Human septin–septin interactions as a prerequisite for targeting septin complexes in the cytosol

Constantino MARTÍNEZ*, Miguel A. SANJUAN†, Judith A. DENT*, Lars KARLSSON† and Jerry WARE*¹

*The Roon Research Center for Arteriosclerosis and Thrombosis, Division of Experimental Hemostasis and Thrombosis, Department of Molecular and Experimental Medicine The Scripps Research Institute, La Jolla, CA 92037, U.S.A., and †Johnson & Johnson Pharmaceutical Research & Development, L.L.C., La Jolla, CA 92121, U.S.A.

Septins are a cytosolic GTP-binding protein family first characterized in yeast, but gaining increasing recognition as critical protagonists in higher eukaryotic cellular events. Mammalian septins have been associated with cytokinesis and exocytosis, along with contributing to the development of neurological disorders. Ten different septins, divided into four groups, have been identified in mammals, and individual septins are capable of interacting with each other to form macromolecular complexes. The present study characterizes the structural requirements for human septin–septin interactions using a yeast two-hybrid system. We focus on three septins that are highly expressed in platelets and neurons, SEPT4 [previously designated H5, CDCrel-2 (cell-division-control-related-2), PNUTL2], SEPT5 (CDCrel-1, PNUTL1) and SEPT8 (KIAA0202). Each of these three septins contains a characteristic domain structure consisting of unique N- and C-termini, and a central core domain conserved among the family of proteins. The yeast two-hybrid system yielded data consistent with a model

where each of the three septins can interact with itself (homotypic assembly) or with one of the other septins (heterotypic assembly). For SEPT5 and SEPT8, the results illustrate a model whereby heterotypic septin assembly is dependent on the conserved central core domain and homotypic interactions require the N- and C-termini of each protein. We also characterized a model in which the proper cellular localization of SEPT5 and SEPT8 requires concomitant expression of both proteins. Co-transfection of SEPT5 and SEPT8 results in both proteins targeted to a vesicular-like location. Therefore the cellular repertoire of human septins has an impact on function by targeting septin macromolecular complexes to specific cellular locations.

Key words: heterotypic septin–septin interaction, homotypic septin–septin interaction, macromolecular complex, septin group, vesicle, yeast two-hybrid system.

INTRODUCTION

Septins are conserved cytosolic GTP-binding proteins first described in yeast as important regulators of the budding processes [1–4]. More recently, septins have gained recognition as a family of proteins also present in higher eukaryotes, and are most commonly associated with biological events where active membrane movement occurs, such as cytokinesis or secretion [5–10]. *In vivo*, mammalian septins are found as heterooligomeric complexes assembled from different members of the septin family. *In vitro*, studies have shown that these complexes can spontaneously form and polymerize into filaments [11,12], yet little is known about how these filaments assemble or about how their assembly is spatially and temporally regulated.

Each member of the septin family is composed of a highly conserved central core domain flanked by divergent C- and N-termini that vary in length. Ten different mammalian septins have been identified and a unifying nomenclature has been adopted [13]. Based on sequence similarity, mammalian septins can be classified into three groups [10], or by phylogeny into four closely related ‘subfamilies’ (Figure 1) [14,15]. A unifying structural feature of most septins is a predicted C-terminal coiled-coil domain, a structure which suggests an involvement in septin–protein interactions [4,16]. Several reports have implicated the coiled-coil domain in intermolecular septin interactions and, as such, important for the formation of septin macromolecular complexes [17–19].

At their N-terminus, septins contain different regions that can potentially regulate their function. Most septins contain a polybasic region located in the N-terminus that can bind phosphoinositides [19,20], but, again, the function and physiologic role of the polybasic region is unknown. One report showed that SEPT4 (septin 4) [also known as H5, CDCrel-2 (cell-division-control-related-2), PNUTL2] specifically binds PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, and this binding is inhibited by GTP binding and hydrolysis [20]. Recently, Casamayor and Snyder [19] showed that the polybasic region of yeast septins plays a role in their function and proper localization. In addition, they also showed that yeast septins bind preferentially to PtdIns(4)P and to PtdIns(5)P, and they hypothesized that PtdIns(4)P contributes to septin organization. Septins also feature a GTPase-binding domain and its physiological relevance in budding yeast is for the structural integrity of the septin [21]. Several studies have shown that mutations within the GTP-binding domain of mammalian septins alter their spatial organization and function [6,7].

Recently, a report by Blaser et al. [22] showed that human SEPT5 (previously designated CDCrel-1, PNUTL1), a septin involved in platelet and neuron exocytosis, binds to SEPT8 (KIAA0202). The physiological relevance of SEPT8 has yet to be defined, but the interaction of SEPT5 and SEPT8, and the fact that they are concomitantly expressed [23], suggests that SEPT8 may also play an important role in platelets and neurons. In an attempt to define further the interaction of SEPT5 and SEPT8, we studied the assembly of three human septins, SEPT4, SEPT5

Abbreviations used: CDCrel-1/2, cell division control-related-1/2; EEA1, early endosomal antigen 1; β -gal, β -galactosidase; GFP, green fluorescent protein; HA, haemagglutinin; SEPT, septin; SV40, simian virus 40; – TL medium, Trp[–]/Leu[–] medium; – TLHA medium, Trp[–]/Leu[–]/His[–]/Ade[–] medium.

¹ To whom correspondence should be addressed at the present address: Department of Physiology and Biophysics, #505, University of Arkansas for Medical Sciences, Little Rock, AR 72223, U.S.A. (email jware@uams.edu).

Figure 1 Mammalian septin classification

The septin primary sequence illustrates a common domain structure of unique N-terminal and C-terminal regions and a conserved central core domain. The latter is highly conserved among septins of the same species with a minimum sequence similarity of 70 %. Septins can be classified into four different groups (groups I to IV) based on the sequence similarity of their C-terminal region [37]. Within each group, septin C-terminal regions share a 50–60 % sequence identity. Dissimilar sequences are shown as a line in the schematic model.

and SEPT8, with each other (heterotypic) and with themselves (homotypic) using a yeast two-hybrid system. We primarily focused on the role of the three major regions of each septin, the C-terminal coiled-coil domains, the N-terminal regions and the central core domain. The results allow the development of an assembly hypothesis where the central core domains are critical for heterotypic assembly, the C-terminal coiled-coil and N-terminal regions are essential for homotypic septin interactions, and septin assembly is preferentially engaged between septins belonging to different groups (Figure 1). The *in vivo* relevance of the results are supported further in a cellular model in which co-expression of SEPT5 and SEPT8 is required to co-localize both proteins into a vesicular-like location.

EXPERIMENTAL

cDNA construct and cloning

In-frame fusions of the *GAL4* DNA-activation domain and the various septin proteins were constructed in the yeast expression vector pGADT7 (Clontech, Palo Alto, CA, U.S.A.). Full-length and deleted variants of the SEPT5 cDNA were generated by a PCR using primers containing *EcoRI* and *SacI* restriction sites at 5' and 3' ends, respectively (Table 1). The primer pairs used were the following: S5-F1 (F designating forward) and S5-B (B designating reverse) for the full-length protein, S5dNt-F and S5-B to construct SEPT5^{Δ1–40}, and S5dNt-F and S5dCt for SEPT5^(41–338). The template DNA corresponded to GenBank[®] accession number NM_002688 [24]. The generation of cDNAs containing SEPT8-encoding sequences was also carried out by a PCR using primers containing *NdeI* and *EcoRI* restriction sites at 5' and 3' ends respectively (Table 1). The template was an EST (expressed sequence tag) clone with the GenBank[®] Accession Number BI603940. The primers used were S8-F and S8-B to obtain full-length SEPT8, S8dNt-F and S8-B for SEPT8^{Δ1–41}, and S8dNt-F and S8dCt-B to construct SEPT8^(42–321). The SEPT4 expression

Table 1 Primers used in the present study

Name ^a	Sequence ^b
S5-F1 ^c	5'-GCGGAATTCATGACACAGGCCTGCGGTACAAG-3'
S5-B ^d	5'-CGGGAGCTCTCACTGGTCTGCATCTGCTGCTTCAT-3'
S5-F2 ^e	5'-CGGGTCGACTCACTGGTCTGCATCTGCTGCTTCAT-3'
S5 ^{Δ339–369} -F ^{d,f}	5'-CCGCTGCCACCCCGGACTAGGAGACTGAGAAGCTT-3'
S5 ^{Δ339–369} -B ^{d,f}	5'-AAGCTTCTCAGTCTCTCAGTCCGGGGTGGGACCGG
S5dNt-F ^d	5'-GAATCAAAGGCTTTGACTTCACACTCATG-3'
S5dCt-B ^d	5'-GTCCGACCTGGGTCAAGTTTGTGGTTCATCTG-3'
S8-F ^d	5'-GAGCATATGATGGCGGCCACCGACCTGGAGCGC-3'
S8-B ^d	5'-ACAGAATTCCTCAGGAATAGTGACACTGTAAAT-3'
S8 ^{Δ322–443} -F ^{d,f}	5'-GATGGTGACAGCCAGCCCTGAAGCCTACAAGAGACATAC-3'
S8 ^{Δ322–443} -B ^{d,f}	5'-GTATGTCTCTTGTAGGCTTCAGGCTGGCTGTCAACATC-3'
S8dNt-F ^c	5'-GAATTCGGCTTCAGCTTCAACATCCTCTGTG-3'
S8dCt-B ^c	5'-GGATCCATCGCTGTCTGAAAGCCCATCTC-3'
S4-F ^d	5'-CATATGATGGACCGTTCACTGGGATGGCAAG-3'
S4-B ^d	5'-GGATCCCTTAATAGTTCTCCTTCATCTGTTTTG-3'
S4 ^{Δ447–478} -F ^{d,f}	5'-GTCCACCCAGGGACATAGCCAGAACTGAGAAG-3'
S4 ^{Δ447–478} -B ^{d,f}	5'-CTTCTCAGTTTCTGGCTATGTCCCTGGTGGGAC-3'
S4dNt-F ^c	5'-CATATGAAAGGCTTTGACTTTACCCCTCATG-3'
S4dCt-B ^c	5'-GGATCCCTTACCACCCAGCGGGTTCATGCT-3'
S5-03/09-F ^{f,g}	5'-CAGTGAGATCCGGAAGCTGATTGACAAGTTTGGGATC-3'
S5-03/09-B ^{f,g}	5'-GATCCCAAACCTGTCAATCAGCTTCCGGATCTCACTG-3'
S5-S38A-F ^h	5'-CAGGTGCACCGCAAGGCGGTGAAGAAGGCTTTG-3'
S5-S38A-B ^h	5'-CAAAGCCTTTCTTACCAGCCTTGGCGTGACCTG-3'
S5-S58N-F ⁱ	5'-GAGTCAGGCGCTGGGGAAGAACACACTGGTCCACAGCCTC-3'
S5-S58N-B ⁱ	5'-GAGGCTGTGGACAGTGTGTTCTTCCCAGGCGTGACTC-3'
S5-KK/AA-F ^j	5'-GTCCACCGCAAGTCGGTGGCGGAGGCTTTGACTTCACATC-3'
S5-KK/AA-B ^j	5'-GAGTGTGAAGTCAAAGCCTGCCGCCACCGACTTGGCGTGAC-3'

^a F is forward primer and B is reverse.

^b Restriction sites used for cDNA cloning are underlined.

^c Primers used for cloning into pGADT7.

^d Primers used for cloning into pGADT7 and pGBKT7.

^e Primer used for cloning into pGBKT7.

^f Primers used for site directed mutagenesis.

^g Primers used for SEPT5^{Δ203–209} construct.

^h Primers used for SEPT5^{S38A} construct.

ⁱ Primers used for SEPT5^{S58N} construct.

^j Primers used for SEPT5^{K40A/K41A} construct.

constructs were generated by a PCR using primers containing *NdeI* and *BamHI* restriction sites at 5' and 3' ends respectively. The template DNA corresponded to GenBank[®] accession number NM_004574 [25]. The primers used were S4-F and S4-B to obtain full-length SEPT4, S4dNt-F and S4-B for SEPT4^{Δ1–140}, and S4dNt-F and S4dCt-B to construct SEPT4^(141–446). The different PCR products were subcloned into pCR2.1 (Invitrogen, Carlsbad, CA, U.S.A.), sequenced to verify the accuracy of the cDNA sequenced and subsequently cloned into pGADT7 for use in yeast two-hybrid experiments.

In-frame fusions of the *GAL4* DNA-binding domain to the various septin proteins were constructed in the yeast expression vector pGBKT7 (Clontech). Similar to the strategy described above, primers were used in a PCR to generate the various full-length and deleted cDNA variants of each septin. However, an alternative set of SEPT5 primers (S5-F1 and S5-F2) were used (Table 1).

cDNA constructs containing internal deletions and point mutations were generated by site-directed mutagenesis using the QuikChange[®] II Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, U.S.A.). Vectors containing the wild-type cDNA were used as template. PCR primer pairs were 5'-TGCGCA-GGTACCCATGGCGGCCACCGACCTGGAG-3' containing a *KpnI* restriction site (underlined) and S8-B (Table 1) for SEPT8. PCR products were digested with *KpnI* and *EcoRI* and ligated in frame to produce the Xpress[™] epitope encoded by

pcDNA3.1/HisA. A full-length SEPT5 cDNA (codons 1–369) lacking the stop codon was cloned in-frame into the *AfeI* restriction site of pEGFP-N1 (Clontech). The expressed polypeptide would contain in an N- to C-terminal direction the complete SEPT5 polypeptide sequence, a short linker sequence followed by GFP (green fluorescent protein). SEPT5^{S58N}/pEGFP-N1 was constructed by site-directed mutagenesis using primers to express GFP-tagged SEPT5 with a Ser⁵⁸ → Asn substitution, SEPT5^{S58N-GFP} (Table 1).

All construct sequences were confirmed by DNA sequencing.

Yeast two-hybrid system

Protein–protein interactions were studied using a MATCH-MAKER™ two-hybrid system (Clontech). Expression plasmids containing the inserted septin cDNA fragments in either pGADT7 or pGBKT7 were transformed into yeast strain AH109 using a yeast-transformation system (Yeastmaker; Clontech). For transformation studies, 2 µg of the pGADT7 cDNA construct and 4 µg of the pGBKT7 cDNA construct were used. The constructs showed no self-activation when assayed for α -galactosidase activity after transformation into AH109 cells and grown on plates containing Trp[−]/Leu[−] drop-out medium (−TL) supplemented with 20 µg/ml X- α -gal (5-bromo-4-chloroindol-3-yl α -D-galactopyranoside). Cells were allowed to grow for 5 days (at 30 °C) on −TL medium and on Trp[−]/Leu[−]/His[−]/Ade[−] drop-out medium (−TLHA). At that point, individual colonies were grown in liquid culture (−TLHA medium) and assayed for the production of β -gal (β -galactosidase) using *O*-nitrophenyl β -D-galactopyranoside (Sigma, St. Louis, MO, U.S.A.) as substrate according to protocol from the manufacturer (Clontech). The positive controls c-Myc-p53 and the SV40 (simian virus 40) large T-antigen were supplied from Clontech. One alteration in the protocol involved permeabilization of cells with 0.2 % *N*-lauroylsarcosine (for 30 min) instead of the recommended freeze–thaw procedure before addition of the substrate [26]. Protein expression was assessed by Western blotting using an anti-HA (haemagglutinin) IgG (Roche Applied Science, Indianapolis, IN, U.S.A.), clone 12CA5, to detect AD-fusion proteins and an anti-c-myc IgG (Clontech) to detect BD-fusion proteins. Yeast protein extracts were generated according to the protocol from the manufacturer (Clontech).

Immunofluorescence and immunoprecipitation

COS-7 cells were seeded at 3×10^5 cells/well in six-well plates. After growth for 18 h, the CsCl-purified plasmid DNAs were transfected using LIPOFECTAMINE™ according to the manufacturer's protocol (Invitrogen). At 24 h post-transfection, cells were washed with PBS and allowed to grow for 6 h in complete medium. Samples were then washed with PBS, fixed with 4 % paraformaldehyde in PBS (at 22 °C for 20 min) and washed again with PBS. Cells were permeabilized and blocked with 2 % BSA/0.05 % Triton X-100 in PBS (for 30 min). All subsequent incubations and washes contained 0.5 % BSA/0.05 % Triton X-100 in PBS.

An anti-Xpress™ mouse monoclonal antibody was purchased from Invitrogen and used at 0.8 µg/ml (1 h incubation). To detect endosomes, an anti-EEA1 (early endosomal antigen 1) monoclonal antibody was used (Transduction laboratories, San Diego, CA, U.S.A.) and FITC–dextran M_r 70000 (Sigma). After two washes, bound antibodies were detected using a Cy3-linked anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA, U.S.A.). Endogenous SEPT5 was detected in rat pheochromocytoma PC12 cells (kindly provided by Dr T. Yagi, The Scripps Research Institute, La Jolla, CA, U.S.A.). Cells were

seeded at 8×10^4 cells/well in six-well plates, and treated for immunofluorescence studies as described above after 48 h of growth. An anti-SEPT5 monoclonal antibody LJ-33 was used at 1.6 µg/ml and secondarily detected using an AlexaFluor® 568-conjugated goat anti-mouse antibody from Molecular Probes (Eugene, OR, U.S.A.) (24). In some experiments, nuclei were stained with propidium iodide. Fluorescence and differential interference contrast were analysed on an Olympus confocal microscope using its associated Fluoview software (Melville, NY, U.S.A.).

COS-7 cells (10^6) were grown in 100-mm dishes and co-transfected with pCDNA3.1-SEPT8 and SEPT5/pGFPN1 or SEPT5^{S58N}/pGFPN1 as described above. Cells were trypsinized and resuspended in lysis buffer (0.1 mM EDTA, 50 mM NaF, 0.4 mM sodium orthovanadate and 1 % Triton X-100 in PBS, pH 7.4) containing protease inhibitors (2 µg/ml leupeptin and 1 mM Pefabloc®). Cells were rotated for 30 min at 4 °C and the lysates were centrifuged at 14000 *g* for 15 min. Supernatants (400 µg) were supplemented with 1 % BSA and incubated with 10 µg LJ-33 (for 2 h at 4 °C). An 80 µl volume of 50 % slurry-immobilized Protein A (Repligen, Waltham, MA, U.S.A.) containing 1 % BSA was added, and the suspension was rotated for an additional 12 h at 4 °C. Agarose beads were rapidly washed five times with lysis buffer, and the pellets were resuspended in Laemmli loading buffer under non-reducing conditions and subjected to SDS/PAGE in 4–20 % gels and subsequently analysed by Western blotting.

RESULTS

Septin–septin interaction requires different regions depending on the heterotypic or homotypic nature of the binding

Homotypic and heterotypic septin interactions were investigated using two human septin proteins, SEPT5 and SEPT8, in a yeast two-hybrid system. Previous studies have shown that SEPT5 interacts tightly with SEPT8 (a heterotypic interaction), as evidenced by the ability of SEPT5 to interact specifically with SEPT8 fusion proteins expressed in cDNA libraries [22]. As presented in Table 2, the heterotypic interaction between the full-length SEPT5 and SEPT8 polypeptides is strong and independent of either septin being fused to the transcription activation domain (pGADT7) or the DNA-binding domain (pGBKT7). The interaction strength between the different pairs was measured through a β -gal assay. β -gal units obtained in the presence of full-length SEPT5 and SEPT8 proteins were equal to that obtained with the positive control interaction of c-Myc-p53 and the SV40 large T-antigen (results not shown). Along with the heterotypic interactions, the strength of homotypic septin interactions, SEPT5–SEPT5 and SEPT8–SEPT8, were also measured. Our results illustrate that both interactions occur, although the strength of the homotypic interaction was less than those obtained in the heterotypic association assays (Table 2).

To examine these interactions further, we analysed the domain structures of SEPT5, SEPT8 and a third septin, SEPT4, using the web-based program, SMART (Simple Modular Architecture Research Tool) [27,28]. This software predicts that SEPT4, SEPT5 and SEPT8 contain a similar domain arrangement consisting of variable N- and C-terminal regions and a conserved central core domain (Figure 2). SMART did not identify specific motifs in any of the N-terminal regions. As expected, a Pfam domain corresponding to a GTP_CDC was found in the central core region of all three septins (Figure 2). This domain contains the GTPase motif common to members of the septin protein family. An alignment of septins reveals that the central core domain spans over 300 residues and shares a minimum of 70 % sequence identity with septins

Table 2 Two-hybrid interactions between SEPT5 and SEPT8: implication of the C-terminal coiled-coil domain

Results are expressed as units of β -galactosidase and are means $\pm$ S.D. for three independent colonies of each strain after growth in – TLHA agar plates for 5 days at 30 °C, and further culture in – TLHA liquid medium for 8 h at 30 °C. nc, no colonies in – TLHA medium; nd, not determined.

pGADT7	pGBKT7					
	SEPT4	SEPT4 Δ 447–478	SEPT5	SEPT5 Δ 339–369	SEPT8	SEPT8 Δ 322–483
SEPT4	19.4 $\pm$ 0.4	5.4 $\pm$ 0.6	nd	nd	nd	nd
SEPT5*	14.5 $\pm$ 0.5	10.1 $\pm$ 0.2	13.2 $\pm$ 1	2.3 $\pm$ 1.9	40.8 $\pm$ 1.5	30.8 $\pm$ 0.7
SEPT5 Δ 339–369	nd	nd	nc	nc	41.2 $\pm$ 3.9	33.1 $\pm$ 3
SEPT8	38.6 $\pm$ 0.7	34.1 $\pm$ 0.5	42.9 $\pm$ 1.9	51.3 $\pm$ 0.6	2.9 $\pm$ 0.2	nc
SEPT8 Δ 322–483	nd	nd	41.3 $\pm$ 10.2	48.9 $\pm$ 4.7	0.9 $\pm$ 0.1	nc

* Non-specific interaction between pGADT7-SEPT5 and a control vector pGBKT7-53 = 0.8 $\pm$ 0.6 β -gal units in – TL medium.

Figure 2 Septin polypeptide organization

A linear representation of septin domain arrangements is shown. Septins are composed of three main regions, an N-terminal region, a central core domain and a C-terminal region with a predicted coiled-coil structure. Mutants of SEPT4, SEPT5 and SEPT8 polypeptides were generated via site-directed mutagenesis, and the corresponding positions of each mutation are shown as they align to the full-length polypeptides. Additional point mutations were generated in the SEPT5 cDNA to obtain polypeptides with mutations within a GTP-binding domain (black box, G1) and polybasic region (black box, PBR). SEPT5 Δ 203–209 has a deletion in a low-complexity region (LCR) located within the central core domain. SEPT5^{S58N} has a Ser⁵⁸ → Asn mutation in the GTP-binding domain. SEPT5^{S38A} has a Ser³⁸ → Ala mutation in a potential phosphorylation site. SEPT5^{K40A;K41A} has Lys⁴⁰ → Ala and Lys⁴¹ → Ala mutations in the polybasic region.

from the same species [29] (Figure 2). Finally, the analysis also predicts the boundaries of a coiled-coil domain within the C-terminal region of each septin (Figure 2). SEPT4 and SEPT5 are two phylogenetically related members of the septin family that have been subclassified together into the same group (group III) [10,13]. According to the same classification scheme, SEPT8 belongs to group II. Both SEPT4 and SEPT5 share 76 % iden-

Figure 3 Septins expressed in the yeast two-hybrid system

Yeast strain AH109 was transformed with plasmids expressing different septins used in the yeast two-hybrid system. Cell extracts were probed by immunoblotting with anti-HA antibodies (lanes 1–8) or anti-c-myc antibodies (lanes 9 and 10). Lane 1, SEPT4; lane 2, SEPT5; lane 3, SEPT5 Δ 339–369; lane 4, SEPT5 Δ 1–40; lane 5, SEPT5^(41–338); lane 6, SEPT8; lane 7, SEPT8 Δ 322–483; lane 8, SEPT8^(42–321); lane 9, SEPT5^{S58N}; lane 10, SEPT5 Δ 203–209. The position of a 60 kDa protein is indicated.

tity within the central core domain and the C-terminal region. However, their N-terminal domains are very different in size (140 residues for SEPT4 and 40 residues for SEPT5) and do not share significant sequence similarity.

Mutants of SEPT4, SEPT5 and SEPT8 were generated to test in a yeast two-hybrid system the role of individual domains for septin–septin interaction. Figure 2 schematically depicts the mutants and Figure 3 confirms the expression of each mutant polypeptide in the yeast lysates. Deletion of the predicted C-terminal coiled-coil domain of SEPT4 (residues 447–478), SEPT5 (residues 339–369) or SEPT8 (residues 322–443) did not alter the heterotypic assembly between SEPT4 and SEPT5 with SEPT8 (Table 2). However, removal of the coiled-coil domain abolished the homotypic assembly of SEPT5–SEPT5 or SEPT8–SEPT8, as evidenced by the lack of viable yeast colonies, and strongly altered the homotypic interaction of SEPT4 (Table 2). Our results do demonstrate that the coiled-coil domains of SEPT4, SEPT5 and SEPT8 are not required for heterotypic assembly between them (Table 2). In addition, given that the different mutants expressed in the present study displayed an interaction with one or several other mutants or full-length septins, it seems that these mutants are stable and correctly folded. However, additional constructs expressing an isolated coiled-coil domain of SEPT5 or SEPT8 failed to self-associate, suggesting that homotypic interactions require other regions of the protein, or the isolated coiled-coil domains do not have an intrinsic ability to fold into a correct tertiary structure (results not shown).

SEPT5 and SEPT8 mutants lacking N-terminal domains were also generated and tested for their ability to assemble into homotypic or heterotypic complexes. Deletion of the SEPT5 N-terminal domain (residues 1–40) had little effect on the ability to interact

Table 3 Two-hybrid interactions between SEPT5 and SEPT8: implication of the N-terminal domain

Results are expressed as units of β -gal and are means $\pm$ S.D. for three independent colonies of each strain after growth in – TLHA agar plates for 5 days at 30 °C, and further culture in – TLHA liquid medium for 8 h at 30 °C. nc, no colonies in – TLHA medium.

pGADT7	pGBKT7	
	SEPT5	SEPT8
SEPT5 Δ 1–40	nc	34.9 $\pm$ 0.5
SEPT5 ^(41–338)	nc	21.1 $\pm$ 0.8
SEPT8 Δ 1–40	45.4 $\pm$ 2.7	nc
SEPT8 ^(42–321)	42.4 $\pm$ 3.5	nc

Table 4 Effect of point mutations in SEPT5 homotypic and heterotypic interactions

Results are expressed as units of β -gal and are means $\pm$ S.D. for three independent colonies of each strain after growth in – TLHA agar plates for 5 days at 30 °C, and further culture in – TLHA liquid medium for 8 h at 30 °C. nc, no colonies in – TLHA medium.

pGADT7	pGBKT7		
	SEPT5 ^{S38A}	SEPT5 ^{K40A;K41A}	SEPT5 ^{S58N}
SEPT5	11.8 $\pm$ 1.6	7.9 $\pm$ 2.5*	nc
SEPT8	35 $\pm$ 0.8	48.1 $\pm$ 3	nc

* Colonies were typically visible after 2 days of growth at 30 °C. In the case of SEPT5^{K40A;K41A}, visible colonies did not appear until 4 days and β -gal activities were not determined until after an 8 day growth.

with SEPT8, whereas the ability for homotypic assembly was abolished (Table 3). Similarly, deletion of the SEPT8 N-terminal domain (residues 1–41) abolished SEPT8 homotypic assembly, but had no effect on the heterotypic interaction between SEPT5 and SEPT8 (Table 3). Finally, the expression of an isolated central core domain of SEPT5 (residues 41–338) or SEPT8 (residues 42–321) supported heterotypic assembly with the corresponding full-length polypeptide, but was unable to support a homotypic assembly (Table 3). Thus the heterotypic assembly of SEPT5 and SEPT8 is a property of the central conserved domain of each protein, whereas homotypic assembly requires the unique sequences found in the flanking N- and C-terminal domains of SEPT5 and SEPT8.

Septin consensus motifs and septin–septin assembly

The N-terminal domain of SEPT5 contains two remarkable motifs, a potential site of phosphorylation, Ser³⁸, and a polybasic motif located between His³⁵ and Lys⁴¹ (H³⁵RKSVKK⁴¹). To determine if either of these motifs was involved in homotypic septin interactions, we engineered two different mutants containing alanine substitutions within each motif, SEPT5^{S38A} and SEPT5^{K40A;K41A}. The later mutation has been shown to ablate the binding of recombinant GST–SEPT4 with PtdIns(4,5)P₂ [20]. Our results demonstrate that Ser³⁸ does not play a role in the homotypic or heterotypic interactions of SEPT5 (Table 4). We also observed that the replacement of lysine residues at positions 40 and 41 did not affect the heterotypic interaction between SEPT5 and SEPT8, but did alter homotypic assembly of SEPT5 (Table 4). Colonies co-transformed with SEPT5^{K40A;K41A} in the activation domain vector, pGADT7, and full-length SEPT5 fused to the DNA-binding-domain vector, pGBKT7, produced visible colonies on day 4 after transformation, and β -gal assays were

carried out on day 8, generating similar results to those obtained with the wild-type SEPT5.

We studied the effect of GTP binding on homotypic and heterotypic interactions by inserting a mutation, Ser⁵⁸ $\rightarrow$ Asn, known to abrogate GTP binding to SEPT5 [20]. Our results show that SEPT5^{S58N} failed to bind to wild-type SEPT5 and to SEPT8, suggesting that the integrity of the GTP-binding domain is necessary for SEPT5 interactions with itself and SEPT8.

Besides the well-known structures that characterize septins, SEPT5 contains a non-globular region of low compositional complexity (I¹⁹⁸RKLKERIREEDK²¹²) as detected using SMART [27,28] (Figure 2). For other proteins, such regions have been associated with protein interactions and function [30,31]. To determine if this region plays a role in heterotypic SEPT5 interactions, we constructed a SEPT5 mutant lacking a segment of this domain between amino acids 203 and 209, SEPT5 Δ 203–209 (Figure 2). Deletion of this segment completely abolished the interaction between SEPT5 and SEPT8 and homotypic interactions, as well. However, subsequent refinement with paired alanine substitutions throughout the entire sequence (residues 203–209) did not abolish homotypic or heterotypic interactions (results not shown). Thus the integrity of residues 198–212 is necessary for both homotypic and heterotypic interaction.

SEPT5–SEPT8 complexes relocate to vesicle-like structures

Our results suggest a strong interaction between SEPT5 and SEPT8 in the yeast two-hybrid system. To examine the association of SEPT5 and SEPT8 further, we studied whether or not that interaction takes place in a mammalian cell system. A GFP-tagged version of SEPT5 in COS-7 cells showed SEPT5 to be equally distributed throughout the cytoplasm and poorly represented inside the nucleus, since no co-localization was found with the fluorescence generated by the nucleic acid intercalating agent, propidium iodide (red) (Figure 4A, panels a and b). In a similar manner, SEPT8 was present as a diffuse cytoplasmic protein when expressed alone (Figure 4A, panels c and d). When COS-7 cells are co-transfected with both SEPT5 and SEPT8 cDNAs, SEPT5 is redistributed to vesicular-like structures (Figure 4A, panels e and f). According to our two-hybrid experiments, if an interaction between SEPT5 and SEPT8 occurs, then both proteins should co-localize. As shown in the merged image of Figure 4(B), the majority of the SEPT5 (green) and SEPT8 (red) protein has a similar cytoplasmic location. Phase-contrast images show that the diameter of these ‘vesicles’ ranges from 1.5 to 2 μ m (Figure 4C). We also performed an immunoprecipitation study using a monoclonal antibody directed against SEPT5, LJ-33, and the lysate from COS-7 cells expressing SEPT5^{GFP} and SEPT8. The results show that immunoprecipitation of SEPT5^{GFP} co-purifies SEPT8, validating the immunofluorescence data (Figure 5). As a negative control, we studied the interaction of SEPT8 with SEPT5^{S58N–GFP}. As shown in Figure 5, SEPT5^{S58N–GFP} failed to co-purify SEPT8, confirming the results obtained with the yeast two-hybrid system. In an attempt to identify the nature of the vesicle-like structures, we used markers such as the early endosomal marker EEA1 and FITC–dextran. Our results consistently showed a negative co-localization between SEPT5–SEPT8 complexes and endosome markers (results not shown). To test the specificity of these results, we performed indirect immunofluorescence assays to detect SEPT5 in rat pheochromocytoma PC12 cells. PC12 cells express SEPT5 together with a wide range of septins [7]. Detection of endogenous SEPT5 in PC12 cells, using the LJ-33 antibody, shows a distribution in vesicular-like structures, demonstrating that these macromolecular complexes are specific and seem to

Figure 4 SEPT5 and SEPT8 organized into macromolecular complexes around cytosolic vesicle-like organelles

(A) COS-7 cells were transfected with SEPT5/pEGFP-N1 and/or SEPT8/pcDNA3.1-His cDNAs. Transfection of a SEPT5 cDNA alone (a and b) revealed a diffuse cytoplasmic location of SEPT5 protein, as detected by a GFP tag. In these images, nuclei were stained with propidium iodide (red). Transfection of a SEPT8 cDNA alone (c and d) revealed a similar diffuse cytoplasmic location, as detected by the presence of a C-terminal XpressTM epitope and immunofluorescence produced by anti-XpressTM antibodies (red). COS-7 cells were cotransfected with both SEPT5/pEGFP-N1 and SEPT8/pcDNA3.1-His cDNAs and nuclei were stained with propidium iodide, and SEPT5 was detected by green fluorescence (e and f). (B) COS-7 cells were co-transfected with both SEPT5/pEGFP-N1 and SEPT8/pcDNA3.1-His cDNAs. SEPT5 and SEPT8 were detected as described above and the images were merged to reveal co-localization. (C) COS-7 cells were co-transfected with both SEPT5/pEGFP-N1 and SEPT8/pcDNA3.1-His. SEPT5 was detected as described above. Cells were observed by differential interference contrast microscopy, and SEPT5 epifluorescence images were overlaid. Arrows indicate example of co-localization between SEPT5–SEPT8 complexes and vesicle-like organelles. (D) Rat pheochromocytoma cells (PC12) were labelled with the anti-SEPT5 monoclonal antibody, LJ-33. The bound monoclonal antibody was detected with an AlexaFluor[®] 568-conjugated goat anti-mouse polyclonal antibody. Scale bars, 20 μ m.

represent the physiological state of SEPT5 in mammalian cells expressing two or more septins (Figure 4D).

DISCUSSION

Understanding the formation of macromolecular septin complexes and how their assembly is regulated *in vivo* is necessary to help define septin function. In the present study, we focused

on three septins, SEPT4, SEPT5 and SEPT8, and examined how the predictive domain structure of each polypeptide contributes to septin–septin assembly. The SEPT5–SEPT8 interaction was originally identified by Blaser et al. [22] using a yeast two-hybrid system. In their study, a full-length SEPT5 protein was used as bait and showed a remarkable preference for SEPT8 clones in either foetal brain or heart cDNA libraries. Indeed, in both the Blaser study [22] and the data we report in the present paper, the strength of the interaction between SEPT5 and SEPT8, measured with a

Figure 5 Co-immunoprecipitation of SEPT5 and SEPT8 expressed in COS-7 cells

SEPT5/pEGFP-N1 and SEPT8/pcDNA3.1-His cDNAs were co-transfected into COS-7 cells. Lysates were immunoprecipitated using the anti-SEPT5 antibody, LJ-33, and the immunoprecipitates were analysed by non-reducing SDS/PAGE and immunoblotting. The presence of SEPT8 was detected using an anti-XpressTM antibody recognizing the XpressTM epitope present on the SEPT8 polypeptide. Lane 1, immunoprecipitation products from cells expressing SEPT5^{GFP} and SEPT8; lane 2, immunoprecipitation products from cells expressing SEPT5^{S58N-GFP} and SEPT8. Molecular-mass sizes (in kDa) are indicated to the left of the blots.

β -gal assay, is equal to the interaction between p53 and the large T-antigen. Nevertheless, a detailed study of the domain requirement for such an interaction has never been undertaken.

All septin members share a conserved central core domain flanked by two variable regions. With the exception of septin members from group I, all the septins display a coiled-coil domain in the C-terminal region (Figure 1). Our results showed that the central core domain is sufficient for the heterotypic interaction between SEPT5 and SEPT8, whereas homotypic interaction involving the same proteins requires, in addition, the structural integrity of both the N-terminal and C-terminal regions (Tables 2 and 3). The central core domain of septins contains a GTPase motif common to that of the GTPase superfamily. This septin GTPase motif hydrolyses GTP and is related to the ATP-binding domain of the protein kinase superfamily [32]. Members of the protein kinase superfamily homodimerize or heterodimerize, and this interaction is required for recognition of the nucleotide [33]. Our results demonstrate that the septin family is not an exception to this rule, since septin monomers also homodimerize and heterodimerize. We attempted to confirm if a septin GTPase domain was essential for septin assembly by introducing the Ser⁵⁸ → Asn mutation into SEPT5 [7]. For SEPT5, this sequence appears to be important for its binding with SEPT8 and itself, but the results may also be suggesting that GTP-binding is crucial for normal folding of the SEPT5 central core domain. This hypothesis is sustained further by a new report suggesting that GTP binding plays a structural role rather than a regulatory role in septins [21].

A definitive role for the GTPase domain is still less than clear. SEPT9 (MSF-A or MLL septin-like fusion-A) with a mutation analogous to SEPT5^{S58N} is able to form filaments in COS cells [34]; a similar mutation in SEPT2 significantly inhibits filament formation in HeLa cells [6]. Mendoza et al. [35] have shown that filament formation in the *Xenopus* septin, SEPT2, requires GTP binding, but not GTP hydrolysis. Together, these results are suggestive of highly unique roles for individual septins where specific regions, such as the GTPase domain, may or may not be required for normal function [12].

As discussed above, the N- and C-terminal regions of the examined proteins are required for homotypic interactions. To characterize further the involvement of the motifs located within the N- and C-terminal regions, we mutated Ser³⁸ in the N-terminal region of SEPT5. This residue is the only potential protein kinase C phosphorylation site of SEPT5 located outside the central core domain. In platelets, SEPT5 is phosphorylated upon activation

with different agonists [8]. Our results do not support phosphorylation of SEPT5 at Ser³⁸ as being critical for any of the examined septin–septin interactions. The situation appears to be dynamic in yeast, where septin phosphorylation can modulate macromolecular complex formation [36,37].

Another important domain at the N-termini of most septins is a polybasic region located upstream to the G1 box of the GTP-exchange domain. SEPT4 interacts with different phosphoinositides through the polybasic region, suggesting that this motif would be important for the interaction between septins and their target membranes. A double mutation in this motif completely blocks binding to PtdIns(4,5)P₂ [20]. We decided to test whether this motif would have an additional role in septin–septin interaction by engineering a homologous double mutation in SEPT5, SEPT5^{K40A;K41A}. This mutation was unable to block the SEPT5 homotypic interaction (Table 4), showing that the polybasic region is not directly involved in homotypic assembly.

We also characterized mutations within the C-terminal domain of each septin, a region predicted to contain a coiled-coil motif. The results suggested the participation of the C-terminal domain in homotypic assembly, yet we were unable to demonstrate a direct homotypic interaction with isolated polypeptides spanning the same region. Although SEPT4, SEPT5 and SEPT8 coiled-coils are not sufficient in establishing a direct homotypic interaction, the coiled-coil domain of the yeast septin Cdc12 has been reported to be sufficient, indicating that our results cannot be generalized to all the septin family members [17]. The coiled-coil deletion of any of the three septins assayed in this work also demonstrates that this domain is dispensable for heterotypic assembly. However, this result contrasts with a new study implicating coiled-coil domains in the heterotypic interactions for mammalian septins SEPT6 and SEPT7 [18]. Although our results shed light on the role of SEPT4, SEPT5 and SEPT8 coiled-coil domains, more studies will be required to elucidate the specific mechanisms involved. For example, the importance of the N- and C-terminal regions in homotypic interactions may be that these regions help to create new tertiary motifs, not required for heterotypic interactions, but essential in the formation of homotypic dependent quaternary structures.

Measured by β -gal levels, the SEPT5–SEPT8 and SEPT4–SEPT8 interactions are stronger than the SEPT4–SEPT5 interaction. In fact, the strength of the SEPT4–SEPT5 interaction was similar to those obtained for the SEPT4, SEPT5 and SEPT8 homotypic interactions. SEPT5 and SEPT4 belong to group III of the septin family, while SEPT8 belongs to group II (Figure 1). Septin heterotypic assembly has been documented among different species where three to four different septin polypeptides can associate [5,38,39], and, *in vitro*, where heterotypic septin complexes polymerize into filaments [11,40]. Although potential heterotypic interaction between members of the same group have been documented to occur *in vivo* [41,42], our data, together with those of others, suggest that septins from one given group interact preferentially with members that belong to a different group. For instance, different reports have shown that SEPT9 (group I), SEPT6 (group II), SEPT2 (group III) and SEPT7 (group IV) are co-immunoprecipitated from an NIH3T3 cell lysate [39] and from a HeLa cell lysate [42,43]. Sheffield et al. [18] have studied the interaction between SEPT2, SEPT6 and SEPT7 further. Their study reported that these three septins form a heterotrimer, and that, taken in pairs, these septins can also form heterodimers. In *Drosophila*, four septins have been described, Pnut, Sep1, Sep2 and Sep5. *Drosophila* Sep2 and Sep5 share sequence similarity with members of the mammalian group II [10], Sep1 with mammalian group III, and Pnut with group IV. Different studies have reported that Pnut, Sep1 and Sep2 co-localize at sites of

embryo cellularization [5,44]. However, the steps leading to septin macromolecular complexes assembly and/or filament formation have to be investigated in more detail. Septin complexes immunoprecipitated from mouse brain contain at least eight different septin monomers, but the exact composition of a single complex has yet to be defined [42]. Thus studying how homotypic and heterotypic interactions are temporally regulated during human septin assembly becomes a first step in understanding septin function.

In platelets, SEPT5 has been localized to membranous areas around the α -granule [8], and in neurons, SEPT5 is co-purified with the sec6–sec8 complex and synaptic vesicles [7,45]. In both cases, SEPT5 has been shown to have a regulatory role in the exocytic process associated with platelet activation or neurotransmitter release. When co-expressed, SEPT5 and SEPT8 relocate from a cytoplasmic localization to a punctate distribution. Sheffield et al. [18] have recently shown that Borg3, a small adapter protein that is downstream of the Cdc42 GTPase, specifically binds to SEPT6–SEPT7 heterodimers and to the heterotrimer SEPT2–SEPT6–SEPT7, but not to isolated septin monomers. In the case of the SEPT5–SEPT8 assembly, their association may be critical for recruiting other proteins to the vesicle membrane that have been shown to bind SEPT5, such as the sec6–sec8 complex and syntaxins, which are important members of the vesicle transport machinery [7,8,45]. Thus the macromolecular assembly of different septin complexes appears to be a prerequisite for specific cellular localization and may play a role in the recruitment of additional effectors that support various cellular events.

Future studies on the biochemical properties and regions involved in septin homotypic and heterotypic interactions will give new insights into the mechanisms involved in macromolecular complex formation. Indeed, our results demonstrate that heterotypic assembly is critical for specific cellular localization of complexes. We also document homotypic assembly, but whether or not this process is essential for septin function has not been established. Further studies will define and validate the existence and physiological relevance of heterotypic and homotypic septin interactions, and their prerequisite formation that leads to higher-order complexes.

This work was funded by a grant from the NHLBI (National Heart, Lung, and Blood Institute) of the National Institutes of Health, HL69951. C. M. received a postdoctoral fellowship from the Spanish Ministerio de Educación y Ciencia. The administrative assistance of Pamela Fagan is greatly appreciated. The authors acknowledge the Sam and Rose Stein Charitable Trust for the establishment of the DNA Core Facility within the Department of Molecular and Experimental Medicine at The Scripps Research Institute.

REFERENCES

- Hartwell, L. H., Culotti, J., Pringle, J. R. and Reid, B. J. (1974) Genetic control of the cell division cycle in yeast. *Science* **183**, 46–51.
- Byers, B. and Goetsch, L. (1976) A highly ordered ring of membrane-associated filaments in budding yeast. *J. Cell Biol.* **69**, 717–721.
- Fares, H., Goetsch, L. and Pringle, J. R. (1996) Identification of a developmentally regulated septin and involvement of the septins in spore formation in *Saccharomyces cerevisiae*. *J. Cell Biol.* **132**, 399–411.
- Longtine, M. S. and Bi, E. (2003) Regulation of septin organization and function in yeast. *Trends Cell Biol.* **13**, 403–409.
- Neufeld, T. P. and Rubin, G. M. (1994) The *Drosophila* peanut gene is required for cytokinesis and encodes a protein similar to yeast putative bud neck filament proteins. *Cell* **77**, 371–379.
- Kinoshita, M., Kumar, S., Mizoguchi, A., Ide, C., Kinoshita, A., Haraguchi, T., Hiraoka, Y. and Noda, M. (1997) Nedd5, a mammalian septin, is a novel cytoskeletal component interacting with actin-based structures. *Genes Dev.* **11**, 1535–1547.
- Beites, C. L., Xie, H., Bowser, R. and Trimble, W. S. (1999) The septin Cdc42 binds syntaxin and inhibits exocytosis. *Nat. Neurosci.* **2**, 434–439.
- Dent, J., Kato, K., Peng, X.-R., Martínez, C., Cattaneo, M., Poujol, C., Nurden, P., Nurden, A. T., Trimble, W. S. and Ware, J. (2002) A prototypic platelet septin and its participation in platelet secretion. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 3064–3069.
- Trimble, W. S. (1999) Septins: a highly conserved family of membrane-associated GTPases with functions in cell division and beyond. *J. Membr. Biol.* **169**, 75–81.
- Kartmann, B. and Roth, D. (2001) Novel roles for mammalian septins: from vesicle trafficking to oncogenesis. *J. Cell. Sci.* **114**, 839–844.
- Field, C. M., al Awar, O., Rosenblatt, J., Wong, M. L., Alberts, B. and Mitchison, T. J. (1996) A purified *Drosophila* septin complex forms filaments and exhibits GTPase activity. *J. Cell Biol.* **133**, 605–616.
- Field, C. M. and Kellogg, D. (1999) Septins: cytoskeletal polymers or signalling GTPases? *Trends Cell Biol.* **9**, 387–394.
- Macara, I. G., Baldarelli, R., Field, C. M., Glotzer, M., Hayashi, Y., Hsu, S. C., Kennedy, M. B., Kinoshita, M., Longtine, M., Low, C. et al. (2002) Mammalian septins nomenclature. *Mol. Biol. Cell* **13**, 4111–4113.
- Sui, L., Zhang, W., Liu, Q., Chen, T., Li, N., Wan, T., Yu, M. and Cao, X. (2003) Cloning and functional characterization of human septin 10, a novel member of septin family cloned from dendritic cells. *Biochem. Biophys. Res. Commun.* **304**, 393–398.
- Kinoshita, M. (2003) Assembly of mammalian septins. *J. Biochem. (Tokyo)* **134**, 491–496.
- Longtine, M. S., DeMarini, D. J., Valencik, M. L., Al-Awar, O. S., Fares, H., De Virgilio, C. and Pringle, J. R. (1996) The septins: roles in cytokinesis and other processes. *Curr. Opin. Cell Biol.* **8**, 106–119.
- Lee, P. R., Song, S., Ro, H. S., Park, C. J., Lippincott, J., Li, R., Pringle, J. R., De Virgilio, C., Longtine, M. S. and Lee, K. S. (2002) Bni5p, a septin-interacting protein, is required for normal septin function and cytokinesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **22**, 6906–6920.
- Sheffield, P. J., Oliver, C. J., Kremer, B. E., Sheng, S., Shao, Z. and Macara, I. G. (2003) Borg/Septin interactions and the assembly of mammalian septin heterodimers, trimers, and filaments. *J. Biol. Chem.* **278**, 3483–3488.
- Casamayor, A. and Snyder, M. (2003) Molecular dissection of a yeast septin: distinct domains are required for septin interaction, localization, and function. *Mol. Cell. Biol.* **23**, 2762–2777.
- Zhang, J., Kong, C., Xie, H., McPherson, P. S., Grinstein, S. and Trimble, W. S. (1999) Phosphatidylinositol polyphosphate binding to the mammalian septin H5 is modulated by GTP. *Curr. Biol.* **9**, 1458–1467.
- Vrabioiu, A. M., Gerber, S. A., Gygi, S. P., Field, C. M. and Mitchison, T. J. (2004) The majority of the *Saccharomyces cerevisiae* septin complexes do not exchange guanine nucleotides. *J. Biol. Chem.* **279**, 3111–3118.
- Blaser, S., Jersch, K., Hainmann, I., Wunderle, D., Zgaga-Griesz, A., Busse, A. and Zieger, B. (2002) Human septin–septin interaction: CDCrel-1 partners with KIAA0202. *FEBS Lett.* **519**, 169–172.
- Blaser, S., Jersch, K., Hainmann, I., Zieger, W., Wunderle, D., Busse, A. and Zieger, B. (2003) Isolation of new splice isoforms, characterization and expression analysis of the human septin SEPT8 (KIAA0202). *Gene* **312**, 313–320.
- Zieger, B., Hashimoto, Y. and Ware, J. (1997) Alternative expression of platelet glycoprotein Ib mRNA from an adjacent 5' gene with an imperfect polyadenylation signal sequence. *J. Clin. Invest.* **99**, 520–525.
- Zieger, B., Tran, H., Hainmann, I., Wunderle, D., Zgaga-Griesz, A., Blaser, S. and Ware, J. (2000) Characterization and expression analysis of two human septin genes, *PNUTL1* and *PNUTL2*. *Gene* **261**, 197–203.
- Kippert, F. (1995) A rapid permeabilization procedure for accurate quantitative determination of β -galactosidase activity in yeast cells. *FEMS Microbiol. Lett.* **128**, 201–206.
- Schultz, J., Milpetz, F., Bork, P. and Ponting, C. P. (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5857–5864.
- Letunic, I., Goodstadt, L., Dickens, N. J., Doerks, T., Schultz, J., Mott, R., Ciccarelli, F., Copley, R. R., Ponting, C. P. and Bork, P. (2002) Recent improvements to the SMART domain-based sequence annotation resource. *Nucleic Acids Res.* **30**, 242–244.
- Cooper, J. A. and Kiehart, D. P. (1996) Septins may form a ubiquitous family of cytoskeletal filaments. *J. Cell Biol.* **134**, 1345–1348.
- Sonhammer, E. L. and Wootton, J. C. (2001) Integrated graphical analysis of protein sequence features predicted from sequence composition. *Proteins* **45**, 262–273.
- Wright, P. E. and Dyson, H. J. (1999) Intrinsically unstructured proteins: re-assessing the protein structure–function paradigm. *J. Mol. Biol.* **293**, 321–331.
- Hanks, S. K. and Hunter, T. (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* **9**, 576–596.
- Rao, V. D., Misra, S., Boronnikov, I. V., Anderson, R. A. and Hurley, J. H. (1998) Structure of type II β phosphatidylinositol phosphate kinase: a protein kinase fold flattened for interfacial phosphorylation. *Cell* **94**, 829–839.

- 34 Nagata, K., Kawajiri, A., Matsui, S., Takagishi, M., Shiromizu, T., Saitoh, N., Izawa, I., Kiyono, T., Itoh, T. J., Hotani, H. and Inagaki, M. (2003) Filament formation of MSF-A, a mammalian septin, in human mammary epithelial cells depends on interactions with microtubules. *J. Biol. Chem.* **278**, 18538–18543
- 35 Mendoza, M., Hyman, A. A. and Glotzer, M. (2002) GTP binding induces filament assembly of a recombinant septin. *Curr. Biol.* **12**, 1858–1863
- 36 Tang, C. S. and Reed, S. I. (2002) Phosphorylation of the septin cdc3 in g1 by the cdc28 kinase is essential for efficient septin ring disassembly. *Cell Cycle* **1**, 42–49
- 37 Dobbelaere, J., Gentry, M. S., Hallberg, R. L. and Barral, Y. (2003) Phosphorylation-dependent regulation of septin dynamics during the cell cycle. *Dev. Cell* **4**, 345–357
- 38 Longtine, M. S., Fares, H. and Pringle, J. R. (1998) Role of the yeast Gin4p protein kinase in septin assembly and the relationship between septin assembly and septin function. *J. Cell Biol.* **143**, 719–736
- 39 Joberty, G., Perlungher, R. R., Sheffield, P. J., Kinoshita, M., Noda, M., Haystead, T. and Macara, I. G. (2001) Borg proteins control septin organization and are negatively regulated by Cdc42. *Nat. Cell Biol.* **3**, 861–866
- 40 Frazier, J. A., Wong, M. L., Longtin, M. S., Pringle, J. R., Mann, M., Mitchison, T. J. and Field, C. (1998) Polymerization of purified yeast septins: evidence that organized filament arrays may not be required for septin function. *J. Cell Biol.* **143**, 737–749
- 41 Peng, X.-R., Jia, Z., Ware, J. and Trimble, W. S. (2002) The septin CDCrel-1 is dispensable for normal development and neurotransmitter release. *Mol. Cell Biol.* **22**, 378–387
- 42 Kinoshita, M., Field, C. M., Coughlin, M. L., Straight, A. F. and Mitchison, T. J. (2002) Self- and actin-templated assembly of mammalian septins. *Dev. Cell* **3**, 791–802
- 43 Surka, M. C., Tsang, C. W. and Trimble, W. S. (2002) The mammalian septin MSF localizes with microtubules and is required for completion of cytokinesis. *Mol. Biol. Cell* **13**, 3532–3545
- 44 Adam, J. C., Pringle, J. R. and Peifer, M. (2000) Evidence for functional differentiation among *Drosophila* septins in cytokinesis and cellularization. *Mol. Biol. Cell* **11**, 3123–3135
- 45 Hsu, S. C., Hazuka, C. D., Roth, R., Foletti, D. L., Heuser, J. and Scheller, R. H. (1998) Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments. *Neuron* **20**, 1111–1122

Received 8 March 2004/3 June 2004; accepted 23 June 2004

Published as BJ Immediate Publication 23 June 2004, DOI 10.1042/BJ20040372