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New Insights into the Phylogenetic Distribution and Evolutionary Origins of the Septins

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Abstract

Until recently, it had appeared that the septin family of proteins was restricted to the opisthokont eukaryotes (the fungi and animals and their close relatives the microsporidia and choanoflagellates). It has now become apparent that septins are also present in several other widely divergent eukaryotic lineages (chlorophyte algae, brown algae, and ciliates). This distribution and the details of the non-opisthokont septin sequences appear to require major revisions to hypotheses about the origins and early evolution of the septins.

Keywords

Brown algae; chlorophyte algae; ciliates; cytokinesis; green algae; guanine-nucleotide-binding proteins

Introduction

The septins were first identified in the late 1980s by molecular analyses of a set of cytokinesisdefective mutants in the budding yeast *Saccharomyces cerevisiae* (reviewed by Longtine et al., 1996; Pringle, 2008). This was followed within a few years by their identification also in the fission yeast *Schizosaccharomyces pombe*, *Drosophila melanogaster*, mice, and humans, so that by the year 2000 (when the *Caenorhabditis elegans* septins were described), it seemed clear that these proteins were ubiquitously present in fungi and animals. However, despite a variety of efforts, no septins had been found in plants or indeed anywhere else in the eukaryotic tree of life outside of the fungi and animals. In 2005, Versele and Thorner (who had gotten an early look at the genome sequences) noted briefly that the chlorophyte algae *Chlamydomonas reinhardtii* and *Nannochloris bacillaris* contain genes that "...encode bona fide septin orthologs" (Versele and Thorner, 2005), but they provided no actual sequences or other details. Presumably for this reason and because the relevant genome sequences were not publicly available until late 2007 (Merchant et al., 2007), these algal sequences were not included in the otherwise-comprehensive phylogenetic analysis of the septins performed by Momany and co-workers (Pan et al., 2007; Momany et al., 2008). Similarly, a paper reporting the presence of septins in the ciliates *Tetrahymena thermophila* and *Paramecium tetraurelia* (Wloga et al., 2008) did not appear until after Momany and co-workers had completed their study. Thus, it has continued to be widely believed that the septins were confined to the opisthokonts (the fungi, animals, and their close relatives the microsporidia and choanoflagellates: Figure 1A). This in turn has led to the hypothesis that the septins evolved by horizontal transfer of an ancestral bacterial GTPase (Leipe et al., 2002) into the opisthokont lineage after this had

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already diverged from the other major eukaryotic lineages (Pan et al., 2007; Momany et al., 2008).

The explosion of genome-sequence information during the past few years suggested that it was time to revisit these questions. Remarkably, a survey of the available sequences has found unequivocal septin sequences not only in *C. reinhardtii*, *N. bacillaris*, *T. thermophila*, and *P. tetraurelia*, but also in two other chlorophyte algae and in the very distantly related brown algae. These observations appear to require major revisions of views on the origins and early evolution of the septins.

Results

We used the *S. cerevisiae* Cdc3 sequence as a query to search the publicly available sequence databases as described in Materials and Methods. This search revealed septin genes in *C. reinhardtii* and *N. bacillaris* (a single gene in each case); in two other chlorophyte algae, *Volvox carteri* (one gene) and *Chlorella variabilis* (two genes); in the brown alga *Ectocarpus siliculosus* (five genes); and in two ciliates, *T. thermophila* (two or three genes; see Materials and Methods) and *P. tetraurelia* (five to seven genes; see Materials and Methods). These groups represent lineages that diverged from each other, and from the opisthokonts, very early in eukaryotic evolution (Figure 1B–D).

With a few possible exceptions as noted below, these genes all appear to encode *bona fide* septins, as the proteins contain not only well conserved G1, G3, and G4 elements of the guanine-nucleotide-binding site but also most of the other sequence motifs that were shown by Momany and co-workers to be characteristic of the 162 opisthokont septins that they examined (Pan et al., 2007; Momany et al., 2008) (Figure 2 and data not shown). Perhaps most significantly, all of the non-opisthokont septins show good conservation of the "septin-unique element" as recognized originally by Versele et al. (2004) and subsequently shown by Sirajuddin et al. (2007) to be involved in forming the interfaces through which septin monomers assemble into protofilaments. Interestingly, although about two thirds of opisthokont septins contain a recognizable "polybasic region" upstream of the G1 element (Pan et al., 2007; Momany et al., 2008), this feature is absent in all of the non-opisthokont septins except for three (or perhaps four, depending on the stringency of definition) of the five chlorophyte-algal proteins (Figure 2 and data not shown). Similarly, although most opisthokont septins contain a strongly predicted coiled-coil domain at or very near their C-termini (Longtine et al., 1996; Pan et al., 2007; Momany et al., 2008), this feature is absent in all of the non-opisthokont septins except for *C. variabilis* EFN57048 and possibly *P. tetraurelia* CAI38985 (Figure 3). Interestingly, all of the chlorophyte-algal, brown-algal, and *T. thermophila* septins, and one of the *P. tetraurelia* septins, share a feature that opisthokont septins lack, namely a hydrophobic patch of 17–36 amino acids (Figure 4 and data not shown). This patch lies at various distances from the C-terminus but is always just a short distance C-terminal of the septin-unique element. It was originally noted in the *T. thermophila*, *C. reinhardtii*, and *N. variabilis* septins by Wloga et al. (2008), who interpreted it as a transmembrane domain, but for various reasons (too short or too long, insufficient hydrophobicity, and/or presence of helix-breaking prolines, plus some difficulty in visualizing the role and evolution of a transmembrane domain in these proteins) we suggest that this feature may have another role (see Discussion).

To gain more insight into the relationships of the non-opisthokont septins to each other and to the opisthokont septins, and thus into septin evolution, we performed phylogenetic analyses as described in Materials and Methods. Using either a Bayesian (Figure 5) or maximum-likelihood method (data not shown), we found that the opisthokont septins placed by Momany and co-workers into their Groups 1A, 1B, 2A, 2B, 3, and 4 separated out

cleanly as a clade distinct not only from those containing the Group 5 fungal septins (Pan et al., 2007; Momany et al., 2008), but also from the clades containing all of the non-opisthokont septins (Figure 5). The latter clades were interdigitated with those containing the Group 5 fungal septins (Figure 5), suggesting a relatively close relationship that is also consistent with the absence of C-terminal coiled-coil domains in the Group 5 fungal septins and all but one or two of the non-opisthokont septins (Pan et al., 2007; Figure 3). In other features of interest, the *P. tetraurelia* septins form a clade that is closely related to, but seemingly distinct from, the clade containing the *T. thermophila* septins; the five chlorophyte-algal septins form a distinct clade; and the brown-algal septins also form a distinct clade (Figure 5).

Discussion

Until recently, it had appeared that the septins had evolved in (or been horizontally transferred into) the opisthokont lineage only after it had separated from other eukaryotic lineages. The identification of unequivocal septins in three other evolutionarily distant groups, the chlorophyte algae, brown algae, and ciliates, makes this model untenable and suggests instead that a septin gene and protein were present in a very early and basal eukaryotic ancestor. During the proliferation and divergence of eukaryotic lineages, this gene and protein appear to have been lost in some lineages and to have proliferated into a gene/protein family in others. Importantly, these conclusions do not depend on a judgment as to whether the *Giardia* "septin-like sequence" is a *bona fide* septin relative or not (from our own examination of the sequence, we suggest not), as it had appeared when Momany and co-workers performed their analyses (Pan et al., 2007; Momany et al., 2008). Further insight into the origins of the septins may come when genome sequences become available for groups where there currently are none (Figure 1).

Our analyses also suggest other conclusions about septin evolution and the roles of various septin structural elements, as follows. First, the interdigitation of the clades containing Group 5 fungal septins with those containing the non-opisthokont septins suggests that rather than being a late-arising fungal invention (Pan et al., 2007), the Group 5 septins are actually relicts of the ancestral septin that have been retained in some fungi but not in other fungi or animals.

Second, as a corollary, the general absence of C-terminal coiled-coil domains in the Group 5 and non-opisthokont septins (Figure 3; Pan et al., 2007; Momany et al., 2008) suggests that this structural feature was not present in the ancestral septin but was evolved later and probably in more than one evolutionary line [given its presence in one chlorophyte-algal septin and, less robustly, in one ciliate septin and several Group 5 fungal septins (Figure 3; Pan et al., 2007)]. The non-basal nature of the coiled coil seems consistent with the now-overwhelming evidence that it is less important for the assembly of septin monomers than had earlier been thought (Mendoza et al., 2002; An et al., 2004; Sirajuddin et al., 2007; Bertin et al., 2008; Onishi et al., 2010). Even if these views are correct, it remains possible that the Group 1A fungal and animal septins might have secondarily lost a C-terminal coiled-coil domain that had been present in a fungal/animal precursor septin (Pan et al., 2007; Momany et al., 2008).

Third, the observation that the hydrophobic (possibly transmembrane) domain is present, with just one exception, only in non-opisthokont septins that lack the coiled-coil domain (Figures 3 and 4), suggests that these motifs represent alternative evolutionary solutions to the same problem in protein anchoring and/or protein-protein interaction.

Fourth, the observations that the polybasic domain is present in some, but not all, non-opisthokont septins, some, but not all, Group 5 septins, and some, but not all, Groups 1–4 septins (Figure 2; Pan et al., 2007; Momany et al., 2008; and data not shown) make it difficult to say whether this feature was present in the ancestral septin and then lost in multiple derived lineages or separately evolved in multiple lineages, although we think that the former hypothesis is somewhat more likely.

Fifth, in contrast, the consistent presence of the septin-unique element (Versele et al., 2004) in both opisthokont and non-opisthokont septins (Pan et al., 2007; Figure 2; data not shown) suggests strongly that this feature was present in the ancestral septin, which in turn suggests that polymerization into filaments (in which the septin-unique element is intimately involved: Sirajuddin et al., 2007, 2009) is an ancestral septin feature. In this regard, it will be interesting to determine if the single septins of *C. reinhardtii*, *V. carteri*, and *N. variabilis* form homopolymeric filaments *in vivo*. Structural studies suggest strongly that this should be possible (John et al., 2007; Sirajuddin et al., 2007; Bertin et al., 2008; Sirajuddin et al., 2009), and the formation of homopolymeric filaments *in vitro* was shown for a single recombinant *Xenopus laevis* septin (Mendoza et al., 2002).

Sixth, given that guanine-nucleotide binding and the presence of the associated G1, G3, and G4 motifs are generally considered defining septin features, it is surprising that three of the non-opisthokont septins lack one or more of these motifs: *C. variabilis* EFN57048 lacks a normal G3 motif, although the G1 and G4 motifs look normal; *T. thermophila* EAS04011 lacks a normal G1 motif, although the G3 and G4 motifs look normal; and *P. tetraurelia* CAK85597 lacks normal G1 and G4 motifs although the G3 motif looks normal (data not shown). However, in this regard it should be noted that abundant evidence indicates that *S. pombe* Spn7 is a full-fledged member of the septin complex in sporulating cells despite its lacking the G1, G3, and G4 motifs (Onishi et al., 2010). Structural and functional studies on these (presumably) guanine-nucleotide-free septins should be of great interest.

Seventh, it is interesting that most of the proliferation of non-opisthokont septin types appears to have occurred after the organismal lineages had diverged. In particular, there appears to have been a single ancestral chlorophyte-algal septin, a single ancestral brown-algal septin, and a single ancestral ciliate septin (which apparently did not begin to proliferate until after the *P. tetraurelia* and *T. thermophila* lines had diverged). This observation probably reflects mainly the antiquity both of the ancestral septin and of the divergence of these major eukaryotic lineages, but it remains of great interest, and still perplexing, why the septins have proliferated so greatly in some lineages but not in others, a question that arises equally forcefully in relation to the opisthokont septins.

Finally, it is of great interest to ask about the cellular process(es) in which the non-opisthokont septins are involved. Given the universal (or nearly so) involvement of the opisthokont septins in cytokinesis, it will be surprising if there is no such role for the nonopisthokont septins, but the only experimental evidence to date is for roles of the *T. thermophila* septins in mitochondrial, endoplasmic-reticulum, and nuclear-envelope stability (Wloga et al., 2008). As most chlorophyte algae, brown algae, and ciliates have ciliated cells, and roles in ciliary organization and function have been observed in vertebrates (Ihara et al., 2005; Kissel et al., 2005; Hu et al., 2010; Kim et al., 2010), the non-opisthokont septins may have such a role, but it should be noted that many other groups with ciliated cells appear to have no septins (Figure 1). Further functional studies should be deeply revealing about the ancestral and fundamental modes of septin function.

Materials and Methods

Database search for septin genes

To search for septin genes in non-opisthokont organisms, the *S. cerevisiae* Cdc3 sequence was used as the query for a PSI-BLAST search (Altschul et al., 1997) against non-redundant protein sequences from which those in the Fungi/Metazoa (taxid:33154) and choanoflagellate (taxid:28009) groups were excluded using the ‘Organism’ box at the NCBI BLAST website (<http://blast.ncbi.nlm.nih.gov/>). As of April 15, 2011, this search returned 20 annotated sequences of interest, as follows: five sequences from four species of chlorophyte algae [one each from *C. reinhardtii* (Accession Number EDP03113), *N. bacillaris* (BAD42341), and *V. carteri* (EFJ52266), and two from *C. variabilis* (EFN51664 and EFN57048)]; five sequences from one species of brown alga, *E. siliculosus* (CBJ31641, CBJ31642, CBJ31643, CBN74009, and CBN74010); and ten sequences from two species of ciliates [*T. thermophila* (EAS01103, EAS04011, and EAS02194) and *P. tetraurelia* (CAI38983, CAI38984, CAI38985, CAI38986, CAK85597, CAK86889, and CAK94988)]. We included all seven *P. tetraurelia* genes in our subsequent analyses even though one may possibly be a pseudogene (Wloga et al., 2008) and two pairs (CAI38984 and CAK86889; CAI38983 and CAK94988) are so closely related in sequence as to raise doubts as to whether they are really distinct genes. However, we excluded *T. thermophila* EAS02194 from our analyses because the inconsistency between the sequence described by Wloga et al. (2008) and that of the current database entry left us uncertain of its status.

Sequence analyses

Multiple-sequence alignments were performed using MUSCLE (Edgar, 2004) operated either in R (<http://www.r-project.org/>) with the bio3D package (for Figure 2) or in the Geneious software (Biomatters Ltd., New Zealand; Drummond et al., 2010) using the default settings (for the line-up used as starting point for phylogeny analysis). The coiled-coil probabilities shown in Figure 3 were predicted using the COILS program (Lupas et al., 1991; see http://www.ch.embnet.org/software/COILS_form.html), using a window of 28 residues. Another program, Paircoil2 (McDonnell et al., 2006), also predicted no coiled coils near the C-termini of non-opisthokont septins other than *C. variabilis* EFN57048. Kyte-Doolittle hydrophathy plots (Kyte and Doolittle, 1982) were calculated using DNA Strider 1.4f1 (CEA, France).

Phylogenetic analysis

First, a multiple-sequence alignment was performed for 65 septin proteins, including the 19 non-opisthokont proteins (see above) plus 19 animal septins [P40797 (Pnut), NP_523430 (Sep1), NP_524417 (Sep2), NP_728003 (Sep4), and NP_651961 (Sep5) from *Drosophila melanogaster*; and NP_443070 (Sept1), NP_006146 (Sept2), NP_663786 (Sept3), NP_004565 (Sept4), NP_002679 (Sept5), NP_055944 (Sept6), NP_001779 (Sept7), NP_001092281 (Sept8), AAF23374 (Sept9), NP_653311 (Sept10), NP_060713 (Sept11), NP_653206 (Sept12), XP_001133108 (Sept13), and NP_997249 (Sept14) from *Homo sapiens*] and 27 fungal septins [DAA09624 (Cdc3), DAA07481 (Cdc10), DAA08862 (Cdc11), DAA06801 (Cdc12), DAA11640 (Shs1), DAA08155 (Spr3), and DAA12061 (Spr28) from *S. cerevisiae*; O36023 (Spn1), Q09116 (Spn2), P48008 (Spn3), P48009 (Spn4), P48010 (Spn5), Q09883 (Spn6), and O60165 (Spn7) from *S. pombe*; CBF77039 (AspA), CBF71251 (AspB), CBF74054 (AspC), CBF87584 (AspD), and CBF76739 (AspE) from *Aspergillus nidulans*; AAL18019 (Sep4) from *Coccidioides immitis*; EAL22862 (Hyp5) from *Cryptococcus neoformans*; XP_388886 (Hyp5) and XP_385568 (Hyp6) from *Gibberella zeae*; EAA47383 (Hyp5) and EAA56370 (Hyp6) from *Magnaporthe grisea*; and XP_329188 (Hyp5) and XP_326269 (Hyp6) from *Neurospora crassa*]. These fungal septins

included all septins from *S. cerevisiae*, *S. pombe*, and *A. nidulans* and all of the Group 5 septins, but none of the Groups 1–4 septins, from the other five species.

This alignment was used as input for Bayesian phylogenetic analysis using Markov-chain Monte-Carlo simulations by MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). The parameters used were: lset rates = invgamma; prset aamodelpr = fixed(blosum); samplefreq = 100; nchains = 4; temp = 0.1; savebrlens=yes. The motor domain of the *S. cerevisiae* Type V myosin Myo2 (aa 64–782) was used as outgroup. The analysis was terminated after 400 000 generations with average standard deviation of split frequencies <0.025.

Maximum-likelihood analysis was performed using PhyML v.2.0.7 (Guindon and Gascuel, 2003) with the WAG substitution model and approximate likelihood ratio test. The parameters used are as follows: proportion of invariable sites, estimated; number of substitution rate categories, 4; gamma distribution parameter, estimated; optimization, tree topology and branch length.

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Figure 1. Distribution of septins in eukaryotes

A simplified version of the eukaryotic tree of life proposed by (Baldauf, 2003) is shown. Magenta, taxonomic groups that are known to include organisms with septin genes; green, groups for which no septin genes are found in the annotated sequence databases although some members have had their whole genomes sequenced; black, groups for which the presence or absence of septin genes cannot be determined because of a lack of whole-genome sequences.

Dme Pnut	MNSPRSNVNGSGGGAISALP	STLAQLA	RD	QQAASA	SASSATNGSSGSES	LVGVGGRP	PNQPPSV	PVAASG	KLDT	77
Pte CAI38984										35
Esi CBN74009										25
Cre EDP03113										42
Dme Pnut	SGGGS	NGDS	NKLTHDLQE	KEHQQAQKPKQ	PP	PVR				144
Pte CAI38984										106
Esi CBN74009										38
Cre EDP03113										112
Dme Pnut	LMVVGASGLGKSTLINS	MFL								210
Pte CAI38984										177
Esi CBN74009										115
Cre EDP03113										183
Dme Pnut	GDAVDNS	NCW	VPI	EVVDSKYE						276
Pte CAI38984										249
Esi CBN74009										179
Cre EDP03113										251
Dme Pnut	SDKVN	VPVIAKADTMT	PDEVH	LKKQI	NETAQ					339
Pte CAI38984										311
Esi CBN74009										248
Cre EDP03113										318
Dme Pnut	PFAVVGANTII									404
Pte CAI38984										383
Esi CBN74009										319
Cre EDP03113										387
Dme Pnut										473
Pte CAI38984										410
Esi CBN74009										389
Cre EDP03113										435
Dme Pnut	HEER	KALELQIRELEEKR	REFEREKKE	WED	VNH					539
Pte CAI38984										451
Esi CBN74009										481
Cre EDP03113										

Figure 2. Alignment of the amino-acid sequences of three non-opisthokont septins with that of a canonical animal septin

The sequence of *D. melanogaster* (Dme) Pnut was aligned with those of representative septins from the ciliate *P. tetraurelia* (Pte), the brown alga *E. siliculosus* (Esi), and a chlorophyte alga (*C. reinhardtii*: Cre) as described in Materials and Methods. Four manual gap adjustments were made near the C-terminal end of the septin-unique element to improve the alignment in that region. Numbers indicate amino acid positions. Amino acids that are identical or similar (L,I,V; S,T; D,E; K,R; N,Q) between Pnut and one of the other proteins are shaded in black and gray, respectively (with black overriding gray in the Pnut sequence). The dashed box indicates the approximate position of the polybasic region that is found in many septins. The G1, G3, and G4 motifs of the predicted guanine-nucleotide-binding sites are indicated by black arrows. White boxes and letters therein indicate motifs and single residues that are highly conserved in opisthokont septins (Pan et al., 2007; Momany et al., 2008), including the "septin-unique element" (Versele et al., 2004). The C-terminal six amino acids (EQARPT) of the Esi sequence are omitted (#) to save space.

Figure 3. Absence of C-terminal coiled-coil domains in most non-opisthokont septins

Coiled-coil probabilities were calculated as described in Materials and Methods and are shown for *D. melanogaster* Pnut, *S. cerevisiae* Cdc3; the septins from *C. reinhardtii* (Cre), *E. siliculosus* (Esi), *P. tetraurelia* (Pte), and *T. thermophila* (Tth); and one of the septins from *C. variabilis* (Cva). The other *C. variabilis* septin and those from *V. carteri* (Vca) and *N. bacillaris* (Nba) contained no predicted coiled coils (data not shown).

Figure 4. Hydropathy plots of septin proteins

Hydropathy values were calculated as described in Materials and Methods using a window size of 19 with the exception of Tth_EAS01103, for which a window of 9 was used to make the C-terminal hydrophobic patch easier to see. Species names are abbreviated as in Figure 3. Black bars represent septin core domains (G1, G3, and G4 motifs plus the septin-unique element). Hydrophobic patches just C-terminal to the core domains are shaded black. A second hydrophobic patch in Esi_CBJ31641 is shaded gray.

Figure 5. Phylogeny of septin proteins

A majority consensus phylogeny for 65 septins was inferred by Bayesian analysis as described in Materials and Methods. The septins analyzed included 19 non-opisthokont proteins (species names are abbreviated as in Figure 3) plus 46 animal and fungal septins (see list in Materials and Methods). The 37 of these animal and fungal septins that were previously assigned to Groups 1A, 1B, 2A, 2B, 3, and 4 by Momany and co-workers (Pan et al., 2007; Momany et al., 2008) also formed a single clade (with multiple branches) in our analysis and are represented here by the line with black triangle. Posterior probabilities are

indicated on selected branches. The *S. cerevisiae* (Sce) Myo2 motor domain served as outgroup. The scale bar represents evolutionary distance in substitutions per residue.