Identification of a novel alternatively spliced septin

Eric T. Fung, Richard H. Scheller*

Howard Hughes Medical Institute, Department of Molecular and Cellular Physiology, Stanford University Medical School, Stanford, CA 94305, USA

Received 6 April 1999

Abstract Septins are a family of cytoskeletal proteins involved in cytokinesis, targeting of proteins to specific sites on the plasma membrane, and cellular morphogenesis. While many aspects of their function in cytokinesis in yeast cells have been investigated, the function of septins in mammalian cells is less well understood. For example, septins are present in post-mitotic neurons, suggesting they have other roles in, for example, establishing cell polarity. The full extent of the septin gene family is not known in mammalian cells. To better understand the septin gene family, we have cloned and characterized a novel mammalian septin.

Key words: Septin; Sec 6/8 complex; Exocyst

1. Introduction

Septins are a family of cytoskeletal proteins first identified in yeast (reviewed in [1,2]). The septin yeast mutants *CDC3, CDC10, CDC11*, and *CDC12* demonstrated defects in the cell division cycle whose phenotype consisted of defects in bud morphogenesis, cytokinesis and localization of chitin deposition [3]. The defective chitin deposition seen in the original mutants has since been traced to the role of septins in the proper targeting of Chs3p, the catalytic subunit of chitin synthase III, to the ring at the base of the bud [4]. The defective bud morphogenesis is related to the septins' role in localizing membrane proteins such as Bud3p and Bud4p to sites at which budding should occur [2]. Septin proteins have subsequently been found to be present in most cell types of multicellular organisms and appear to play a role in cytokinesis in these cells. For example, in *Drosophila*, the septin Pnut localizes to cleavage furrows of dividing cells, and flies deficient for Pnut die as pupae with small imaginal discs [5]. Similar cytokinesis defects have been seen in flies mutant for a second *Drosophila* septin, Sep1 [6]. At the ultrastructural level, septins localize to a ring of 10 nm filaments seen at the bud neck of dividing yeast cells [7,8]. Septins have been shown in several systems to associate with each other and to form filaments in vitro [9]

It is likely that the ability of septins to recruit proteins to specific sites on the plasma membrane during cytokinesis is important for their participation in processes such as cellular morphogenesis and the development of cellular polarity. For example, septins have been implicated in the morphogenetic response to pheromones in yeast via their interaction with Afr1p [10]. Septins have also been found in neurons, which are polarized but mitotically inactive, and their function in these cells remains to be determined [11]. Recently, it was found that some septins are associated with the sec 6/8 complex purified from brain, suggesting a role for these proteins in the recruitment or docking of vesicles to specific sites on the plasma membrane [12].

The four original yeast septins were characterized by Pringle and co-workers, and seven mammalian septins have been characterized. All septins characterized to date contain a P loop nucleotide binding sequence near the N-terminus as well as other domains contained in members of the GTPase superfamily, suggesting that septins may have GTPase catalytic activity. Indeed, several septins have been shown to have such activity [9] and mutations in the GTP binding site alter septin localization in mammalian cells [13]. We report here the characterization of a novel septin that is alternatively spliced.

2. Materials and methods

2.1. Cloning of cDNAs encoding eseptin

mRNA from various rat tissues was purified using the Qiagen RNEasy kit. cDNA was synthesized from the mRNA using Moloney murine leukemia virus reverse transcriptase (Promega) and the cDNA from brain was used as a template for polymerase chain reaction. Degenerate oligonucleotides were synthesized and used as primers. PCR products were cloned into pCR2.1 and sequenced. The clone encoding a fragment of septin was labeled using the High Prime kit (Stratagene) and used to screen a lambda-zap rat brain library. For 5′ RACE PCR, nested antisense oligonucleotides were synthesized and used as primers. Marathon cDNA (Clontech) was used as a template and PCR products were cloned into pCR2.1 and sequenced. Northern blot analysis was done on a purchased Northern blot (Clontech) according to the manufacturer's instructions. Site directed mutagenesis of the GTP binding site was done using the Quik-Change protocol (Stratagene).

2.2. GTP binding assay

Glutathione *S*-transferase fusion proteins of eseptin were expressed in BL21 or AB1899 cells and purified according to the manufacturer's instructions (Pharmacia). Approximately 2 μg of fusion protein bound to glutathione agarose was incubated with binding buffer (50 mM Tris 7.4, 2 mM $MgCl_2$, 2 mM DTT, 100 μM ATP, 0.3% Tween) containing 1 μCi/ml [^{32}P]α-GTP at 4°C for 1 h. Beads were washed with binding buffer and bound radioactivity was measured by scintillation counting.

2.3. Tissue culture and immunofluorescence

COS cells were maintained in DMEM containing 10% fetal bovine serum with penicillin-streptomycin. Transfection was done using TransIt reagent (Panvera). Forty-eight hours after transfection, cells grown on glass coverslips were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min at room temperature and permeabilized in 0.5% Triton in PBS. To reduce background, coverslips were incubated in 10% normal goat serum in PBS at room temperature for 1–2 h. Cells were then incubated with a monoclonal anti-myc antibody (Santa Cruz Biotechnology) diluted in 4% normal goat serum in PBS at room temperature for 1 h, washed in PBS, and then incubated with Texas red-conjugated anti-mouse antibody. Cells were washed in PBS and then mounted using Vectashield.

*Corresponding author. Fax: (1) (650) 725-4436.
E-mail: scheller@cmgm.stanford.edu

PII: S0014-5793(99)00559-1

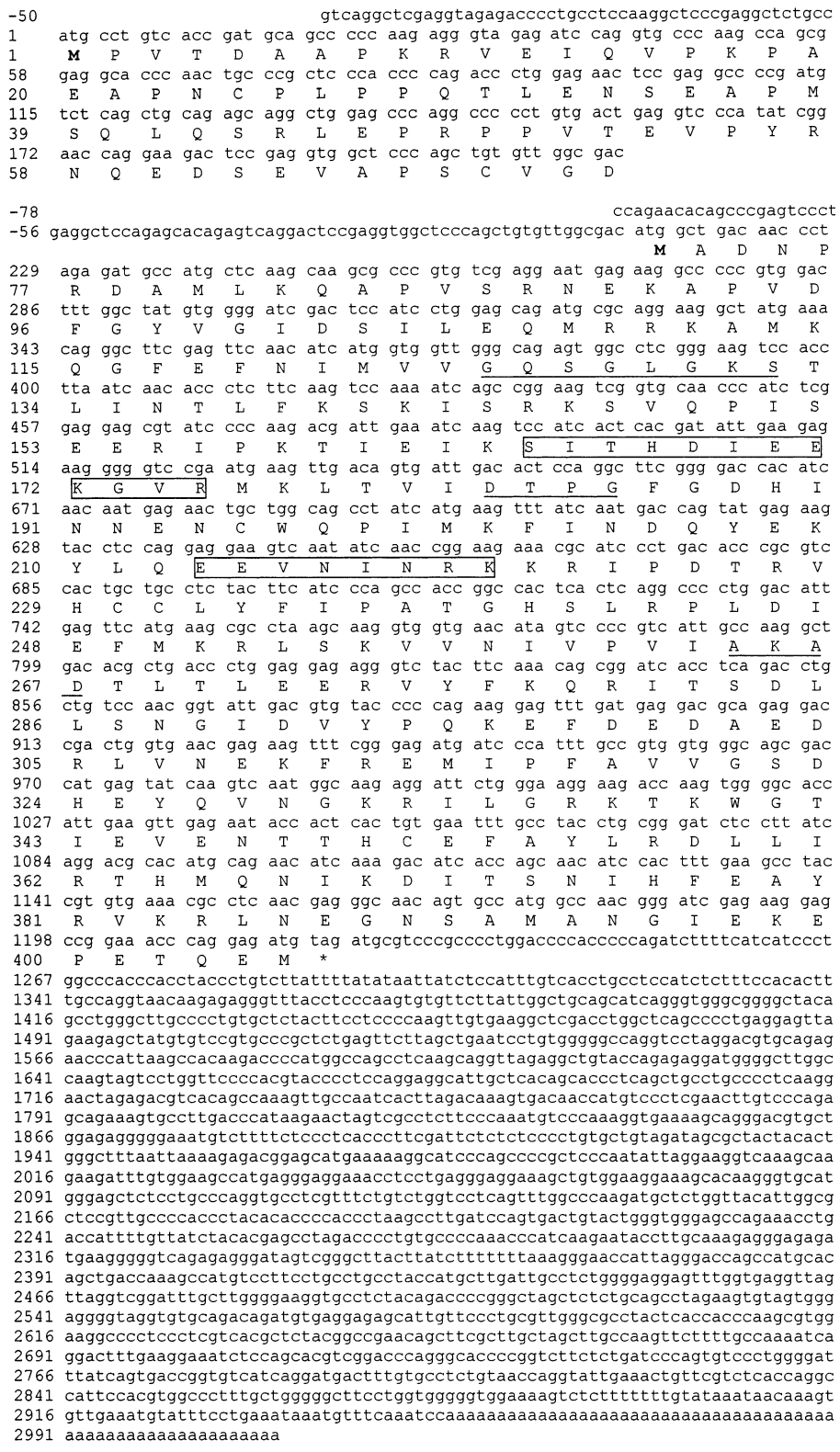

Fig. 1. Nucleotide and amino acid sequence of eseptins. The two start methionines are in bold. The peptide sequences from which the pair of degenerate oligonucleotides were generated are boxed. Underlined are the three consensus sequences for GTP binding. The long form continues with the start methionine of the short form, and the numbering on the left corresponds to that of the long form, except for the 5′ untranslated region of the short form.

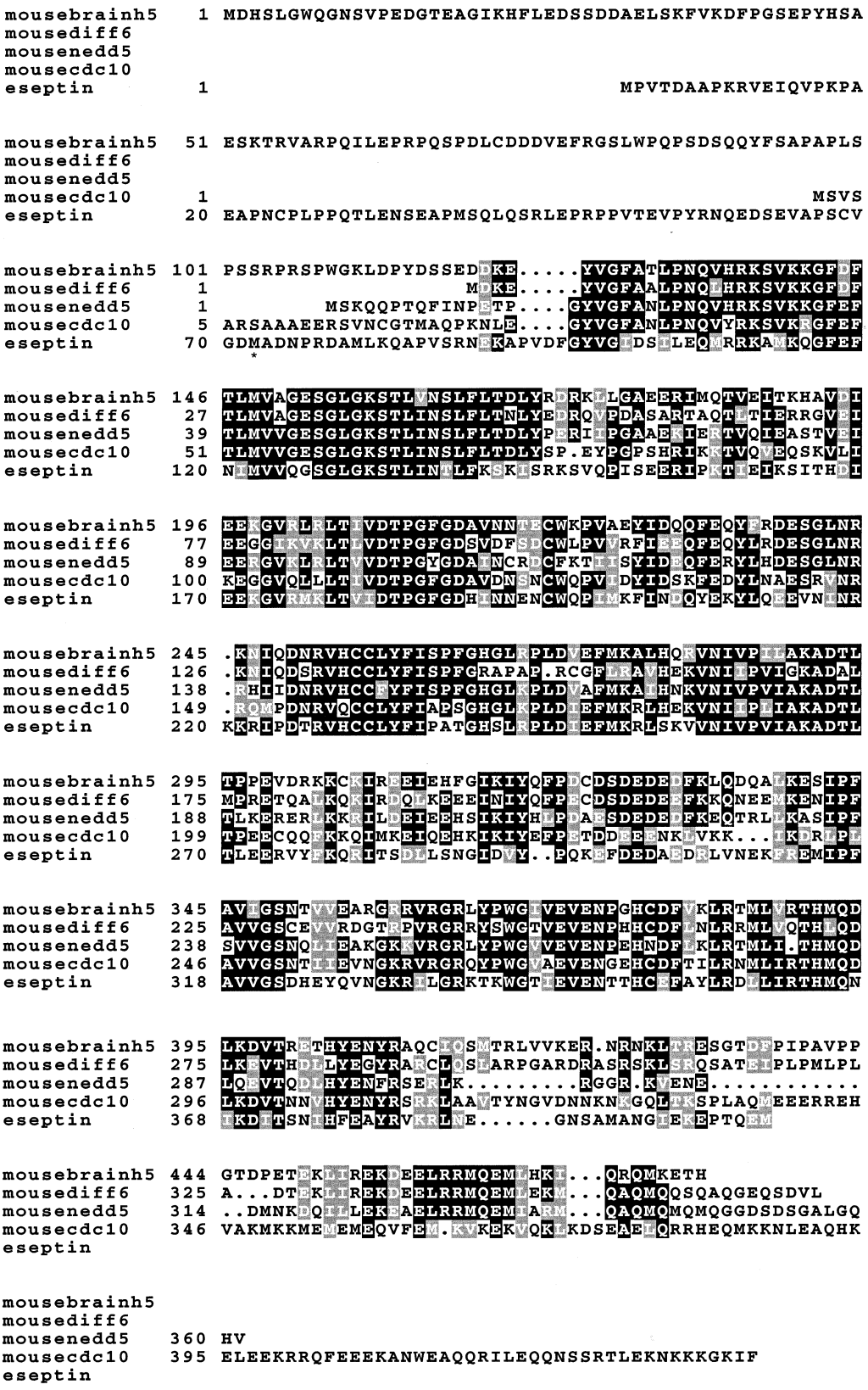

Fig. 2. Alignment of the eseptins with other mouse septins. The eseptins demonstrate high homology with other mouse septins in their central portion. Neither eseptin contains a carboxy-terminal extension, while the short eseptin has virtually no N-terminal extension. The GTP binding motifs are strongly conserved. The asterisk indicates the start methionine of the short eseptin. Dark shading indicates identical residues and light shading indicates conserved residues.

3. Results

Immunoprecipitation of the sec 6/8 complex from brain revealed several associated proteins [12]. Peptide sequencing of co-immunoprecipitated protein bands led to the identification of several septins, including DIFF6, CDC10, H5, and KIAA0128, that were associated with the sec 6/8 complex. An additional band of ~46 kDa yielded peptides which did not correspond to any known protein. To characterize this protein, degenerate oligonucleotides were made to several of these peptides and used as primers for PCR using rat brain cDNA as template. Two pairs of oligonucleotides yielded PCR products with an open reading frame with high homology to the known septins. The longer PCR product was used to screen a recombinant brain cDNA library. Because no obvious start methionine was present, 5′ RACE was performed. Two pools of RACE products with different 5′ ends were identified (Figs. 1 and 2). The shorter product contained an in-frame stop codon, but repeated rounds of RACE failed to identify an in-frame stop codon for the longer product. However, the predicted molecular weight of the protein encoded by the open reading frame is 47 kDa, in agreement with the size of the protein band from which the novel septin was cloned. This novel septin was designated eseptin for eighth septin, since a seventh homolog of septin was recently identified [14].

The long form of eseptin is 405 amino acids long while the short form is 334 amino acids long (Fig. 2). The long form appears to be an N-terminal extension of the short form, since it includes the entire coding region of the short form. Analysis of the primary sequence of eseptin reveals a nucleotide binding site and additional sequences required for GTPase activity (underlined in Fig. 1). Much of the central portion of the protein is conserved with that of other septins, while the amino-terminus is divergent from the hitherto cloned septins. There is no carboxy-terminal extension containing a predicted coil-coil domain, as is the case for some septins [1]. No other functional domains are identified in the eseptins. A Northern blot of various rat tissues reveals the presence of the two forms of eseptin (Fig. 3). These two forms are expressed at different ratios in various tissues. For example, testes, kidney, and liver express almost exclusively the long form. Heart ex-

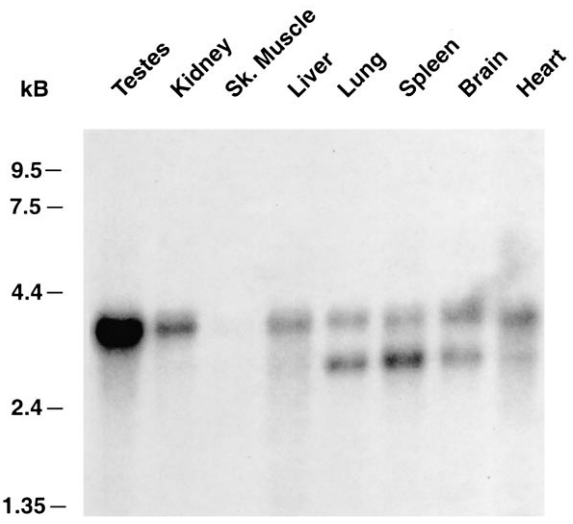

Fig. 3. Northern blot analysis of rat tissues. A Northern blot containing mRNA from adult rat tissues was probed with the long form of eseptin. The long eseptin transcript is approximately 4.0 kb and the short eseptin transcript is approximately 3.7 kb. The Northern blot shows a wide distribution of eseptin, although it appears to be absent from skeletal muscle. The long and short forms are not expressed at equal levels in all tissues.

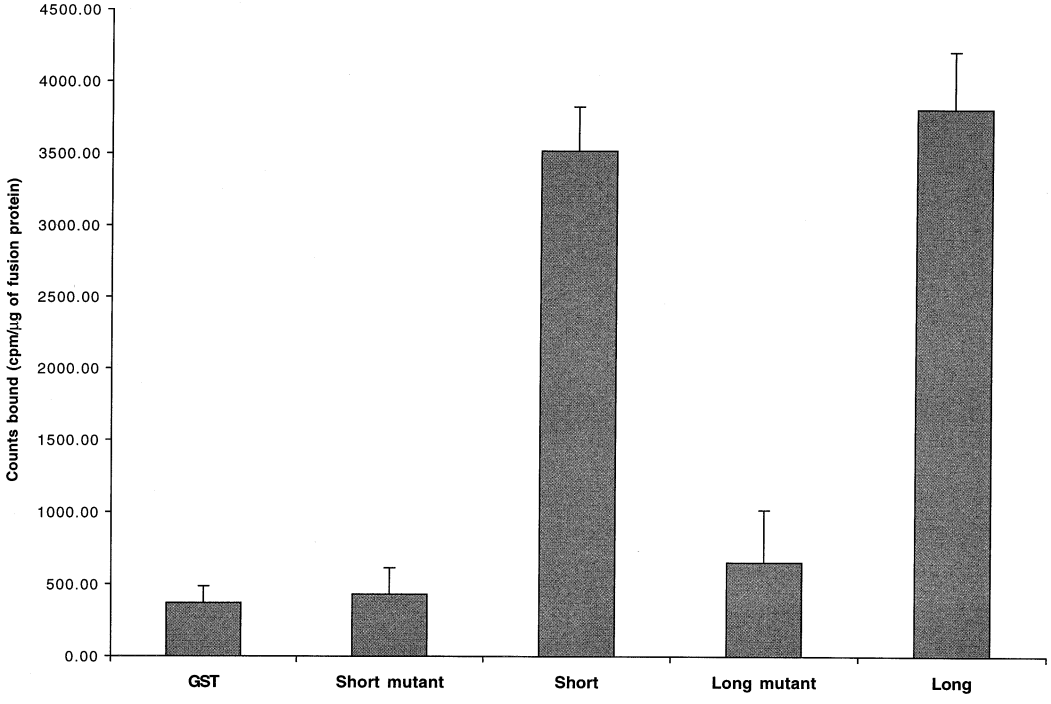

Fig. 4. GTP binding of eseptins. Wild type full length eseptins and mutant eseptins (GQSGLGKS was changed to GQSVLGKS) were used in this assay. Glutathione *S*-transferase fusion proteins bound to glutathione agarose were incubated with [γ-^{32}P]GTP, washed, and bound radioactivity was assayed by scintillation counting. Data shown are the average of assays done in triplicate.

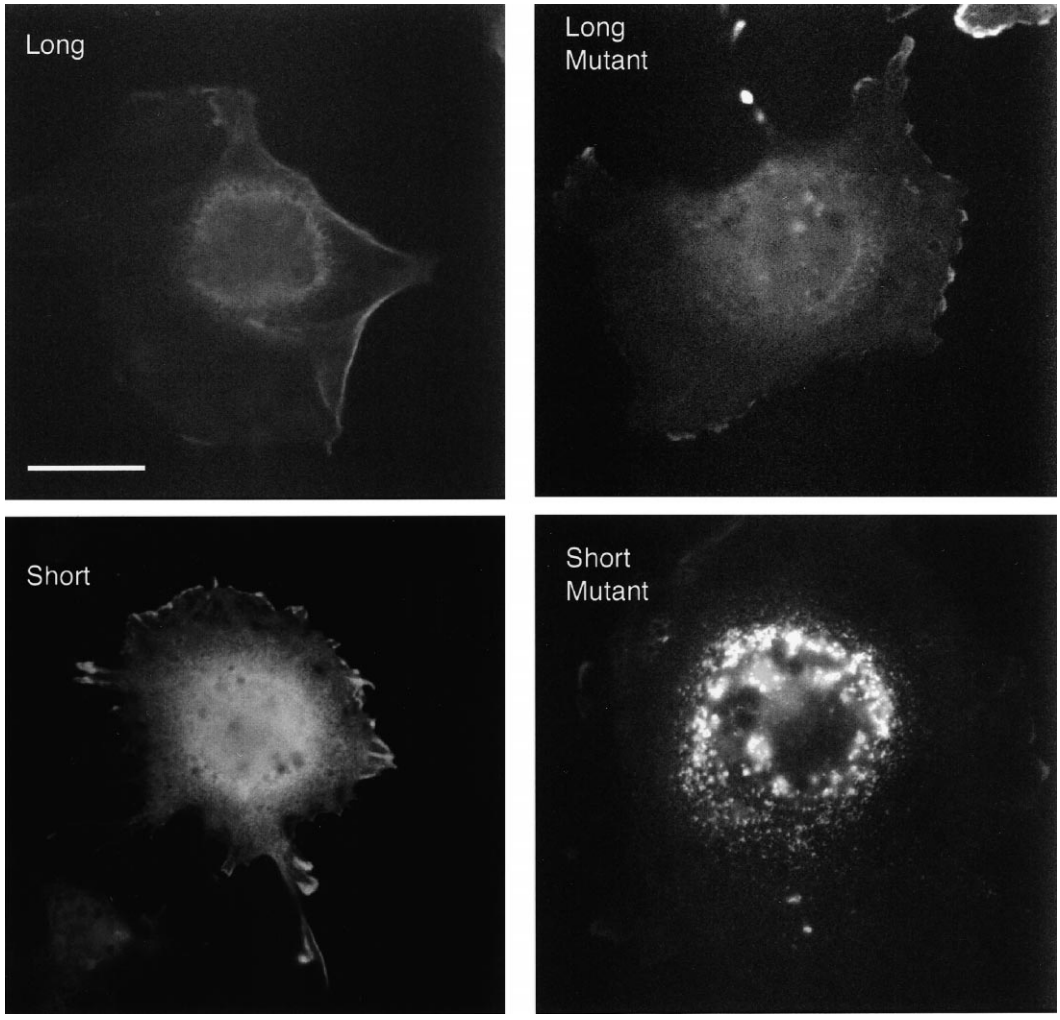

Fig. 5. Localization of heterologously expressed eseptins. Myc epitope tagged eseptins were transfected into COS cells, and expressed protein was detected using a monoclonal antibody to the myc epitope. Scale bar, 4 μm.

presses predominantly the long form, while lung, spleen, and brain express almost equal amounts of both forms. These septins do not appear to be expressed in skeletal muscle.

To examine the ability of eseptin to bind GTP, fusion proteins of the short and long forms were incubated with [^{32}P]α-GTP (Fig. 4). In addition, fusion proteins whose consensus GTP binding motif GQSGLGKS was mutated to GQSVLGKS were also tested. Both the long and short forms of eseptin bound GTP to an equal extent. The mutants did not bind, as expected.

To examine the subcellular distribution of eseptin, both the long and short forms were expressed in COS cells with a myc epitope tag (Fig. 5). While both wild type forms were distributed to the plasma membrane, the short form also had a more tubular and vesicular, perinuclear distribution as well. A GTP binding mutant form of the long eseptin did not have altered subcellular distribution, but the same mutation in the short eseptin led to a distinct staining pattern. Eseptin immunoreactivity in cells transfected with the mutant short form of eseptin was predominantly perinuclear. These results suggest that the long and short forms of eseptin have different functions and that the N-terminal extension of long eseptin may have specific binding partners that alter its function, stability, or subcellular distribution.

4. Discussion

In this report we describe the cloning of a novel septin. Eseptin is an alternatively spliced septin that is expressed in a variety of tissues, although it is notably absent from skeletal muscle. Like a number of other septins, both forms of eseptin bind GTP. While both forms are localized to the plasma membrane, the short form also has a distribution suggestive of a vesicular membrane compartment, possibly the endoplasmic reticulum. This distribution needs to be confirmed by localizing endogenous protein. While abolishing the GTP binding capability of the long form does not appear to alter its distribution at the light microscopic level, the same is not true of the short form. Mutant short eseptin expressed in COS cells causes the protein to be localized in a more perinuclear fashion. Mutant long eseptin did not have a dramatically changed distribution. The short eseptin is comprised of the central conserved domain and lacks carboxy- or amino-terminal extensions, suggesting that it may have limited ability to bind non-septin partners. Assuming that the signals for septin assembly are contained within their central conserved region, the short eseptin may be restricted to septin filaments, and the GTP binding mutation may completely disrupt its ability to assemble into these filaments. In the absence of other binding

partners to stabilize or target the mutant short eseptin, it may form intracellular aggregates. Future studies to identify the functions of the two forms will help expand our understanding of septins in general.

It has been shown recently that the septins associate with the sec 6/8 complex [12]. The sec 6/8 complex is the mammalian counterpart to the yeast exocyst. In yeast, the exocyst plays a role both in the secretory pathway and during cytokinesis [15]. Members of the sec 6/8 complex show homology to components of the yeast exocyst, and the overall biophysical characteristics of the sec 6/8 complex suggest that it probably has similar functions to that of the yeast complex [16]. It has recently been shown that the sec 6/8 complex has a periodic distribution in the growing axons of cultured hippocampal neurons [17]. While the mechanism by which the complex is selectively placed in these specific sites is unknown, the interaction between the complex and the septins may play a role. A major question to be answered is whether septins form a nucleation site on the plasma membrane about which the sec 6/8 complex localizes, or whether their association occurs at earlier stages of sec 6/8 complex targeting within the cell. Since septins have already been shown to localize several associated proteins to specific sites on the plasma membrane [2,4,18,19], it is tempting to postulate that the sec 6/8 complex is another substrate for septin localization. One question is how the septins can target such a variety of proteins with no apparent homology to each other. There are now at least eight characterized mammalian septins, five of which appear to interact with the sec 6/8 complex. It is likely that the subunit composition of septin filaments and the stoichiometry of the individual septins within these filaments determine the substrate specificity for targeting. Further studies of the septin gene family are required to better understand the functions of these proteins in processes as diverse as cytokinesis, exocytosis, and the establishment of cell polarity.

Acknowledgements: The authors thank Jason Bock for helpful discussion and reading of the manuscript.

References

[1] Longtine, M.S., DeMarini, D.J., Valencik, M.L., Al-Awar, O.S., Fares, H., De Virgilio, C. and Pringle, J.R. (1996) Curr. Opin. Cell Biol. 8, 106–119.
[2] Chant, J. (1996) Cell 84, 187–190.
[3] Hartwell, L.H. (1971) Exp. Cell Res. 69, 265–276.
[4] DeMarini, D.J., Adams, A.E., Fares, H., De Virgilio, C., Valle, G., Chuang, J.S. and Pringle, J.R. (1997) J. Cell Biol. 139, 75–93.
[5] Neufeld, T.P. and Rubin, G.M. (1994) Cell 77, 371–379.
[6] Fares, H., Peifer, M. and Pringle, J.R. (1995) Mol. Biol. Cell 6, 1843–1859.
[7] Kim, H.B., Haarer, B.K. and Pringle, J.R. (1991) J. Cell Biol. 112, 535–544.
[8] Byers, B. and Goetsch, L. (1976) J. Cell Biol. 69, 717–721.
[9] Field, C.M., al-Awar, O., Rosenblatt, J., Wong, M.L., Alberts, B. and Mitchison, T.J. (1996) J. Cell Biol. 133, 605–616.
[10] Giot, L. and Konopka, J.B. (1997) Mol. Biol. Cell 8, 987–998.
[11] Caltagarone, J., Rhodes, J., Honer, W.G. and Bowser, R. (1998) Neuroreport 9, 2907–2912.
[12] Hsu, S.C., Hazuka, C.D., Roth, R., Foletti, D.L., Heuser, J. and Scheller, R.H. (1998) Neuron 20, 1111–1122.
[13] Kinoshita, M., Kumar, S., Mizoguchi, A., Ide, C., Kinoshita, A., Haraguchi, T., Hiraoka, Y. and Noda, M. (1997) Genes Dev. 11, 1535–1547.
[14] Mino, A., Tanaka, K., Kamei, T., Umikawa, M., Fujiwara, T. and Takai, Y. (1998) Biochem. Biophys. Res. Commun. 251, 732–736.
[15] TerBush, D.R., Maurice, T., Roth, D. and Novick, P. (1996) EMBO J. 15, 6483–6494.
[16] Hsu, S.C., Ting, A.E., Hazuka, C.D., Davanger, S., Kenny, J.W., Kee, Y. and Scheller, R.H. (1996) Neuron 17, 1209–1219.
[17] Hazuka, C.D., Foletti, D.L., Hsu, S.C., Kee, Y., Hopf, F.W. and Scheller, R.H. (1999) J. Neurosci. 19, 1324–1334.
[18] Carroll, C.W., Altman, R., Schieltz, D., Yates, J.R. and Kellogg, D. (1998) J. Cell Biol. 143, 709–717.
[19] Longtine, M.S., Fares, H. and Pringle, J.R. (1998) J. Cell Biol. 143, 719–736.