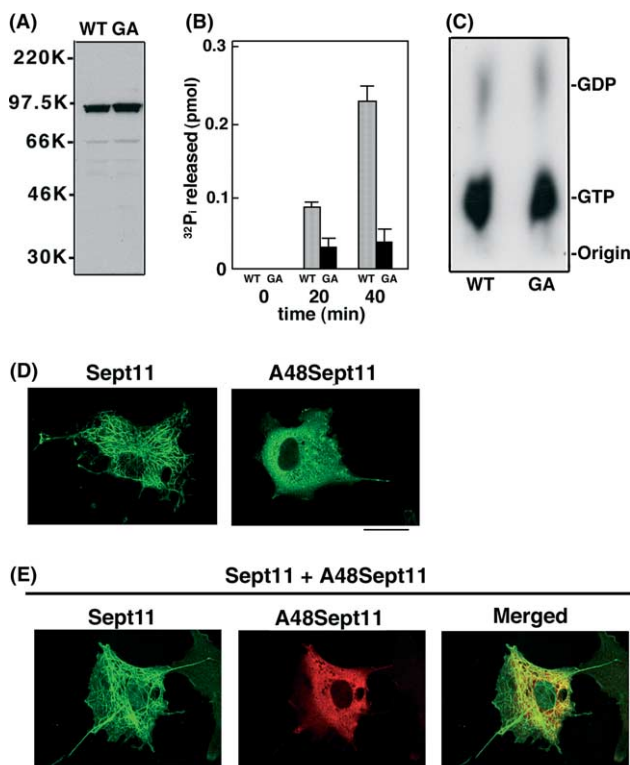


Fig. 2. Effects of a GTPase-deficient mutation in G1 box on the filament-forming ability of Sept11. (A) Coomassie blue staining of purified MBP-Sept11 (WT) and MBP-A48Sept11 (GA). Molecular size markers (Da) were shown on the left. (B) Time course of in vitro GTPase activity of Sept11 (WT) or A48Sept11 (GA) was examined. Data are means \pm S.D. of three separate experiments. (C) Purified recombinant Sept11 (WT) and A48Sept11 (GA) ($1 \mu\text{M}$) were labeled with $10 \mu\text{M}$ [α - 32 P]GTP and the bound radioactive nt were eluted from the proteins, resolved by thin-layer chromatography and counted. The positions of GDP and GTP standards as well as the origin are indicated. The results shown are representatives of three independent experiments. (D) COS7 cells were transfected with pRK5-Myc-Sept11 or -A48Sept11. After 24 h of transfection, these cells were stained using 9E10 antibody. (E) COS7 cells were double-transfected with pRK5-Myc-Sept11 and pRK5-Flag-A48Sept11. After 24 h of transfection, cells were double-stained using the anti-Myc polyclonal (left) and M2 antibodies (middle). The merged image is also shown (right). Bar, $20 \mu\text{m}$.

3.3. Detection of Sept11 in various cell lines using a specific antibody

We next prepared a rabbit polyclonal antibody, anti-Sept11-C, against the C-terminal 66 aa of Sept11, and affinity-purified it on a column to which the antigen had been conjugated. Specificity of anti-Sept11-C was confirmed with various septin proteins overexpressed in COS7 cells. As shown in Fig. 3A, anti-Sept11-C specifically recognized Sept11 in Western blot analyses. As other septins including Sept6 and Sept8, which are highly homologous to Sept11 (Fig. 1C), were not recognized by the antibody, we concluded that the antibody is specific for Sept11. We next did a Western blot analysis to detect endogenous Sept11 in rat brain homogenates and lysates of various mammalian cell lines, including HeLa, fibroblast REF52 and mammary gland HMEC cells (Fig. 3B). In the cell lysates tested, a protein with molecular mass of 48 kDa, coincident with that of Sept11, was observed. The high expression of Sept11-mRNA in kidney and relatively low

² Localization of Sept11 in COS cells, and characterization of anti-Sept6 and anti-Sept7 will be described in detail elsewhere.

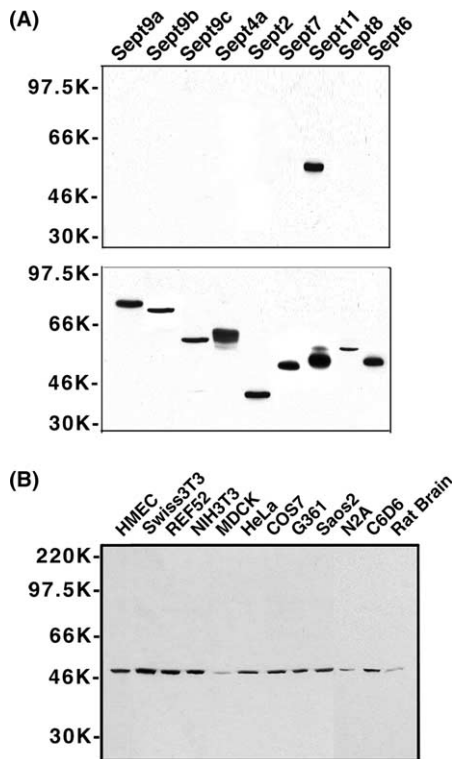


Fig. 3. Characterization of an affinity-purified antibody for Sept11. (A) Lysates from COS7 cells transiently expressing Myc-tagged Sept9a, Sept9b, Sept9c, Sept4a, Sept2, Sept7, Sept11, and Sept8 were separated on a 10% polyacrylamide gel and then subjected to Western blotting, using anti-Sept11-C (upper panel) or 9E10 (lower panel). (B) Lysates (30 μ g of each) of HMEC, Swiss3T3, REF52, NIH3T3, MDCK, HeLa, COS7, G361, Saos2, Neuro2a (N2A), C6D6 cells and rat brain were subjected to SDS-PAGE (10% gel) followed by Western blotting using anti-Sept11-C. Molecular size markers were shown on the left side of each panel.

Sept11 protein expression in a kidney epithelial cell line, MDCK, imply some role of Sept11 in the kidney functions such as tubule transport and glomerular ultrafiltration. In Fig. 3A, anti-Sept11-C did not visualize endogenous Sept11 in COS cells under the condition used, since the expression levels of Myc-Sept11 were very high. Preincubation of the antibody with recombinant Sept11 selectively inhibited the immunoreactivity (data not shown).

3.4. Analyses of septin complexes immunisolated with anti-Sept11-C or anti-Sept9 antibody

Presence of a variety of septins in brain implies the presence of various functional septin complexes containing different polypeptides. Respective septin complexes may be localized in different intracellular places and are assembled to accomplish different biological processes. We thus tested if CNBr-activated Sepharose 4B beads coupled with anti-Sept11-C or anti-Sept9 immunisolate different septin complexes from the porcine brain high speed supernatant prepared as described [8]. As shown in Fig. 4A, silver-staining patterns of the protein complexes isolated with anti-Sept11-C–Sepharose 4B and anti-Sept9–Sepharose 4B were similar but clearly different. Especially, a protein band with a molecular mass of \sim 75 kDa was detected only in anti-Sept9–Sepharose 4B-binding complexes (Fig. 4A). When Western blot analysis of the isolated immunocomplexes was done, the \sim 75-kDa band was found to con-

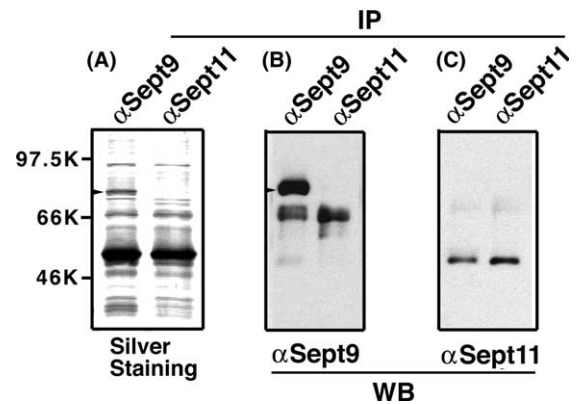


Fig. 4. Immunisation of Sept11- and Sept9-containing septin complexes from porcine brain high speed supernatant. Septin complexes isolated with Sepharose 4B beads coupled with anti-Sept9 antibody (left lane of each panel) and anti-Sept11-C antibody (right lane of each panel) were analyzed by silver-staining (A) or Western blotting with anti-Sept9 (B) and anti-Sept11-C (C).

tain a Sept9 isoform (Fig. 4B). A Sept9 variant with \sim 66 kDa and Sept11 was detected in both immunisolated complexes (Fig. 4B and C). These results suggest that there are at least two different septin complexes in porcine brain tissues, although the possibility that a region unique to the Sept9 isoform with a higher molecular mass masks the Sept11 epitope in the septin complexes cannot be excluded. The major bands with \sim 50 kDa observed in the silver-stained gel are likely to be composed of septins or septin-binding protein(s) yet unidentified in the present study. Actually, Sept6 and Sept7 that constitute the main septin framework in mouse brain and HeLa cells [13] were detected in the major band by Western blotting analyses¹ (data not shown). On the other hand, rabbit IgG released from the antibody-coupled beads was not detected in Western blotting with anti-rabbit IgG raised in donkey (data not shown).

The Sec6/8 (or exocyst) complex is thought to be essential for secretory vesicle targeting and docking, and has so far been reported to form large complexes with septin filaments [14]. Sept5 was also reported to bind directly with a SNARE protein syntaxin 1 and regulate exocytosis [15]. We thus asked if Sec6, Sec8 and syntaxin are included in the septin complexes isolated with anti-Sept9- or anti-Sept11-C-conjugated beads. When Western blot analyses were performed with specific antibodies for Sec6 (mouse monoclonal 9H5 clone, Calbiochem Inc.), Sec8 (mouse monoclonal 14G1 clone, Stressgen Biotech. Corp.) and syntaxin (goat polyclonal, Santa Cruz Inc.), these proteins were not detected in the isolated septin complexes (data not shown), maybe due to the possibly stringent purification procedures used in the present study. Alternatively, Sec6/8 and syntaxin may interact with septin complexes different from those isolated here.

3.5. Sept11 colocalizes with cytoskeletons in cell type-specific manner

Mammalian septin organization is thought to be related to actin filament structures and functions. Several septins have been reported to localize along with actin filaments [11,13,16,17], while Sept9 distributes along microtubules as well as actin filaments [7,17]. We examined the subcellular localization of Sept11 in HeLa, HMEC and REF52 cells, using a confocal microscope. Interestingly, the localization patterns

of Sept11 were distinct among cell types tested. In HeLa cells, Sept11 was present in short filamentous structures and partly colocalized with stress fibers (Fig. 5A). This localization was similar to that of Sept2 in NIH3T3 cells [13]. Double-staining with tubulin revealed some overlap between Sept11 and the cytoskeleton (Fig. 5A). In HMEC cells, significant colocalization of Sept11 with microtubule networks was observed (Fig. 5B,a–c), while characteristic localization of Sept11 along with actin stress fibers was seen in REF52 cells (Fig. 5C,a–c). We next asked if Sept11 colocalizes with another major cytoskeleton, vimentin, in HeLa, HMEC and REF52 cells. Double-staining showed no apparent codistribution of Sept11 with vimentin filaments in these cells (data not shown). Since septin filaments are composed of hetero-septin molecules, it is tempting to speculate that Sept11 functions, in harmony with other septin molecules, to control actin- and/or tubulin-dependent cellular events.

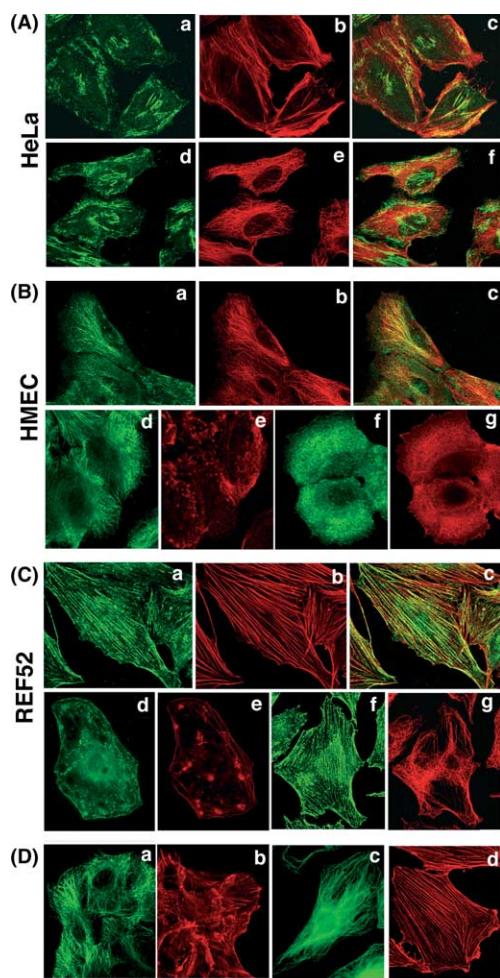


Fig. 5. Localization of Sept11 in HeLa, HMEC and REF52 cells. (A) HeLa cells were double-stained using anti-Sept11-C (a,d) and rhodamine-phalloidin (b) or an anti-tubulin antibody (e). The merged image was also shown (c,f). (B,C) HMEC cells (B) and REF52 cells (C) were double-stained using anti-Sept11-C (a) and an anti-tubulin antibody (B,b) or rhodamine-phalloidin (C,b). The merged image was shown (c). Effects of treatment with cytochalasin B (d,e) or demecolcine (f,g) on the filamentous distributions of Sept11 (d,f), F-actin (e) and microtubules (g) in the cells. (D) Staining patterns of microtubules (a,c) and F-actin (b,d) in the untreated HMEC (a,b) and REF52 (c,d) cells. Bar, 20 μ m.

The structural relationships among actin filaments, microtubules and Sept11 were further examined using reagents known to disrupt microtubule or actin filament structure. When HMEC cells were treated for 15 min with 5 μ M cytochalasin B (Sigma), a specific inhibitor of actin polymerization, the fibrous distribution of Sept11 was still observed under the conditions where actin filaments were disrupted (Fig. 5B,d and e). When the cells were treated for 100 min with 10 ng/ml demecolcine (Sigma), a specific inhibitor of tubulin polymerization, the fibrous distribution of Sept11 as well as microtubules was disrupted (Fig. 5B,f and g). These results suggest that Sept11 interacts with microtubules in HMEC cells as is the case of Sept9 [7]. On the other hand, treatment of REF52 cells with cytochalasin B induced disruption of Sept11-containing septin filaments as well as stress fibers (Fig. 5C,d and e). When the cells were treated with demecolcine, the fibrous distribution of Sept11 was still observed under the conditions where microtubules were disrupted (Fig. 5C,f and g). These results suggest that Sept11 interacts with stress fibers in REF52 cells. It is tempting to speculate that cell- or tissue type-specific interaction between Sept11-containing septin complexes and cytoskeletons might affect the cytoskeletal dynamics and/or septin functions in individual cells or tissues. In Fig. 5D, staining patterns of microtubules (a and c) and F-actin (b and d) in the untreated HMEC (a and b) and REF52 (c and d) cells were shown.

In the present study, we found that a yet uncharacterized member of septin family, Sept11, is widely expressed in mammalian tissues and distributes along with cytoskeletons in cell type-specific manner. The function of Sept11 remains to be clarified, although some septins have been reported to be involved in cytokinetic events [7,11,16,17]. The presence of a variety of septins in brain means that they are likely to play some roles in yet unidentified various cellular processes. In this context, accumulating data on mammalian septins indicate that some such as Sept5, Sept4 and Sept2 function not only in vesicle fusion processes [14,15] but also apoptosis [18] and neurodegeneration [19–21], although the precise molecular mechanisms of these processes are largely unknown.

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