Microtubules support a disk-like septin arrangement at the plasma membrane of mammalian cells

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ABSTRACT Septin family proteins oligomerize through guanosine 5’-triphosphate–binding domains into core heteromers, which in turn polymerize at the cleavage furrow of dividing fungal and animal cells. Septin assemblies during the interphase of animal cells remain poorly defined and are the topic of this report. In this study, we developed protocols for visualization of authentic higher-order assemblies using tagged septins to effectively replace the endogenous gene product within septin core heteromers in human cells. Our analysis revealed that septins assemble into microtubule-supported, disk-like structures at the plasma membrane. In the absence of cell substrate adhesion, this is the predominant higher-order arrangement in interphase cells and each of the seven to eight septin family members expressed by the two analyzed cell types appears equally represented. However, studies of myeloid and lymphoid cell model systems revealed cell type–specific alterations of higher-order septin arrangements in response to substrate adhesion. Live-cell observations suggested that all higher-order septin assemblies are mutually exclusive with plasma membrane regions undergoing remodeling. The combined data point to a mechanism by which densely arranged cortical microtubules, which are typical for nonadhered spherical cells, support plasma membrane–bound, disk-like septin assemblies.

INTRODUCTION

Septins comprise a family of guanosine 5’-triphosphate (GTP)-binding proteins implicated in membrane compartmentalization, cell polarity, and cytokinesis in fungal and animal cells [reviewed in Oh and Bi (2011)]. The deciphering of molecular mechanisms has been hampered by the multitude of septin genes, with seven in most yeast species and nine to 17 in vertebrates (Pan et al., 2007). Animal septin family members fall into independent evolutionary groups (Kinoshita, 2003b). Mammals have 13 genes encoding both ubiquitous and tissue-specific septins (SEPT1-12, and SEPT14 in humans; note that the former SEPT13 is pseudogene SEPT7P2), which have been classified into four homology subgroups named for the founding members SEPT2, SEPT3, SEPT6, and SEPT7 (Kinoshita, 2003a). All four subgroups appear to be represented in vertebrates but not in animals belonging to nonchordate phyla (Cao et al., 2007).

Septins assemble into core heteromer units, which in turn serve as building blocks of higher-order structures (reviewed in Weirich et al. (2008)). When coexpressed in Escherichia coli, human SEPT2, SEPT6, and SEPT7 assemble into apolar six-subunit rods, arranged as a dimer of trimers with SEPT7 at the ends. In a manner similar to authentic core heteromers, these six-subunit rods polymerize into filaments at low ionic strength (Sheffield et al., 2003; Sirajuddin et al., 2007). Recombinant coexpression of four specific septins of budding yeast has resulted in the characterization of a cognate eight-subunit core heteromer arranged as a perfect palindrome (Bertin et al., 2008). Crystals of bacterially expressed human septins as building blocks of higher-order structures (reviewed in Weirich et al. (2008)). When coexpressed in Escherichia coli, human SEPT2, SEPT6, and SEPT7 assemble into apolar six-subunit rods, arranged as a dimer of trimers with SEPT7 at the ends. In a manner similar to authentic core heteromers, these six-subunit rods polymerize into filaments at low ionic strength (Sheffield et al., 2003; Sirajuddin et al., 2007). Recombinant coexpression of four specific septins of budding yeast has resulted in the characterization of a cognate eight-subunit core heteromer arranged as a perfect palindrome (Bertin et al., 2008). Crystals of bacterially expressed human septins have identified universal oligomerization interfaces—denoted as the G-interface and the NC-interface—that are located on opposite sides of the conserved GTP-binding domain (G-domain). These
interfaces mediate assembly into both core heteromers and extended filaments (Sirajuddin et al., 2007, 2009).

All the essential functions of septins in budding yeast depend on filament formation (McMurray et al., 2011). Moreover, septins bind phosphatidylinositol-4,5-bisphosphate (PIP2; Zhang et al., 1999; Casamayor and Snyder, 2003), which may direct filament formation onto the inner leaflet of the plasma membrane (Tanaka-Takiguchi et al., 2009; Bertin et al., 2010). In budding yeast, septins assemble into elaborate lateral diffusion barriers and molecular scaffolds at the bud neck (reviewed in Caudron and Barral (2009)). These higher-order filamentous arrangements are dynamically modified through each cell division cycle and involve both longitudinal and lateral interactions between heteromers. At cell cycle entry, septins establish polarity by forming a ring-like structure at the incipient bud site, which is rearranged into an hourglass-shaped collar around the emerging bud neck, which is followed by the “hourglass-to-split rings” transition during cytokinesis. This transition involves rapid disassembly of paired filaments running parallel to the growth axis, which is followed by reassembly perpendicular to the growth axis (Vrabioiu and Mitchison, 2006; DeMay et al., 2011). While the best understood septin arrangement by far is that of filaments at the fungal bud neck, septins localize to the cleavage furrow during cytokinesis in all cell types across kingdoms, which suggests a conserved role during late stages of cell division (Caudron and Barral, 2009).

Mammalian septins have been ascribed diverse roles during the interphase of the cell cycle, including DNA damage response, chromosome segregation, and exocytosis, as well as tumor and neurological diseases states (reviewed in Peterson and Petty [2010]). Consistent with compartmentalization of the plasma membrane, septins have been shown to localize to the annulus of spermatozoa and at the base of cell appendages, such as cilia and neural spines (Ihara et al., 2005; Kissel et al., 2005; Tada et al., 2007; Xie et al., 2007; Hu et al., 2010). Mammalian septins have been reported to associate to locations at the cell cortex, as well as to actin bundles, phagocytic vesicles, and/or microtubules (Kinosita et al., 2002; Surka et al., 2002; Nagata et al., 2003; Vega and Hsu, 2003; Martinez et al., 2006; Huang et al., 2008). Septins may also form free-floating cytoplasmic rings shortly after drug disruption of actin bundles (Kinoshita et al., 2002). It is also noteworthy that individual septins have been assigned a diverse array of scaffolding functions at locations distal from the cell cortex (Kremer et al., 2005, 2007; Spiliotis et al., 2005; Mostowy et al., 2010). Thus the links between higher-order filamentous assemblies and proposed septin functions in interphase or postmitotic mammalian cells remain poorly understood.

Most mammalian cells express a tissue-specific collection of multiple members and/or isoforms of each of the SEPT2, SEPT3, and SEPT6 subgroups, but a single subgroup member, SEPT7, appears expressed in all cell types (Cao et al., 2007). This has evoked questions concerning the degree of combinational diversity and whether homology-based subgroups predict septin-pairing preferences. By analyzing human cell lines lacking or overexpressing selected septins, we have recently derived a generic model for how subgroup-specific properties direct the subunit arrangement within heteromers (Sellin et al., 2011). That study suggested that individual septins exist solely in the context of stable six- to eight-subunit core heteromers, all of which contain variable SEPT2- and SEPT6-subgroup members together with SEPT7. The subpopulation of eight-subunit heteromers was found to also contain SEPT9—the sole SEPT3-subgroup member expressed by the analyzed cell types. Thus native septin filaments are predictively built from an array of core heteromers, all of which contain SEPT7, and with the variable septin subunits arranged according to subgroup (see Figure 1A, a model in part based on Sirajuddin et al. [2007]).

In this paper, we describe essential premises for live-cell imaging of the septin system in mammals. Our approach involved stably expressing Aequorea coerulescens green fluorescent protein (AcGFP)-fusion derivatives of septins belonging to each one of the four homology subgroups. We noted many caveats of septin-AcGFP reporters, such as an aberrant heteromeric context and formation of ectopic filaments. However, by optimizing conditions for effective replacement of endogenous SEPT7 with SEPT7-AcGFP within heteromers, we achieved specific visualization of various types of heteromer assemblies. Our data, combined with previous studies, suggest a general model for assembly of higher-order septin arrangements at the locations providing the greatest opportunity for binding cooperativity, which depends on both the cell type and external cues.

**RESULTS**

**Septin core heteromers do not exchange their subunits**

As outlined in the Introduction, individual septins in human cell lines appear to exist solely in the context of six- to eight-subunit heteromers in which the subunit arrangement coincides with homology-based subgroups (see Figure 1A). To address whether preexisting heteromers exchange their septin subunits, the heteromeric context of expressed His-tagged SEPT7 (His-SEPT7) was analyzed over time. For regulatable expression in stably transfected human cells, we used the episomally replicating pMEP vector system and the K562 erythroleukemia cell line. By initial suppression followed by induction, a transient burst of expression from the hMTIIa promoter can be achieved (Melander Gradin et al., 1997; Sellin et al., 2008a). Western blots showed inductive expression of His-SEPT7, which migrates above endogenous SEPT7 due to a His tag (Figure 1B). Quantification of Western blots showed that the total amounts of soluble SEPT7 protein—that is, His-SEPT7 (shaded bars) together with endogenous SEPT7 (open bars)—was transiently increased three- to fourfold within 4 h of induction (Figure 1C).

Artificially expressed SEPT7 is known to form insoluble aggregates (Kinosita, 2003a; Sheffield et al., 2003), which we confirmed by boiling insoluble proteins in SDS sample buffer. Western blotting revealed insoluble His-SEPT7 between 4 and 72 h after the induced burst of expression, but not at the final time point on day 4 (Supplemental Figure S1). Thus, at most time points only about one-half of the expressed His-SEPT7 protein is soluble, but insoluble His-SEPT7 aggregates appeared essentially degraded after 4 d.

It is evident from Figure 1, B and C, that induced His-SEPT7 expression caused a gradual decline of the endogenous gene product, while SEPT2, SEPT6, and SEPT9 remained constant. This is consistent with excess His-SEPT7 competing for limited amounts of heterooligomerization partners, and with the nonheteromeric SEPT7 protein being unstable, as previously found (Sellin et al., 2011). Given that K562 cells double approximately every 20 h, endogenous SEPT7 declined at a rate compatible with dilution by newly synthesized and successfully heterooligomerized His-SEPT7 during each cell division. This suggests that the core heteromer assembly process is cotranslational and irreversible.

To determine the heteromeric context of His-SEPT7 over time, we fractionated crude cell extracts by density-gradient centrifugation. High-salt cell extraction buffers and gradients were prepared that disassembled all higher-order septin structures in K562 cells into pools of soluble six- to eight-subunit heteromers (Sellin et al., 2011). By this means, the fraction of His-SEPT7 containing heteromers could be determined. Figure 1D shows that the initial accumulation...
Differential assembly states of expressed AcGFP-tagged, homology-based subgroup representatives

The above data show that unbalanced septin expression may result in aggregation and that a tagged septin derivative cannot be expected to become incorporated into preexisting heteromers. Thus live-cell imaging of the septin system requires fluorescent septin reporters to be expressed at modest levels over many cell doublings. Accordingly, the fluorescent reporters analyzed in Figure 2, which includes a representative of each of the four septin homology-based subgroups tagged at the C-terminus with AcGFP, were expressed by the low basal activity of the hMTIIa promoter of the replicating pMEP vector over a time period corresponding to at least seven cell doublings. Under these circumstances, the contents of SEPT2-, SEPT6-, SEPT7- and SEPT9-AcGFP in cleared lysates of the transfected K562 cell lines appear roughly the same (Figure 2A).

On the basis of mRNA analysis of all septin family members, we have found that K562 cells express significant levels of seven endogenous septins (Sellin et al., 2011). As indicated in Figure 2A, these include two and three members of the SEPT2 and SEPT6 subgroup, respectively. It is noteworthy that SEPT6-AcGFP expression caused a decreased content of all the endogenous SEPT6 subgroup members, that is, SEPT6/8/11, while other septins remained unaltered. Moreover, SEPT7-AcGFP expression caused a selective decrease of the single subgroup member SEPT7. Consistent with our previous report (Sellin et al., 2011), these data suggest subgroup-restricted competition for limiting amounts of heterooligomerization partners. However, expression of SEPT2-AcGFP or SEPT9-AcGFP had little or no effect on the levels of any of the endogenous septins (Figure 2A), which suggests nonsuccessful competition with the cognate endogenous septins.

Endogenous septins appear to exist solely in the context of six- to eight-subunit heteromers (Sellin et al., 2011), which are resolved as a single peak by the present density-gradient centrifugation protocol (note coinciding peaks in Figure 2B). On the basis of detection of representatives of each of the four homology subgroups, as well as the AcGFP-reporter, we found that heteromers derived from SEPT7-AcGFP–expressing cells sedimented similarly to heteromers in untransfected control cells, that is, as single overlapping peaks (compare Figure 2, B and E; note that a 26.9-kDa AcGFP reporter is too small to alter heteromer peak positions). In contrast, however, other AcGFP-tagged septins also exist in a nonheteromeric context and SEPT9-AcGFP (Figure 2F) almost completely failed to assemble with endogenous septins. The hydrodynamic parameters (i.e., the Stokes’ radius and sedimentation coefficient, as described in Sellin et al. [2011]) of the nonheteromeric forms indicate that SEPT2-AcGFP exists as a homodimer, while SEPT6- and SEPT9-AcGFP exist as monomers (unpublished data).
Figure 2 shows that AcGFP-fused septin derivatives differ in their proficiency in competing with cognate endogenous septins for heterooligomerization partners, as well as in their persistence in a non-heteromeric context. According to these data, only SEPT7-AcGFP qualifies as a bona fide specific reporter of the localization of endogenous core heteromers. Hence SEPT7-AcGFP can be expected to visualize the complete core heteromer pool without altering the composition of other septins. Epifluorescence analysis of the SEPT7-AcGFP-expressing K562 cell line described in Figure 2 revealed localization of septin assemblies that seemed consistent with immunostaining of SEPT7 in methanol-fixed, nontransfected cells, but with superior resolution and sensitivity (Figure 3, A and B). Thus SEPT7-AcGFP localized mainly to the cleavage furrow during cytokinesis and appeared as distinct cortical assemblies in interphase cells. In contrast, the other AcGFP-reporters described in Figure 2 were prone to form ectopic filaments and aggregates, and/or appeared as nonlocalized in the cytosol (Figures S2 and S3).

Previous reports on septin localization have focused on adhesion substrate-dependent cell types. However, K562 cells lack functional integrins on the surface and are consequently nonadherent and have a uniform spherical shape (Muller et al., 1995), which provides spatial separation across the cortex. This simplified characterization of cortical septin assemblies, which were the predominant higher-order septin arrangement detected in interphase cells. Optical sectioning of live cells revealed disk-like submembranous structures (compare the section at the cell equator with the section parallel to the plasma membrane in Figure 3B). These structures appear less distinct in fixed and immunostained cells (compare Figure 3, A and B). Inspection of live cells revealed a central clearing in many of the disk-like structures (see enlargement in Figure 3B and intensity profiles in Figure S4A), that is consistent with closed septin filaments. The disk-like structures appeared uniformly sized ($\sim$0.8 μm; Figure 3C), and optical sectioning of interphase cells suggested an even cortical distribution (Figure S4B). Based on analysis of consecutive optical sections, the number of disk-like structures was estimated to be 141 ± 29 per cell (Figure S4C).

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consistent with septin filament rearrangement prior to cytokinesis and the gradual reassembly of cortical disk-like structures after each cell division.

The present evaluation of reporter systems for imaging of septins in human cells revealed several examples of maladies associated with artificially expressed septin-fusion derivatives, which are discussed in the description of the data in Figures S2 and S3. However, our protocol for effective replacement of endogenous SEPT7 with SEPT7-AcGFP provides a system that fulfills the essential criteria of a specific reporter system. This facilitated detection of uniformly sized structures that, because of the impression of disks attached flat against the plasma membrane, are hereafter referred to as “septin disks.”

The stability of septin disks is dependent on intact microtubules
In most cell types, septin assemblies appear to localize in conjunction with the plasma membrane, but cytosolic localization in association to actin bundles and/or microtubules has also been reported. Interphase K562 cells are spherical and have a dense array of microtubules that extend all along the cortex (optical sections at either the equatorial plane or parallel to the plasma membrane are shown in Figure 4A, Control). Moreover, filamentous actin localizes to both the cortex and cytosolic structures surrounding the Golgi apparatus (Figure S5). Still, we detected localization of septins to punctuate cortical assemblies only in interphase K562 cells (Figures 3 and S4). Interestingly, we found that nocodazole-mediated depolymerization of microtubules caused a gradual disintegration of these septin assemblies (Figure 4). As a consequence, most cells did not display any clearly resolved punctuate assemblies after 30 min, and septins appeared relatively evenly distributed at the cortex at this stage. A view perpendicular to the plasma membrane gave an impression of a diffuse array of minute septin assemblies (unpublished data), which suggests that most core heteromers at the cell cortex retain some higher-order filamentous structure.

Mammalian microtubules are renowned to be unstable at low temperatures and, similar to nocodazole treatment, cooling on ice was found to cause both microtubule depolymerization and disintegration of septin disks (Figure 4A). Under these conditions, the septins also remained localized at the cell cortex. For comparison, cytochalasin D–treated K562 cells were also analyzed and showed that septin disks remain intact after disruption of the filamentous actin that surrounds the cortex (Figure S5). Thus microtubules, but not filamentous actin, are essential for septin disks at the cell cortex of nonadherent K562 cells.

To explore the interdependency of the microtubule and septin systems, K562 cells were depleted of their respective protomer units. Our experimental strategies relied on Epstein-Barr virus (EBV)-based replicating vectors directing expression of either a short-hairpin RNA (shRNA) targeting SEPT7 mRNA (shRNA-SEPT7) or a cDNA encoding the tubulin heterodimer disrupting protein cofactor E (pMEP-TBCE-FLAG; Bhamidipati et al., 2000). All native heteromers in K562 cells contain SEPT7, and we have found that SEPT7 depletion results in unstable aberrant complexes of SEPT2 and SEPT6-subgroup members and monomeric SEPT9 (Sellin et al., 2011). Consistent with that report, Figure 5A shows that 1 wk of shRNA-SEPT7 expression depletes >90% of SEPT7; this depletion was associated with decreased content of all other septins, as would be anticipated. However, analyses of microtubule content and stability in these cells did not reveal any effects of septin depletion (note indistinguishable nocodazole dose responses in Figure 5B), which contrasts to shRNA-mediated depletion of either of the two well-characterized microtubule regulatory proteins oncoprotein 18/Stathmin (Op18) or microtubule-associated protein 4 (MAP4; Figure S6). We also analyzed consequences of artificial overexpression, but observed no effect of excessive septin levels on microtubules (Figure S7). In contrast, depletion of tubulin heterodimers, as observed within 8 h of induced TBCE expression (Figure 5C), resulted in complete disintegration of punctuate cortical septin assemblies in most cells (Figure 5D). Thus, while confirming the microtubule dependence of septin disks, the present data do not hint at a reciprocal dependency.

Microtubule stabilization preserves septin disks in permeabilized cells
The microtubule-stabilizing drug Taxol promotes rapid polymerization, as well as a gradual accumulation of large microtubule bundles in K562 cells (Holmfeldt et al., 2002). Figure 6A shows that Taxol rapidly increased the fraction of polymerized tubulin heterodimers in K562 cells to nearly 100%. In observing SEPT7-AcGFP localization in Taxol-treated cells, we did not note any clear-cut, immediate effects (Figure 6B, 3 min Taxol), but a gradual clustering of septin disks to one side of the spherical cortex became noticeable within 60 min (Figure 6B, 60 min Taxol). Thereafter, an increasing fraction of cells appeared in which all SEPT7-AcGFP was localized to elongated structures (Figure 6, A and B, 4 h Taxol). Co-staining of tubulin revealed that these obviously aberrant structures consisted of the extensive microtubule bundles that gradually form during prolonged Taxol treatment (Figure S8). This is indeed consistent with septin localization to microtubule bundles in Taxol-treated epithelial cells (Bowen et al., 2011), as well as localization to the circumferential
Septin assemblies in mammalian cells

Punctuate cortical septin assemblies accumulate in the uropod during amoeboid movement

To address septin localization and dynamics in a physiological context, we used the SEPT7-AcGFP reporter in combination with the Jurkat cell line, which provides a well-characterized T-lymphocyte model system. Phase-contrast and fluorescence microscopy of albumin-plated, live Jurkat cells—that is, nonmotile control cells—revealed a spherical shape with uniformly sized punctuate septin assemblies (Figure 8A). Similar to K562 (Figures 3 and S4B), inspection parallel to the plasma membrane of interphase cells showed cortical septin disks (Figure 8A). Moreover, analyses of permeabilized Jurkat cells confirmed that these septin assemblies are preserved by microtubule stabilization (Figure S10). Thus the ø-0.8-μm disk-like arrangement characterized in undifferentiated K562 cells is also predominant in a nonactivated differentiated cell type that grows in suspension.

Plating on invasin, an integrin-activating bacterial protein, stimulates polarization and amoeboid movement of T lymphocytes (Arenencia et al., 1997). Figure 8B, left, shows a phase-contrast image of a Jurkat cell migrating on an invasin-coated surface. Letters indicate the trailing uropod (U), which lacks substrate microtubule coat in platelets (Martinez et al., 2006). Thus septins may have an innate affinity for microtubules (or associated proteins), which can be expected to gain in avidity with multiple-bond interactions on bundles.

Permeabilization of K562 cells at physiological ionic strength results in disassembly of both the microtubule and septin systems, and their respective protomer units are released into the media (Sellin et al., 2011). To further address the microtubule dependence of septin disks, we analyzed the effect of a brief Taxol exposure prior to permeabilization of K562 cells. Epifluorescence analysis of permeabilized control cells showed the expected release of all cell-associated SEPT7-AcGFP, as well as tubulin (compare fixation prior or subsequent to permeabilization in Figure 7A, Control). Significantly, Taxol exposure prior to permeabilization not only resulted in the expected stabilization of microtubules, but also in preservation of septin disks (Figure 7A, 3 min Taxol).

Taxol-mediated preservation of septin disks in permeabilized cells provided a means for a semiquantitative analysis of their septin composition. Accordingly, K562 cells were permeabilized in the absence and presence of Taxol and the partitioning of individual septins between soluble (i.e., released) and insoluble (i.e., cell-associated) states was evaluated by Western blotting (Figure 7B). The data show that, while most tubulin was released by permeabilized non-treated cells, a brief Taxol exposure was sufficient for recovery of most tubulin in the cell-associated fraction. Significantly, while completely released by nontreated cells, ~50% of all septins were recovered in the cell-associated fraction in the presence of Taxol (Figure 7B). Quantification of Western blots by serial dilution of cell lysates suggested that all individual septins are indistinguishable with respect to their partitioning between soluble and insoluble states (Figure 7C).

The present data suggest that all septin family members contribute equally to microtubule-supported septin disks. In line with this notion, our analysis of cells depleted of selected septins (by means of shRNA vectors) failed to identify any individual septin of particular significance for microtubule-dependent stabilization of septin disks (Figure S9). Thus these microtubule-supported septin assemblies appear independent of the specific septin composition of K562 cells.
attachment, and the substrate-attached pseudopodium (P) at the leading edge. The fluorescence image of the same cell reveals the absence of septin assemblies within the pseudopodium and accumulation of punctuate structures at the cortex of the uropod (a section across the uropod is shown in Figure 8B). Owing to being crowded in a confined area, septin assemblies cannot be readily distinguished as discrete structures. Inspections of optical sections did not provide any evidence for extended filaments in the uropod or at any other cellular locations (unpublished data).

Amoeboid movement is characterized by dynamic, actin-rich protrusions at the leading edge. To visualize actin dynamics, we used the actin filament–binding Lifeact peptide fused to DsRed monomer (Riedl et al., 2008). Coexpression of this red fluorescence reporter with SEPT7-AcGFP confirms abundant actin filaments in pseudopodial protrusions at the leading edge, that is, spatially exclusive with higher-order septin structures, which accumulate at the cortex of the trailing uropod.

Cell adhesion and spreading transforms septin disks into heterogeneous assemblies

K562 lacks cell surface β1 integrins, which can be amended by introducing an appropriate α chain. To analyze the fate of septin disks after cell spreading on a surface coated with integrin-activating invasin, we therefore used K562 transfected with an integrin α8 chain (termed K562-KA8), which reconstitutes a functional integrin complex (Mueller et al., 1995). SEPT7-AcGFP–expressing K562-KA8 cells appeared indistinguishable from parental K562 with respect to spherical shape and even distribution of septin disks when plated on bovine serum albumin (BSA; unpublished data). However, K562-KA8 cells spread and become flattened if plated on invasin (Holmfeldt et al., 2002). This is shown in Figure 9A, which presents a phase-contrast image, as well as an adhesion-substrate proximal section of a SEPT7-AcGFP–expressing K562-KA8 cell.

Inspection of septin assemblies revealed that the great majority of septin disks transformed into a variety of arrangements along cell edges and adjacent to the substrate attachment. The enlarged view in Figure 9A also shows that the punctuate assemblies were heterogeneous in size and shape, and in most cases smaller than the uniformly sized septin disks. Some of the punctuate assemblies had a central clearing, but these structures were also heterogeneous in size and shape (Figure S11). Thus septin assemblies in invasin-plated cells resembled the heterogeneous peripheral and punctuate septin arrangements previously described in adhesion substrate–dependent cell lines (reviewed in Lindsey and Momany [2006]).

K562-KA8 cells plated on an invasin-coated surface remained nonmotile, but had abundant filopodia at cell edges (see phase-contrast image in Figure 9A). These were continuously sprouting and readily visualized by a yellow fluorescent protein (YFP)–actin reporter, but inspection of individual filopodia in SEPT7-AcGFP–expressing cells revealed the absence of detectable septin assemblies (Figure 9B).

The pseudopodia of migrating Jurkat cells lacked septin assemblies at edges adjacent to the invasin attachment substrate, which contrasted to prominent septin assemblies along the edges of K562-KA8 cells (compare Figures 8 and 9). This may reflect that K562-KA8 cells are nonmotile and that extensive membrane remodeling is confined to locations of sprouting filopodia. To directly explore the relationship between membrane dynamics and the localization of containing SEPT7-AcGFP fluorescence (Figure 8D). Thus, during amoeboid movement, regions characterized by dynamic actin polymerization and consequent plasma membrane remodeling appear mutually exclusive with higher-order septin structures, which accumulate at the cortex of the trailing uropod.
higher-order septin filamentous structures, we studied K562-KA8 cells in which filopodia were visualized by labeling the outside of cells with a red fluorescence–labeled lectin. As is evident in the enlarged view in Figure 9C, the bases of filopodia were not surrounded by septins. Instead, it appears that filopodia only emerged at locations devoid of higher-order septin filamentous structures (note alternating green and filopodia-associated red fluorescence along the cell edge in the overlay shown in Figure 9C). Thus all the septin arrangements observed upon invasin-plating of Jurkat and K562-KA8 cells appeared mutually exclusive with plasma membrane regions undergoing extensive remodeling.

**Septin disks appear excluded from endocytotic vesicles**

For simultaneous visualization of septin disks and endocytosis via clathrin-coated pits in living cells, Alexa Fluor 594–labeled transferrin (red fluorescence) was added to SEPT7-AcGFP–expressing K562 cells. As expected, epifluorescence microscopy revealed continuous formation of endocytotic vesicles, and vesicles gathered at the area of the Golgi apparatus within a few minutes (Figure 10A). In contrast, septin disks were only detected at the cell cortex. As anticipated from the presented image, real-time observation of cells did not reveal any vesicles containing detectable SEPT7-AcGFP fluorescence (unpublished data).

To explore whether internalization of septin disks can be provoked by artificial clustering of an abundant transmembrane cell surface protein, we exposed K562 cells to anti-CD46 antibodies for 40 min, and then incubated them with cross-linking DyLight 549–labeled secondary antibodies at 37°C. Figure 10B shows that essentially all the secondary antibodies were residing in endocytotic vesicles within 30 min but, even under these cross-linking conditions, we only detected SEPT7-AcGFP fluorescence at the cell cortex. Thus septin assemblies were not under any circumstances detected in association with endocytotic vesicles.

**DISCUSSION**

Previous reports on the organization of the mammalian septin system have focused on polarized and/or adhesion substrate–dependent cell types. This study examines two adhesion substrate–independent human cell model systems—the K562 myeloblastic/erythroblastic and the Jurkat T-lymphoblastic leukemia. In the absence of adhesion substrate attachment, these grow in suspension and lack actin bundles during interphase of the cell cycle. Under such conditions, microtubule-supported septin disks are the predominant higher-order septin arrangement, but these transform into a variety of arrangements upon cell spreading on an adhesion substrate. Moreover, all the septin arrangements observed in the present cell model systems appear mutually exclusive with plasma membrane regions undergoing remodeling. In this section we discuss the evidence that supports the model in Figure 11, which shows how microtubules extending along the cortex support plasma membrane–bound, disk-like septin assemblies in nonadhered cells.

**Septin-reporter caveats**

Fungal and animal septin core heteromers resist dissociation in high salt and characterization of recombinant septin heteromers suggests that the assembly is cotranslational (reviewed in McMurray and Thorner [2009]). In addition, attempts to assemble heteromers by combining individual recombinant septin subunits in a test tube have failed (Kinoshita, 2003a). However, there is still ample evidence for subunit exchange within core heteromers during cell cycle and developmental transitions of budding yeast, which suggests that this organism has chaperones or other factors facilitating subunit exchange (McMurray and Thorner, 2008). To address premises for tagging the endogenous mammalian heteromer pool with AcGFP reporters, we monitored the assembly states of epitope-tagged SEPT7 (Figure 1) or SEPT2 and SEPT6 (unpublished data) following a pulse of their expression. Our data support the notion that mammalian core heteromers are stable protein complexes and that heterooligomerization of tagged septins occurs only during de novo synthesis.
Studies of K562, Jurkat, and HeLa cells suggest that endogenous septins exist solely in the context of six-to-eight-subunit core heteromers (Sellin et al., 2011). Despite optimized conditions for heterooligomerization with endogenous septins, only SEPT7 reporters were effectively integrated into core heteromers. It is also noteworthy that both the SEPT2- and SEPT6-reporters are prone to form ectopic filaments, which were found to sequester endogenous heteromers and thereby deplete the native cortical assemblies (Figure S3). Thus, if expressed at nonphysiological levels, these septins are promiscuous with respect to pairing partners, which is indeed as predicted by oligomerization of bacterially expressed septins (Sheffield et al., 2003). This puts previous work based on septin reporters into perspective, since septin localization may not represent the localization of endogenous core heteromers. These issues are expanded on and further discussed in the Supplemental Material.

Microtubule-supported organization of submembranous closed septin filaments

The most common higher-order septin arrangement across kingdoms is a circular structure in the size range \(0.5-3.5 \mu\text{m}\), which is thought to function in scaffolds and/or lateral diffusion barriers in both fungi and animals (reviewed in Gladfelter [2010]). Moreover, hyphal fungi contain several distinct classes of circular septin structures in a single cell (DeMay et al., 2009). Significantly, actin filament-disrupting drugs cause transformation of the actin bundle–associated septins observed in fibroblasts into free-floating circles \((\sim 0.6 \mu\text{m})\) (Kinoshita et al., 2002; Schmidt and Nichols, 2004). It is also noteworthy that circular \(\sim 0.6-0.8 \mu\text{m}\) filamentous septin bundles are formed spontaneously by recombinant human SEPT2/6/7 hexamers stored under low-salt conditions (Kinoshita et al., 2002). Thus there is ample evidence to suggest that mammalian heteromers have an innate propensity to assemble into \(0.6\)-to-\(0.8\)-\(\mu\text{m}\) continuous filamentous hoops. The \(\sim 0.8\)-\(\mu\text{m}\) structure characterized in this study appears as a disk attached flat against the plasma membrane, many of these structures have a visible central clearing (Figures 3, 8, and S4A). These disks are distinguished from previously described circular structures in animal cells by an exclusive cortical localization and microtubule dependence.

Previous reports on interacting microtubule and septin systems (reviewed in Spiliotis [2010]) provoked the initiation of the present study. On the basis of our experiences in studies on MAP4 (reviewed in Holmfeldt et al. [2009]), we have evaluated previously reported links between MAP4 and septins. However, we have failed to detect any functional links (Figures S6 and S7) or interactions between these proteins (unpublished data). Moreover, while microtubule depolymerization or tubulin heterodimer depletion caused dispersal of septin disks (Figures 4, 5, and 7), we did not detect any effect on the microtubule system caused (bottom panels). Owing to a dense microtubule array, individual microtubules are not clearly resolved. Deconvoluted optical sections at the equatorial plane of representative cells are shown. DNA (red fluorescence) was stained by propidium iodide. Scale bar: 5 \(\mu\text{m}\). (B) K562 cells were either untreated (Control) or Taxol-treated for 3 min and subsequently permeabilized, as in (A). The released (supernatant [S] and cell-associated (pellet [P]) proteins were analyzed by Western blotting. The cytosolic Op18 protein served as control for complete permeabilization in the presence of Taxol. (C) The fraction of individual septins that remained cell-associated in Taxol-treated cells was determined by quantification of Western blots. Bar charts represent the mean \(\pm\) SE of data from duplicate experiments.
by depletion of septins (Figures 5 and S6) or excessive overexpression of SEPT2 (Figure S7). However, we noted accumulation of septins on microtubule bundles in long-term Taxol-treated cells, which suggests an innate propensity of septins to bind microtubules or associated proteins (Figures 6 and S8). Such an innate propensity seems generally consistent with studies on cells containing bundles in a native context.

For example, in platelets (anuclear blood cells), septins localize to a densely packed circumferential cortical coil of microtubules (Martinez et al., 2006).

Septins also appear to interact with the perinuclear microtubule bundles observed during the microtubule remodeling process that is a hallmark of epithelial polarization (Bowen et al., 2011). Moreover, under sporulation-promoting conditions, septins also colocalize with microtubules in budding yeast (Pablo-Hernando et al., 2008). Finally, while septins do not localize to the mitotic spindle of K562 or Jurkat cells (Figure S4D; unpublished data), septins do appear to colocalize with densely arranged microtubules during metaphase as well as in the final phases of cytokinesis in other cell types (Surka et al., 2002; Spiliotis et al., 2005; Zhu et al., 2008).
Septins colocalize with actin bundles at the contractile ring during cytokinesis and at stress fibers during interphase; both processes depend on specific adaptors (Kinoshita et al., 2002). Thus, as evidenced by their localization in intact cells, septins have an apparent innate propensity to bind densely arranged assemblies of either microtubules or actin. Moreover, purified septin heteromers of both yeast and mammalian origins are known to interact with PIP2. This is a compartmentalized phosphoinositide enriched at the inner leaflet of the plasma membrane (reviewed in Varnai and Balla [2007]), which promotes polymerization and organization of purified heteromers on artificial lipid layers (Tanaka-Takiguchi et al., 2009; Bertin et al., 2010). Thus PIP2 interactions may at least in part explain the propensity of septins to localize to the plasma membrane of K562 and Jurkat cells.

It is noteworthy that, while septin disks in nonadherent cells depend on intact microtubules, the diffuse array of minute septin assemblies observed in microtubule-deficient cells are still retained at the plasma membrane (Figure 4). These observations provide the foundation for the model depicted in Figure 11. Thus densely arranged microtubules that extend along the cortex, such as in spherical adhesion substrate–independent cells (Figure 4A), may provide sufficient multiple-bond interactions to cooperate with binding sites at the plasma membrane, such as PIP2, and thereby facilitate assembly into septin disks. In addition, septin localization during cytokinesis of mammalian cells may also be facilitated by an analogous mechanism, namely cooperative binding at the plasma membrane and a septin adaptor such as anillin, which appears to interact with both septins and actin bundles at the contractile ring (Oegema et al., 2000; Kinoshita et al., 2002). In contrast, as shown here, actin filaments involved in remodeling of the plasma membrane during interphase (such as within filopodia or pseudopodia) are mutually exclusive with septin assemblies (Figures 8 and 9).
Evidence that submembranous septin assemblies delineate static plasma membrane regions

Cortical septin disks are the predominant septin assemblies detected in cells lacking substrate attachment (Figures 3 and S4). This, combined with preservation by microtubule stabilization, allowed us to address their composition by Western blotting (Figure 7). The data suggested that all the expressed septins are equally represented, and we obtained no evidence for a variable dependence on specific septins between cell types (Figures 7C, S9, and S10). Analysis of Taxol-treated cells permeabilized over a 5- to 60-min time period revealed only a slow decay of cell-associated septin structures (unpublished data). Given that this analysis implied a dramatic dilution at physiological ionic strength, ~50% of insoluble septins may still represent an underestimate of the fraction of core heteromers assembled into cortical disks.

A K562 cell contains ~180,000 heteromers (~0.1% of total cell proteins, of which approximately two-thirds is six-subunit and one-third is eight-subunit heteromers; Sellin et al., 2011). This number should be compared with ~1 × 10^7 tubulin heterodimers in a K562 cell (~2% of total cell proteins; Sellin et al., 2008a), which implies an ~60-fold molar excess of tubulin heterodimers over septin heteromers. Based on the assumption that most heteromers are assembled into 141 ± 29 septin disks per cell (Figure S4C), it can be deduced that each one contains ~1300 heteromers. Moreover, given an average cell surface area of 850 μm² (depending on cell cycle stage; Figure S4B) and that each septin disk covers a ~0.5-μm² area, these disks occupy roughly 8% of the inner leaflet of the plasma membrane of nonadhered, blood-derived cell types such as K562.

Each septin disk occupies a much larger area than any known functional unit in the plasma membrane of animal cells; in most cases, these areas are referred to as “microdomains” (Lingwood and Simons, 2010). These are most often described as dynamic domains smaller than the resolution of light microscopy, which contrast to the readily resoluted septin assemblies that appear mutually exclusive with membrane remodeling events. Thus analysis of various plasma membrane remodeling events in living K562 or Jurkat cells indicated exclusion of all detectable septin assemblies from dynamic regions of the plasma membrane (Figures 8–10).

As depicted in Figure 11, our results indicate that the septin-disk arrangement depends on cortical microtubules, which are densely arranged along the cortex of nonadhered spherical cells such as K562 and Jurkat (Figure 4A; Holmfeldt et al., 2003a). Such multivalent microtubule interactions combined with cooperative binding at the inner leaflet of the plasma membrane are likely to be of significance for the static nature of cortical septin disks. In the case of the septin arrangements observed in cells adhered to a substrate or during cell division, the model in Figure 11 predicts that other types of multivalent interactions will provide static properties to higher-order septin structures.

Concluding remarks

During ameoboid movement of Jurkat T lymphocytes we found that punctuate septin assemblies accumulate at the cortex of the trailing uropod (Figure 8). This appears generally consistent with published images of immunostained murine T lymphocytes (Tooley et al., 2009), which shows a mix of fibrous and punctuate septins at the uropod cortex. It was stated by the authors that the appearance of septin assemblies varies considerably with the antibody used for detection, and it was specifically noted that in some cells, particularly for detection of Sept6, distributions are predominantly punctuate. Based on the observed cortical localization and enhanced migration of septin-depleted cells through restrictive pores in trans-well assays (Tooley et al., 2009), it was proposed that septin assemblies provide cortical rigidity. However, we have not found that septin depletion of Jurkat T lymphocytes (by means of shRNA-SEPT7 expression; see Figure 5A), facilitates trans-migration through restrictive pores (3- and 5-μm pores tested, with or without chemotactant [unpublished data]). Moreover, we have not detected fibrous septins in the uropod. Thus our observations do not support the idea of a rigidifying “septin corset” at the uropod cortex that consists of fibers oriented perpendicular to the axis of locomotion.

Our studies on K562 and Jurkat cells cannot be directly compared with the inherently polarized cells of budding yeast, that is, the model system in which the significance of septins for membrane dynamics at the bud site has been established. It is still noteworthy that the actin cables that guide vesicle transport into the emerging bud have been shown to be spatially segregated from the septin ring structure that initially marks the bud site and thereafter remains at the base of the bud as a diffusion barrier and scaffold (reviewed in Oh and Bi [2011]). An analogous sequence of events has been observed in hyphal fungi in which the diffuse septin patch at the hyphal tip is transformed to a ring encircling the hyphal cell cortex (reviewed in Gladfelter [2010]). Studies of dendritic spines, flagellum, and cilia in vertebrates also reveal septin assemblies confined to the base of cell appendages (Ihara et al., 2005; Kissel et al., 2005; Tada et al., 2007; Xie et al., 2007; Hu et al., 2010). At this location, septins across kingdoms appear to function as a lateral diffusion barrier, which implies both static features and lateral immobility. Thus our evidence that submembranous septin assemblies in mammalian cells are mutually exclusive with dynamic membrane regions seems completely consistent with studies concerned with a diverse array of cellular structures and organisms.

MATERIALS AND METHODS

DNA constructs, shuttle vectors, and regulated expression from the hMTIIa promoter

Construction of EBV-based shuttle vectors directing constitutive expression of shRNA targeting mRNAs encoding SEPT2, SEPT5, SEPT7, SEPT9, Op18, and MAP4 has been described (Holmfeldt et al., 2007; Sellin et al., 2011). The derivative encoding shRNA-SEPT6 was constructed by an analogous strategy (accession NM_145799.2; targeting sequence: GAGAGACAAAGAGAAAGAAA).

EBV-based pMEP4 shuttle vectors directing inducible expression of SEPT6-FLAG, SEPT7-FLAG, Op18-FLAG, MAP4-FLAG, and TBC1-FLAG, all of which contain a C-terminal eight-aminoc acid FLAG epitope tag, as well as His-SEPT7, which contains an N-terminal 6xHis tag, have been described (Holmfeldt et al., 2002; Sellin et al., 2008b, 2011). The pMEP4 vector directing expression of SEPT2 derivative with an eight amino acid FLAG (SEPT2-FLAG) was constructed by an analogous PCR-based strategy using pK-myc-SEPT2 (NP_001008492.1) as a template (Sheffield et al., 2003). Vectors directing expression of septins fused at their C-terminus with AcGFP were based on either the pMEP4 or pCEP4 shuttle vectors, as indicated in text. The SEPT2-, SEPT6-, SEPT7- and SEPT9-AcGFP (SEPT9_v1-transcript; Nagata et al., 2003) derivatives have the same general design and include the complete open reading frames, with AcGFP located at the C-terminus. Constructions of these reporters, as well as the Lifeact-DsRed monomer reporter (Riedl et al., 2008), are detailed in the Supplemental Material. The general procedure for transfections of K562 or Jurkat with shuttle vectors and subsequent selection of hygromycin has been described (Holmfeldt et al., 2007). A transient burst of maximum expression from the hMTIIa promoter of pMEP4, which peaks during the first 6- to 8-h period of stimulation, was achieved by shifting cells from a medium designed
to suppress expression (containing 25 μM EDTA) to a medium containing 0.25 μM CdCl₂ (Melander Gradin et al., 1997; Sellin et al., 2008a). Constitutive expression from the hMTIα promoter was obtained by cultivation of cells in standard growth medium (RPMI 1640 supplemented with insulin, 5 mg/ml, transferrin, 5 mg/l, sodium selenite, 5 μg/l, and 5% fetal calf serum). Co-transfections of pCEP4, pMEP4, and/or shRNA vectors were performed as described (Melander Gradin et al., 1997; Holmfeldt et al., 2004).

Immunoblotting, antibodies, and determination of tubulin monomer–polymer partitioning

Immunoblotting and subsequent detection using the ECL detection system (GE Healthcare, Waukesha, WI) were performed using Op18, MAP4, α-tubulin, HSP70, and FLAG-tagged antibodies described in Holmfeldt et al. (2002) and the panel of septin antibodies described in Sellin et al. (2011). Moreover, antibodies toward GFP (sc-9996; Santa Cruz Biotechnology, Santa Cruz, CA), CD46 (555948; PharMin-gen, BD Biosciences, San Diego, CA), and SEPT8 (sc-48937; Santa Cruz Biotechnology) were also used. For quantitative analysis of Western blots, the Bio-Rad ChemiDoc (Bio-Rad, Hercules, CA) system was used with the Quantity One 4.4 program. Microtubule content was determined as detailed in Sellin et al. (2008a). To determine the total amount of polymerizable tubulin, cells were treated with the polymerization-promoting drug Taxol (15 min, 2 μg/ml), which was found by quantitative Western blotting to cause essentially complete polymerization (<3% unassembled tubulin) and allowed calculation of tubulin heterodimer–polymer partitioning.

Determinations of protein partitioning between soluble and cell-associated states, and hydrodynamic parameters

The assembly state of septins was evaluated by an assay based on the release of soluble septins after cell permeabilization (Sellin et al., 2011). In brief, cells were gently resuspended in the presence of 0.2% saponin, 10 μg/ml leupeptin in 80 mM PIPES (pH 6.9), 2 mM MgCl₂, and 4 mM ethylene glycol tetraacetic acid (PEM buffer [80 mM PIPES, pH 6.9, 2 mM MgCl₂, 4 mM ethylene glycol tetraacetic acid]). The particulate cell fraction was isolated by centrifugation (0.5 min, 2000 g). The particulate cell fraction was isolated by centrifugation (0.5 min, 2000 g) and cell-associated states, and hydrodynamic parameters allowed calculation of tubulin heterodimer–polymer partitioning.

Examination of live or fixed cells by fluorescence microscopy

Cells were cultured in chamber slides (100 μl/well; LabTek II; Nalge Nunc International, Naperville, IL) coated with BSA or with the bacterial Yersinia pseudotuberculosis invasin protein at 10 μg/ml (Arencibia et al., 1997). Epifluorescence images were acquired on an Olympus CellIR imaging station (Olympus Biosystems, Planegg, Germany) equipped with an inverted microscope (IX81; Olympus), a 100× 1.4 numerical aperture Plan apochromat objective, and a cooled CCD camera (Orca ER, Hamamatsu Photonics, Herrsching am Ammersee, Germany). Microscopy of live cells was performed at 37°C (humidified air with 5% CO₂). Immuno-staining of cells was performed after fixation in either 2% paraformaldehyde (37°C) or methanol (−20°C) as previously described (Holmfeldt et al., 2003b). Methanol- and paraformaldehyde-based fixation protocols resulted in similar appearances of SEPT7-ACGFp visualized structures, but fixation causes blurring of septin disks, and this effect varied between experiments. All immunostaining of SEPT7 was performed on methanol-fixed cells, since the antibody fails in specificity if used in combination with paraformaldehyde fixation. As indicated in the text, some analyses involved permeabilization of cells with 0.2% saponin in PEM buffer prior to fixation, which resulted in release of soluble proteins, such as nonpolymerized septin heteromers or tubulin heterodimers. Antibodies used for immunostaining are described above and Alexa Fluor 594–labeled transferrin (T13343; Molecular Probes, Invitrogen, Carlsbad, CA), Alexa Fluor 568–labeled phalloidin (A12380; Molecular Probes, Invitrogen), and Alexa Fluor 594–labeled wheat germ agglutinin (WGA; W11262; Molecular Probes, Invitro-gen) were used as fluorescent probes for live and/or fixed cells.

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