THE ROLE OF SEPT2 ON NEURONAL DEVELOPMENT

by

HYUN JONG KIM

A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in

Cell and Developmental Biology

written under the direction of

Shu Chan Hsu, Ph. D

and approved by

____________________________________________________

____________________________________________________

____________________________________________________

____________________________________________________

New Brunswick, New Jersey

May, 2009
Septins constitute a family of evolutionarily conserved proteins that were first discovered for their involvement in cell division. Recently, they are also found to be increasingly important in various neurodegenerative states, such as Parkinson’s and Alzheimer’s diseases. Molecular mechanisms underlying the function of septin proteins in the nervous system, however, remain largely unknown. Work from this thesis revealed that a member of the septin family, SEPT2, is important for exocytosis underlying neurite outgrowth in PC12 cells. SEPT2 has a similar tissue distribution and developmental expression profile as that of sec15, a subunit of the exocyst complex which has been found to be required for exocytosis. In undifferentiated PC12 cells, SEPT2 exhibited perinuclear enrichment, as observed for exocyst subunits. Upon neuronal differentiation, SEPT2, like exocyst subunits, redistributes from its perinuclear enrichment to the neurites and the growth cones. Knockdown of SEPT2 disrupted the perinuclear enrichment of exocyst subunits, decreased the protein levels of exocyst subunits. Under this condition, neurite outgrowth and the insertion of protein into the plasma membrane of PC12 cells were inhibited. These results suggest a novel mechanism by which SEPT2 can mediate and/or
regulate exocytosis underlying neurite outgrowth through its direct or indirect association with the exocyst complex.
Acknowledgement

Firstly I would like to thank all the people who have contributed to the fulfillment of this thesis work. Without their generous help, I would not be able to finish all works I have done in this thesis. Above all, I am really grateful to Dr. Shu C. Hsu for giving me the opportunity to work with her during these past years. She has generously spent a lot of time to train me, inspire me, guide me, and advise me both in everyday life and in my PhD study. Without her patient training and guidance, I would not be able to get to where I am now. So from bottom of my heart, I really appreciate her mentoring during my graduation study.

I also want to thank all my committee members (Dr. Robin Davis, Dr. Bonnie Firestein, Dr. Mark Plummer and Dr. Renping Zhou), who spent their precious time in reviewing my thesis and support to my research work. In addition, I would like to thank all the members of the Rutgers community: Dr. Renping Zhou for his generous gift of pAdTrack-CMV vector, Rosa Cortes and Jason Magby for helping me to study calcium current measuring.

The support from the professional level is important for the completion of my thesis work, but without my family’s support, I would not be able to complete this work either. So, I want to thank my wife Heejung Yoo, my son Soowhan, and daughter Rachel. Without her support, encouragement and love, I would not be able to finish my research. I also want to thank to my mother Weeja Son, my mother Jaebum Kim, my brother Hyunjin Kim, mother-in-law Soonhee Jung, and father-in-law Seungryul Yoo for their continuous support and help. And finally, at a spiritual level, I wish to thank God for giving me these precious family members.
# Table of Contents

Abstract...........................................................................................................................................ii

Acknowledgement...............................................................................................................................iv

Table of Contents.................................................................................................................................v

List of Tables........................................................................................................................................viii

List of illustrations...............................................................................................................................ix

Introduction........................................................................................................................................1

A. Neurite outgrowth process.................................................................1

B. Axon//dendrite differentiation.............................................................5

C. Vesicle trafficking underlying neurite outgrowth.................................6

D. Characterization of septin family........................................................13

E. Septin protein SEPT2, formerly known as Nedd5 ...................23

Chapter 1 *In vitro* characterization of septin proteins.................25

Introduction.................................................................................................................................26

Material and methods......................................................................................28

Generation of recombinant septin proteins........................................28

Generation of monoclonal antibodies......................................................28

In vitro protein binding and immunoprecipitation assays...............29

Western blotting.......................................................................................30

Tubulin and septin polymerization assay..............................................31

Negative staining transmission electromicroscopy..........................31

Results........................................................................................................33
A. Production of recombinant septin proteins……………………………33
B. Generation of anti-septin monoclonal antibodies………………….33
C. Characterization of septin protein associations and filament formation
……………………………………………………………………………37
D. Tissue distribution and neuronal developmental expression of septin
proteins…………………………………………………………………39
Discussions…………………………………………………………………..41

Chapter 2 A member of septin family, SEPT2, plays a role in neurite outgrowth……….50

Introduction…………………………………………………………………51
Material and methods ……………………………………………………………53

Cell culture………………………………………………………………53
Sec8 and SEPT2 knockdown……………………………………………..53
Electrophysiological recording………………………………………………54
Patch-clamp recording…………………………………………………54
Data analysis……………………………………………………………54

Antibodies and immunocytochemistry………………………………55

Western blot……………………………………………………56

Results………………………………………………………………………57

A. Endogenous SEPT2 and exocyst show in vivo co-localization in
PC12 cells …………………………………………………………………57
B. Both exocyst and SEPT2 are upregulated upon NGF-induced neuronal
differentiation………………………………………………………….58
C. Exocyst subunit sec8 knock-down does not affect SEPT2 localization
and protein level but SEPT2 knock-down abolishes exocyst perinuclear enrichment and decreases exocyst protein level ……………60

D. Both SEPT2 and exocyst knock-downs inhibit neurite outgrowth ………63

E. SEPT2 and exocyst knock-downs decrease NGF-induced calcium channel insertion into the plasma membrane …………………..64

Discussions …………………………………………………………………………………..68

Conclusion and future directions ………………………………………………….76

References ………………………………………………………………………………..84

Curriculum Vitae ……………………………………………………………………121
Lists of tables

Table 1. Diseases correlated with defective septin function……………………………………..19

Table 2. Septin monoclonal antibodies available for immunoprecipitation (IP), immunocytochemistry (IC), and western blotting (WB) studies……………………………..36
List of illustrations

Fig. 1. A schematic diagram of the secretory vesicle trafficking pathway………………12

Fig. 2. Western blot and electron microscopic analysis of five septin proteins that co-purify and co-immunoprecipitate with the exocyst complex, a protein complex required for exocytosis.................................................................18

Fig. 3. An example of septin cDNA subcloned into the pGEX bacterial vector..........28

Fig. 4. A schematic diagram illustrating the in vitro protein binding assay strategy……30

Fig. 5. Recombinant septin proteins used for immunization.....................................44

Fig. 6. Anti-septin antibodies recognized septin proteins in brain lysates and recombinant septin proteins........................................................................................................45

Fig. 7. Intermolecular association of septin proteins.................................................46

Fig. 8. Electron microscopic analysis of septin filament formation..........................47

Fig. 9. Tissue distribution of septins........................................................................48

Fig. 10. Septin protein levels in brain during development........................................49

Fig. 11. SEPT2 and exocyst subunits co-localize in PC12 cells and show specific distribution, via a filament-like structure, from their perinuclear enrichment to the growth cone.........................................................................................71

Fig. 12. Both exocyst and septin2 are upregulated upon NGF-induced neuronal differentiation..................................................................................................................72

Fig. 13. Exocyst subunit sec8 knock-down does not affect SEPT2 perinuclear localization and protein level but septin2 knock-down abolishes exocyst perinuclear enrichment and decreases exocyst protein level.........................................................73
Fig. 14. Both SEPT2 and exocyst knock-downs by shRNA inhibit NGF-induced neurite outgrowth in PC12 cells.

Fig. 15. SEPT2 and exocyst knock-downs decrease NGF-induced calcium channel insertion into the plasma membrane.

Fig. 16. A working model proposed for potential function of SEPT2 in exocytosis.

Fig. 17. A model of how perturbation of SEPT2 function may affect synaptic protein trafficking in neurons.

Fig. 18. SEPT2 is expressed in hippocampal neurons.

Fig. 19. Manipulation of SEPT2 protein levels by shRNA knockdown and overexpression constructs.
INTRODUCTION

Communication between neurons and their targets is the cellular basis of most, if not all, neuronal function in the body. To achieve a precise and fast communication with their targets, neurons develop long extensions in the form of axons and dendrites. The development of these extensions, also known as the neurite outgrowth process, requires the coordination of multiple biological pathways including protein synthesis, cytoskeletal remodeling and vesicle trafficking. The cross-talking and regulation of these pathways are likely to be controlled by both intracellular and extracellular factors. Understanding the molecular mechanisms and regulation of these pathways should provide important insights into the neurite outgrowth process during neuronal development and regeneration.

A. The neurite outgrowth process

The neurite outgrowth process is a complex phenomenon that can be divided into three temporal stages: neurite initiation, elongation and specialization. Neurite initiation may be triggered either by inherited intrinsic factors such as members of the basic helix-loop-helix (bHLH) transcription factor family or by extracellular factors such as members of the neurotrophic factor family. For neuronal differentiation induced by intrinsic factors, inherited nuclear or cytosolic factors in neuronal progenitor cells activate the transcription of neuron-specific genes and thus initiate the production of neuronal proteins. A well-known example of intrinsic factor is Neuro D. Introduction of Neuro D into epithelial cells can change these cells into neurons. It can also promote premature cell cycle exit and differentiation of neural precursor cells (Lee et al., 1995). Gain-of-function
studies in Xenopus embryos and loss-of-function studies in mice indicate that one of the bHLH transcription factors, Neurogenin (Ngn), can induce the expression of Neuro D (Ma et al., 1996). Although the mechanisms of Neuro D function during neural development is not fully understood, it is reported that activated Neuro D can arrest cell-cycle by activating cyclin-dependent kinase p21 gene promoter (Mutoh et al., 1998), and phosphorylated Neuro D can stimulate dendritic growth via activating CaMKII (Gaudilliere et al., 2004).

For neuronal differentiation induced by extrinsic factors, extracellular factors bind to their respective receptors on the plasma membrane on the neuronal progenitor cell. This interaction initiates a signal cascade that ultimately results in changes in DNA transcription to promote the synthesis of neuronal proteins. A widely studied group of extrinsic factors is the neurotrophic factor family. According to the neurotrophic factor theory, target tissues produce a limited amount of neurotrophic factors to determine the number of surviving neurons innervating the tissues (Korsching, 1993). There are four major neurotrophic factor families: the neurotrophin family, the ciliary neurotrophic factor (CNTF) family, the leukemia inhibitory factor (LIF) family, and the fibroblast growth factor (FGF) family (Korsching, 1993 review). Well known neurotrophic factors involved in neuronal differentiation and development, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, -4, and -5 (NT-3, NT-4, NT-5), belong to the neurotrophin family. In general, the neurotrophins are recognized by the trk family of receptor tyrosine kinases (Cordon-Cardo et al., 1991, Lamballe et al., 1991, Soppet et al., 1991) and/or the p75 neurotrophin receptor (p75NTR) (Dechant and Barde, 2002). Each neurotrophin may bind to one or more Trk receptors. Each Trk receptor may be specific for a neurotrophin or can cross-react to multiple neurotrophins (Chao, 2003). In
general, the affinity of neurotrophins to their respective trk receptors is low, but these interactions can be strengthened by trk receptor dimerization and/or association with the p75NTR co-receptor (Arevalo et al., 2000, Esposito et al., 2001).

The first and the most intensely studied neurotrophin is NGF. Binding of NGF to the Trk receptor A activates the tyrosine-kinase domain of this receptor. The activated tyrosine-kinase domain then initiates multiple signal cascades, which include phospholipase C (PLC) - γ1 recruitment to the plasma membrane and the Ras/ERK (extracellular signal-regulated kinase) signaling pathway (Huang and Reichardt, 2003 review). The recruited active PLC-γ1 subsequently hydrolyzes phosphatidylinositides to generate inositol tris-phosphate and diacylglycerol (DAG) (Vetter et al., 1991). The formation of DAG then stimulates the activity of DAG-regulated protein kinase C (PKC) isoforms. In neuroendocrine pheochromocytoma (PC12) cells, a DAG-regulated PKCδ has been shown to be responsive to NGF activation and is required for activating the Erk signaling cascade to promote neurite outgrowth (Corbit et al., 1999). Also, phosphorylation of Trk A receptor can result in the recruitment and phosphorylation of Src homologous and collagen-like (Shc) adaptor proteins. Phosphorylation of Shc then leads to an increase in the activity of Ras and ERK. These events induce the activation of cyclic AMP-response element binding (CREB) transcription factor (Chao, 2003). CREB has been shown to change cellular transcription activity to regulate many neuronal activities, including neurite outgrowth and synaptic plasticity (Lonze and Ginty, 2002).

Following the onset of neuronal differentiation, neurite outgrowth takes place at a specialized structure called growth cone. Neuronal growth cone is composed of two major
domains: an actin-rich filopodium that forms the outer edge of the growth cone and a microtubule-rich lamellipodium that is located between the cell body (or a growing neurite shaft) and the filopodium, (Dehmelt and Halpain, 2004). The neurite outgrowth phenomenon is believed to involve at least two biological processes: cytoskeletal remodeling and Golgi-to-plasma membrane vesicle trafficking. Two major classes of cytoskeletons, microtubules and actin filaments, show changes in their intracellular distribution and polymerization state upon the initiation of neurite outgrowth. Although the signaling pathways leading to these changes are not yet defined, both microtubules and actin are found to become enriched and stabilized in their polymeric form at the site of neurite outgrowth. Microtubules are observed to advance into the neurite outgrowth initiation site in the lamellipodium of the nascent growth cone. As the newly formed neurite shaft elongates, microtubules become tightly packed into bundles inside the neurite shaft (Dehmelt and Halpain, 2004). Based on these findings, microtubules are believed to serve at least two roles in neurite outgrowth. First, since microtubules are the sturdiest cytoskeleton in neurons and microtubule bundling has often been observed at the site of neurite outgrowth and in the nascent neurite shaft (Gordon-Weeks, 2004), they may function as a mechanical support for deforming plasma membrane to allow neurite outgrowth initiation and for stabilizing the newly formed neurites. The mechanisms underlying this microtubule bundling process, however, are still unknown. Second, microtubules serve as “transportation tracks” for vesicle transport from Golgi to plasma membrane during neurite outgrowth. The mechanisms of vesicle transport by microtubule-associated motors will be discussed in the “neurite elongation” section. The actin cytoskeleton, like microtubules, has also been seen to concentrate near the plasma
membrane at the site of neurite outgrowth (Smith, 1988). Actin polymerization is observed at the tip of filopodia and at the leading edge of lamellipodia, driving membrane protrusion (Tilney et al., 1981). The mechanisms of this site-specific enrichment of actin filament are also not well defined.

The initiation of neurite outgrowth also requires the addition of plasma membrane at specific plasma membrane sites. Currently, no protein or signaling factors have been identified to direct site-specific vesicle trafficking to initiate neurite outgrowth. During neurite outgrowth, plasma membrane addition is believed to take place mainly at the lamellipodium of the growth cone. Vesicles carrying plasma membrane proteins bud off Golgi and are transported to the growth cone via microtubule-associated kinesin motors. Once vesicles are transported to the vicinity of the growth cone lamellipodial plasma membrane, it is believed that they are somehow “transferred” from microtubules to the cortical actin filaments lining the plasma membrane. These Golgi-derived vesicles are subsequently transported via actin-associated myosin motors to the plasma membrane (DePina and Langford, 1999) where fuse via interactions between vesicle and the plasma membrane SNARE proteins (Chen and Scheller, 2001 review).

**B. Axon/dendrite differentiation**

As neurites elongate, they also begin to differentiate into axons and dendrites. The molecular mechanisms underlying axon/dendritic differentiation are best studied in cultured hippocampal neurons. In this *in vitro* neuronal culture, Dotti et al. (1998) observed that in 2 div hippocampal neurons derived from E18 rats, the longest neurites
always differentiate into axons while the remaining neurites from the same neuron develop into dendrites. Recently, it was found that several proteins, including collapsin response mediating protein-2 (CRMP-2) and glycogen synthase kinase-3β (GSK-3β), play a role in neurite elongation and axon formation. CRMP-2 is a 65 kDa protein that has been found to promote neurite outgrowth and to be required for the formation of axons. For its role in promoting neurite outgrowth, it is reported that overexpression of CRMP-2 by adenoviral vector induced neurite elongation. In contrast, the introduction of a dominant negative form of CRMP-2 inhibited neurite formation in dorsal root ganglion organ culture (Suzuki et al., 2003). CRMP-2 is also found to be enriched in the growing axon of cultured hippocampal neuron. For its role in axon determination, overexpression of full-length CRMP-2 induced multiple axons whereas overexpression of truncated form of CRMP-2 inhibited the formation or the growth of primary axons in a dominant negative manner (Inagaki et al., 2001). It has been found that CRMP-2 activity is regulated by GSK-3β, a kinase that was first discovered for its role in glycogen synthesis but has since been found to phosphorylate other substrates including microtubule-association protein 1B (MAP1B), and CRMP-2 (Gordon-Weeks, 2004, Yoshimura et al., 2005). The inhibition of GSK-3β is associated with a reduction in phosphorylation of MAP1B, an increase in the population of stable microtubule in growing axons and growth cones, and reduced axon growth. In contrast, the activation of GSK-3β results in an increased CRMP-2 phosphorylation, which in turn inhibits axonal growth in hippocampal neurons.

C. Vesicle trafficking underlying neurite outgrowth
It is believed that even after axon/dendritic differentiation, the elongation of these two types of neurites is likely to use a very similar, if not identical, plasma membrane addition process. Regulation of this plasma membrane addition process by extracellular factors during neuronal development and regeneration controls both neurite outgrowth and regrowth. In order to elucidate this regulation mechanism, it is first necessary to define the biochemical events underlying the neurite outgrowth process. In this regard, extensive research has been devoted to identify protein players that compose the core vesicle trafficking machinery underlying neurite outgrowth.

The Golgi-to-plasma membrane vesicle trafficking process is best defined at two stages: vesicle budding from Golgi and vesicle fusion at the plasma membrane. Before budding, protein cargos are sorted into different vesicles based on, at least in part, interaction between the cargo proteins and various cytoplasmic adaptor proteins (Herrmann et al., 1999, and adaptor proteins are reviewed in van Vliet et al., 2003). Sometimes, certain cargo proteins can co-segregate with other proteins by adaptor protein-induced protein clustering (Simons and Ikonen, 1997). Vesicle budding is initiated when a group of cytoplasmic proteins called ARFs (ADP-ribosylation factors) bind to cargo proteins on the Golgi membrane. ARF are GTPases that remain the cytoplasm in their GDP-bound form. Upon exchange of GDP for GTP, the ARF proteins undergo conformational changes, leading to their binding to the Golgi membrane (Serafini et al., 1991). The binding of ARFs to the Golgi membrane then recruits coatamer proteins such as COPI, COPII or clathrin to the budding sites (Donaldson et al., 1992, Palmer et al., 1993, Bonifacino and Lippincott-Schwartz, 2003). Coatomers form a rigid cage encompassing the budding site, promoting the deformation of Golgi membrane to allow vesicle formation.
and budding (Orci et al., 1993). After the vesicle pinches off Golgi, coatomer proteins dissociate from the vesicle, followed by the hydrolysis of GTP from ARF and subsequent dissociation of ARFs from the vesicle (Tanigawa et al., 1993).

As the Golgi-derived vesicle arrives at its destination at the plasma membrane, vesicle fusion is mediated by the SNARE (soluble N-ethylmaleimide–sensitive factor attachment protein receptor) proteins. Vesicles derived from different organelles within the cell carry a distinct set of SNARE proteins called v-SNAREs (vesicle SNAREs). Interaction between v-SNAREs with their corresponding t-SNAREs (target SNARES) play a role in enforcing the fusion of vesicles to the correct target membranes, thus ensuring the accuracy of intracellular membrane trafficking, including Golgi-to-plasma membrane vesicle trafficking underlying neurite outgrowth (Rothman, 1994). However since t-SNARE proteins are localized everywhere on the plasma membrane, they cannot determine the vesicle fusion site for neurite outgrowth initiation. It has been hypothesized that proteins that function upstream of the SNARE proteins may play a role in determining vesicle fusion sites.

To date, in addition to microtubules, actin and their associated motor proteins, only one protein complex has been to be required for vesicle trafficking immediately upstream of the SNARE protein function. This protein complex, called exocyst, has been found to be important for neurite outgrowth (Vega and Hsu, 2001, 2003, Murthy and Schwarz, 2004). The exocyst complex is expressed ubiquitously in mammalian tissues, but is enriched in the brain and kidney (Hsu et al., 1996, 1998). Subunits of this complex were first identified by genetic screen looking for mutants defective in secretion in yeast Saccharomyces cerevisiae (Hsu et al., 2004 review) and the complex was later purified.
from yeast by Terbush et al. (Terbush et al., 1996). The exocyst complex is composed of eight subunits: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p. The mammalian homologues of the exocyst proteins were identified by screening human cDNA database with yeast exocyst subunit sequences. The mammalian exocyst complex was also purified from rat brain by column chromatography (reviewed by Hsu et al., 2004). The purified rat brain exocyst complex is very stable and has a molecular weight of 650-700 KDa (Hsu et al., 1996). Pair-wise yeast two hybrid and in vitro protein binding studies found that there is extensive interaction among exocyst subunits (Guo et al., 1999, Matern et al., 2001, and Vega and Hsu, 2001). It was postulated that the stability of the intact complex may be achieved through a series of higher order interactions which is not attainable via pair-wise interactions.

Because defects in every exocyst complex subunit resulted in secretion deficiency in yeast, it is postulated that the intact exocyst complex is required for secretion. In agreement, the introduction of anti-exocyst sec8 antibodies into MDCK epithelial cells disrupted protein targeting to the basolateral plasma membrane domain (Grindstaff et al., 1998). In addition, blocking of the exocyst function with anti-exocyst sec8 antibodies also disrupted the delivery of calcium signaling proteins to the apical plasma membrane domains in pancreatic acinar primary cells (Shin et al., 2000). Finally, Drosophila embryos with sec15 mutation exhibited a loss of Notch signaling. This loss of Notch signaling was postulated to be due to defective vesicle delivery since a vesicular compartment containing Notch, Sanpodo, and endocytosed Delta was found to accumulate in the basal areas of mutant sensory organ (Jafar-Nejad et al., 2005). For its role in neurite outgrowth, the overexpression of an Exocyst subunit sec10 mutant (sec10\textsuperscript{ΔCT}) was found to
inhibit NGF-induced neurite outgrowth in PC12 cells (Vega and Hsu, 2001). In *Drosophila*, embryos with exocyst subunit sec5 knock-out also inhibited neurite outgrowth (Murthy and Schwarz, 2004). Together these results support the hypothesis that the Exocyst complex is required for the trafficking of Golgi-derived vesicles to the plasma membrane in eukaryotic cells.

The molecular mechanisms underlying exocyst function in exocytosis are still under study. Two major hypotheses have been put forward to describe the role of exocyst in mediating plasma membrane addition (Review: Hsu *et al.*, 2004). In the first hypothesis, exocyst is described to regulate and mediate exocytosis by tethering vesicles at the appropriate plasma membrane domain to allow vesicle docking and fusion at that site. According to this model, one half of the exocyst complex would reside on vesicles while the other half of the complex would associate with the plasma membrane. Biochemical studies have found interaction, either direct or indirect, between exocyst and various vesicle cargo proteins including the NMDA receptor (Sans *et al.*, 2003). However, all exocyst subunits have been found to co-immunoprecipitate and co-purify together. There was no evidence that the exocyst complex subunits form two groups within the cell. In the second hypothesis, the exocyst is proposed to modulate the dynamics of microtubules and/or microtubule-associated motors which in turn mediate vesicle trafficking. There are several observations that support a functional relationship between exocyst and the microtubule system. First, the localization of the exocyst complex in pheochromocytoma (PC12) and normal rat kidney (NRK) cells is dependent on microtubule integrity (Vega and Hsu, 2001, Wang *et al.*, 2004). In undifferentiated PC12 cells, the exocyst complex was found largely near or at the microtubule-organizing center. Upon receiving neuronal
differentiation signals, its subcellular localization extended to the growing neurite and the growth cone (Vega and Hsu, 2001). In NRK cells, the exocyst subunits displayed localization similar to that of microtubules. In dividing NRK cells, the exocyst co-localized with spindle pole fibers composed of microtubules but contains no transport vesicles. Also, microtubule-disrupting drug affected the subcellular localization of the exocyst complex in both PC12 and NRK cells. In addition, the exocyst complex subunit Exo70 has been found to affect microtubule dynamics in both in vitro tubulin polymerization assays and in Exo70-overexpressed NRK cells (Wang et al., 2004). However, it is still not clear how exocyst may regulate vesicle trafficking through microtubules. In order to further understand the molecular mechanisms of exocyst, proteins associated with the exocyst complex was isolated. By immunoprecipitating with anti-exocyst subunit sec8 antibody, it was found that a group of five septin proteins SEPT2, SEPT4, SEPT6, SEPT7, and SEPT9 was the major exocyst-associated proteins. In fact, septins co-purified with exocyst during the purification of this complex from rat brain lysate (Hsu et al., 1998). This is a very unusual finding since septins were originally believed to be present only in dividing cells since their main known function is to facilitate cell division.
Fig. 1. A schematic diagram of the secretory vesicle trafficking pathway. Cargoes in the Golgi complex are sorted into different vesicles by tether proteins and adaptor proteins. Vesicles then bud off from the Golgi network and are transported to their destination via microtubule-associated motor proteins. When vesicles arrive at the vicinity of the plasma membrane, they are transported to the plasma membrane via actin-associated motors and fuse with the plasma membrane via SNARE protein-mediated interactions.
D. Characterization of septin family

The septin proteins were originally discovered in *Saccharomyces cerevisiae* as proteins that control the budding yeast cell cycle (Hartwell, 1971). Loss-of-function mutants of yeast septin genes *CDC3*, *CDC10*, *CDC11*, and *CDC12* show a loss of 10 nm filaments at cytoplasmic side of the plasma membrane in the mother-bud neck. Thus the name “septin” was chosen to reflect to the role of these proteins in separating mother-daughter cells at the cleavage furrow or septum. Electron microscopy studies in *S. cerevisiae* showed that septin forms a ring structure at this region (Longtine et al., 1996). Temperature-sensitive (ts) -lethal mutants of septins resulted in defective cytokinesis and in the formation of multi-budded, multinucleated cells at the restrictive temperature (Hartwell et al., 1974). These findings strongly suggest a role for septins in cytokinesis. As the function of septins was further studied, it was discovered that these proteins may have multiple functions in the cell (reviewed by Longtine et al., 1996). Termperature-sensitive mutants of *cdc3*, *cdc10*, *cdc11*, and *cdc12* were found to synthesize chitin early in the cell cycle as in the wild-type yeast, but the synthesized chitin was deposited diffusely in the cell wall of the bud at the restrictive temperature. This observation suggests that septins may play a role in the localization of chitin synthase and/or of relevant activating factors. Also, genetic evidence suggests postential interactions between septins and proteins involved in bipolar budding, such as Spa2p. Deletion of the SPA2 gene was found to result in randomized budding. When SPA2 knock-out was combined with a non-lethal *cdc10* mutation, the double mutation became lethal, suggesting potential interactions between the budding protein Spa2p and the septins.
(Flescher et al., 1993). These findings bring up the possibility that septins may play a role in regulating protein localization/delivery for the establishment of cell polarity.

It is now known that septin proteins are present in all eukaryotic organisms except for plants. Previously, nomenclature of mammalian septins was confusing and chaotic because these proteins were named before they were discovered to be members of a large protein family. Macara et al. proposed a nomenclature system based on the potential evolutionary relatedness of septins that is now widely accepted in the septin field (Macara et al., 2002).

Currently, known septin proteins range from 31,990 kDa to 60,200 kDa in molecular weight. Septin protein sequences are highly conserved evolutionarily and show no homology to other known proteins (Longtine et al., 1996). DNA sequence alignment studies reveal that each septin protein consists of three domains: a N-terminal variable region, a conserved GTPase domain, and a C-terminal region (Field and Kellogg, 1999). The N-terminal variable region is unique to each septin and is assumed to contribute to the functional specificity of each septin. On the contrary, most septins possess a GTP-/APT-binding motif (P-loop motif) in a conserved GTPase domain. In agreement, at least some of the septins from Drosophila, S. cerevisiae, and mammals have been found to bind GTP and GDP (Longtine et al., 1996, Kinoshita et al., 1997, Vega and Hsu, 2003). The C-terminal domains of septins contain a coil-coil structure and are thus a possible region for protein-protein interactions. The GTPase activity has been found to be required for septin filament formation; however, the coiled-coil domain of the C-terminal region is not essential for this process (Mendoza et al., 2002).
Fourteen septin genes have been discovered from human tissues and eight of them are present in mouse and rat. All known mammalian septins can be classified in four subfamily groups - group I (SEPT3, SEPT9, and SEPT12), group II (SEPT6, SEPT8, SEPT10, and SEPT11), group III (SEPT1, SEPT2, SEPT4, and SEPT5), and group IV (SEPT7) (Martinez and Ware, 2004). Like their yeast counterparts, septins in higher organisms are also reported to be involved in cytokinesis and vesicle trafficking. In accordance with this speculation, *Drosophila* septin proteins Pnut and Sep1 were found to colocalize near the contractile ring between two dividing cells (Neufeld and Rubin, 1994, Fares et al., 1995). In fact, a mutation in *pnut* led to a lethal phenotype with multinucleated cells in the larval tissues (Neufeld and Rubin, 1994). In the same manner, SEPT2 in SiHa cell localized to the plasma membrane around the contractile ring early in telophase (Kinoshita et al., 1997). It was also reported that the injection of SEPT2 antibody into HeLa cells in late anaphase to early telophase resulted in binucleated cell phenotype (Kinoshita et al., 1997). In addition, another septin protein, SEPT4, was also reported localize to the cleavage furrow of dividing COS cells (Xie et al., 1999).

Currently, there are also findings that suggest an involvement of septin proteins in other cellular processes. Beites and colleagues found that a septin protein, SEPT5, co-fractionated with synaptic vesicles on a density gradient from rat brain lysates. It also coimmunoprecipitated with a SNARE protein, syntaxin, found on the neuronal plasma membrane. The introduction of a GTPase dominant negative form of SEPT5 into insulin-secreting HIT-T15 cell line enhanced exocytosis whereas the overexpression of wild-type SEPT5 inhibited exocytosis (Beites et al., 1999). In addition, one of the septin protein SEPT4 variant, ARTS (apoptosis-related protein in the TGF-β signaling pathway),
was found to enhance cell death induced by TGF-β (Larish et al., 2000). Finally, septin proteins have also been implicated in neurodegeneration. SEPT1, SEPT2, and SEPT4 are localized to abnormal filamentous structures which are a part of the pathological features of Alzheimer’s disease (Kinoshita et al., 1998). SEPT5 was also observed to be ubiquitinated and degraded by Parkin, a protein involved in Parkinson’s disease (Zhang et al., 2000). Another septin protein, SEPT7, is localized to the dendritic branching points in developing hippocampal neurons. When SEPT7 was downregulated, dendritic branching was impaired (Xie et al., 2007). In addition, a GTPase-defective SEPT2 mutant showed an increased number of neurites and thicker neurites in NGF-induced differentiated neuroendocrine PC12 cells (Irving and Hsu, 2003). In Caenorhabditis elegans, two septin genes, UNC-59 and UNC-61, have also been found to be required for neuronal migration and neurite pathfinding during development (Finger et al., 2003). These results reveal important functions for septins in neuronal development. Studies to date also suggest that there may be some functional redundancy among septin family members. Sept6-deficient mice did not exhibit any observable abnormal phenotype nor caused any additional changes in the Sept4-deficient mice. In addition, the deletion of a closely related homologue of Sept6 did not affect Sept6-null cells in vivo (Ono et al., 2005).

In order to define the molecular mechanisms underlying the diverse array of septin function, the molecular associations of various septin proteins have been studied. Five septin proteins, SEPT2, SEPT4, SEPT6, SEPT7, SEPT9, have been found to copurify and coimmunoprecipitate with the exocyst complex from rat brain lysate (Hsu et al., 1998). As the exocyst complex was known to play a role in exocytic vesicle trafficking, it was hypothesized that at least some septin proteins play a role in vesicle trafficking via their
association with this protein complex. The mammalian septins, like their yeast and fruit fly counterparts, also form 8nm wide filaments (Hsu et al., 1998). Various mammalian septins also showed potential interaction with microtubules and actin cytoskeletons. The treatment of HeLa cells with cytochalasin D, which directly inhibits F actin formation, disrupted the fibrous distribution of SEPT2. Botulinus C3 exoenzyme which disturb F actin also disrupted SEPT2 filament structure in the HeLa cell (Kinoshita et al., 1997). In mouse NIH3T3 fibroblast cells, SEPT2 and SEPT6 colocalized with actin bundles. When actin filaments were disrupted by cytochalasin D, the majority of SEPT2 and SEPT6 ended up as ring structures with an outer diameter of about 0.7nm (Kinoshita et al., 2002). On the other hand, SEPT2 exhibited perinuclear enrichment near the microtubule-organizing center, similar to that shown for microtubules and exocyst complex subunits (Irving and Hsu, 2003). Also, SEPT7 suppression by SiRNA increased microtubule stability in HeLa cells (Kremer et al., 2005). Spiliotis and colleagues found that SEPT2 fibers colocalize with, and are required for polyglutamylated microtubule tracks in MDCK cells (Spiliotis et al., 2008). They also reported that vesicles containing apical or basolateral proteins exited the trans-Golgi network along these SEPT2/polyglutamylated microtubule tracks. These observations bring up the possibility that at least some of the molecular mechanisms underlying septins’ diverse function may be due to their association with ubiquitous cytoskeletons and vesicle trafficking machinery.
Fig. 2. Western blot and electron microscopic analysis of five septin proteins that co-purify and co-immunoprecipitate with the exocyst complex, a protein complex required for exocytosis.

A septin complex composed of five septin proteins: septins 2, 4, 6, 7, and 9, have been shown to co-purify with the exocyst complex. The panel on the right shows a Coomassie blue-stained SDS-polyacrylamide gel of the purified septin complex. The panel on the left shows a scanning electron micrograph of purified exocyst complex (the first two rows and the left panel of the third row), thyroglobulin (a protein complex of approximately 600 kDa as a molecular size control) and the purified septin complex as 8 nm filaments.
Table 1. Diseases correlated with defective septin function

<table>
<thead>
<tr>
<th>Septin</th>
<th>Related Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEPT2</td>
<td>Localized to the paired helical filaments in Alzheimer’s disease brains</td>
<td>Kinoshita et al., 1998</td>
</tr>
<tr>
<td></td>
<td>C-terminal is autoantigen in systemic lupus erythematosus with psychiatric manifestations</td>
<td>Margutti et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Up-regulated in renal cell carcinoma patient</td>
<td>Craven et al., 2006, Craven et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Downregulated in glioblastoma multiform</td>
<td>Khalil 2007</td>
</tr>
<tr>
<td>SEPT3</td>
<td>Different isoform expression in Alzheimer’s disease</td>
<td>Takahashi et al., 2004</td>
</tr>
<tr>
<td>SEPT5</td>
<td>Overexpression of Sept5 lead a progressive loss of nigral dopaminergic neurons and a decline of the striatal dopamine levels</td>
<td>Dong et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Accumulation of Sept5 cause cytotoxic cell death</td>
<td>Son et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Overexpressed in the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder patient</td>
<td>Pennington et al., 2007</td>
</tr>
<tr>
<td>SEPT6</td>
<td>Fusion partner of MLL</td>
<td>Ono et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Upregulated in melanoma cells</td>
<td>Jaeger et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Overexpressed in the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder patient</td>
<td>Pennington et al., 2007</td>
</tr>
<tr>
<td>SEPT7</td>
<td>Downregulated in Down syndrome brain</td>
<td>Engidawork et al.,</td>
</tr>
<tr>
<td>Protein</td>
<td>Function and Characteristics</td>
<td>Year</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>SEPT9</td>
<td>Lost alleles in sporadic ovarian cancers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overexpressed in various cancer cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mutations cause hereditary neuralgic amyotrophy (HNA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Expression level is correlated with susceptibility to microtubule-disrupting agents</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transcription fusion partner of MLL in <em>de novo</em> myelodysplastic syndrome</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Commonly methylated in Head and neck squamous cell carcinoma</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overexpressed in the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder patient</td>
<td></td>
</tr>
</tbody>
</table>
Regulation of septin protein function has also been investigated. Some proteins, such as Borg 3 and rhotekin, have been found to modulate septin filament formation. In mammalian cells, SEPT2-containing filaments and rings are highly dynamic structures that undergo a rapid exchange between cytoplasmic and filamentous SEPT2 pools (Schmidt and Nichols, 2004). Although the significance of this dynamic exchange is not clear, a protein, Borg3, has been implicated to play a role in this dynamics. Joberty and colleagues have shown that Borg3, a downstream effector of Cdc42, directly binds to the mammalian SEPT2-SEPT6-SEPT7 complex and disrupt normal septin organization in MDCK cells (Joberty et al., 2001). Further studies revealed that Borg3 can bind to the SEPT6/7 heterodimer and the SEPT2/6/7 trimer, but not to these septin monomers or to other heterodimers, through its borg-specific BD domain (Sheffield et al., 2003). It is hypothesized that the binding of Borg3 may cover the binding site of septin proteins for other proteins (Sheffield et al., 2003). However, the effect of borg3 on septin dynamics is still unknown. The expression of a constitutively active form of Rho mutant was also found to disrupt the filamentous structure of SEPT9b. In this case, rhotekin, a rho-activating protein has been implicated in regulating septin filament assembly by regulating rho activity (Ito et al., 2005).

A second mechanism that regulates septin function involves the attachment of small ubiquitin-like SUMO proteins to the septin proteins, or sumoylation. In yeast, a SUMO protein, Smt3, was localized to the mother-bud neck between two dividing yeast cells and covalently attaches to the septin Cdc3. This attachment is mediated by the ubiquitination E3 ligase Siz1 (Takahashi et al., 2001 and Makhnevych et al., 2007). The
conjugation of Smt3 to Cdc3 has been postulated to modulate stability of Cdc3 protein during a cell cycle (Takahashi et al., 1999).

The regulation of septin levels in the cell may also affect septin function. In fact, changes in septin levels and mutation have been correlated with the onset of various diseases. In some brain tumor cells, SEPT2 is downregulated as compared to non-tumor cells (Khalil, 2007). Also, SEPT2 upregulation and mutation have been correlated with the occurrence of renal carcinoma (Craven et al., 2006). SEPT6, although exhibited no apparent function in animal knockout studies, was reported to show a decreased expression in fetal brain with Down syndrome (Cheon et al., 2001). Likewise, SEPT7 was also downregulated in cerebral cortex of fetuses with Down syndrome (Engidawork et al., 2006). More strikingly, Son and colleagues showed that the accumulation of Parkin substrate, SEPT5, could cause dopaminergic cell death in transgenic mouse (Son et al., 2005). Other septin-related to diseases are summarized in table 1.

The levels of septin proteins in the cell have been shown to be regulated, at least in part, by ubiquitination. In Drosophila, the overexpression of Septin 4 (orthologues of human SEPT5) caused an age-dependent disruption of dopaminergic neuron integrity in dorsal cluster. This dopaminergic neuronal degeneration was suppressed by the coexpression of Parkin, an E3 ubiquitin ligase, and was enhanced by a reduced Parkin function (Munoz-Soriano and Paricio, 2007). Munoz-Soriano and Paricio showed that Parkin and Septin 4 actually physically interact with each other in vitro. These results strongly suggest that Septin 4 degradation may be, at least partly, controlled by ubiquitination.
E. Septin protein SEPT2, formerly known as Nedd5

SEPT2 was first identified in mouse and has been found to possess GTPase activity (Kinoshita et al., 1997, Huang et al., 2006). Kinoshita and colleagues showed that GTP hydrolysis is required for SEPT2 to form a filament and to interact with actin bundles (Kinoshita et al., 1997). In addition, there is also a potential interaction between SEPT2 and microtubules since SEPT2 was one of the septins that coimmunoprecipitated with tubulin and exocyst complex subunits in brain and neuroendocrine PC12 cells (Vega and Hsu, 2003). When a GFP-tagged GTPase mutant of SEPT2 was introduced into HeLa cells, the mutant SEPT2 protein appeared to form large aggregates instead of filaments. In agreement, the introduction of a nonhydrolyzable GTP analog into HeLa cells also disrupted the fibrous distribution of SEPT2 (Kinoshita et al., 1997).

Originally, the SEPT2 was thought to be only required for mitosis (Kinoshita et al., 1997, Spiliotis et al., 2005). However, the presence of SEPT2 in postmitotic neurons brings up the possibility that it may play a role in neuronal function. In addition, the overexpression of a GTPase-defective SEPT2 into neuroendocrine PC12 cells resulted in more and thicker neurites, as well as larger cell bodies (Vega and Hsu, 2003). These results suggest SEPT2 may play a role regulating neurite outgrowth. To begin investigating the role of SEPT2 in neuronal development and function, two major experimental approaches will be used. In the first approach (Chapter 1), necessary reagents for SEPT studies will be generated. These reagents will be used to characterize the molecular associations and tissue distribution of septin proteins. In the second approach (Chapter 2), we will assess the role of a functional relationship between SEPT2
and its associated exocyst complex in regulating neurite outgrowth. Understanding the role of SEPT 2 in neurite outgrowth is expected to provide important insights into the mechanisms and regulation of neuronal development and function.
Chapter 1 *In vitro* characterization of septin proteins
INTRODUCTION

Septins constitute a family of evolutionarily conserved proteins increasingly found to be important in various neurodegenerative states, such as Parkinson’s and Alzheimer’s diseases. Three members of the mammalian septin family, Nedd5 (SEPT2), Diff6 (SEPT1) and hCDC10 (SEPT7), have been reported to be present in the neurofibrillary tangles of senile plaques in brains affected by Alzheimer’s disease (Kinoshita et al., 1998). Another septin, H5 (SEPT4), was found in the α-synuclein-positive cytoplasmic inclusions associated with Parkinson’s disease as well as other synucleinopathies (Ihara et al., 2003). Co-expression of SEPT4 and α-synuclein induced the formation of Lewy Body-like cytoplasmic inclusions and eventual cell death in NIH3T3 cells. Finally, a neuron-specific septin CDCrel-1 (SEPT5) was shown to be a potential target of parkin, a ubiquitin protein ligase linked to autosomal-recessive juvenile parkinsonism (Zhang et al., 2000). Viral vector-mediated overexpression of this septin in substantia nigra caused death in nigral dopaminergic neurons (Dong et al., 2003). These observations strongly suggest a role of septins in neurodegeneration. As a first step toward understanding the molecular mechanisms of septins in neurodegeneration, it is important to gain insights into their physical properties and molecular associations.

Most septins contain a GTP-binding motif and some of them have been shown to exhibit GTP-binding and GTPase activities. In addition, various septins have also been found to form 8-10 nm filaments in vivo. A key question that has been intensely studied, but has yet to be answered, in the septin field is whether septin filament formation in response to GTP is a requirement for septin function. It is not clear whether septins form
homopolymeric and/or heteropolymeric filaments in the cell. It is also not known whether the septin filament composition determines the function and molecular associations of septins. As a first step to address these questions, four projects will be carried out.

A. Soluble recombinant septin proteins will be generated for both in vitro septin filament assembly studies and monoclonal antibody generation.

B. Monoclonal antibodies against septin proteins will be generated to allow the detection of recombinant and endogenous septin proteins for western blotting and immunofluorescence microscopy assays.

C. Septin protein interactions will be studied using in vitro protein binding and septin filament formation assays.

D. To monitor the tissue distribution and neuronal developmental expression of septin proteins to gain insights into the requirement of septins during brain development.
MATERIAL AND METHODS

GENERATION OF RECOMBINANT SEPTIN PROTEINS

The cDNAs of SEPT 2, 4, 6, 7 and 9 were subcloned into the pGEX-KG bacterial vector and transformed into *E. coli* bacteria. The bacteria were grown at 25 °C and the production of glutathione-S-transferase (GST)-septin proteins in bacteria was induced by the addition of 1mM IPTG. The harvested fusion proteins were purified by binding to glutathione-coupled beads. The GST moiety of these proteins was removed by elution from glutathione beads with cleavage by the thrombin enzyme in the presence of 50 mM Tris, pH 8.0, 300 mM NaCl and 1mM dithiothreitol at 4 °C.

![Fig. 3 An example of septin cDNA subcloned into the pGEX bacterial vector.](image)

The cDNA of SEPT2 protein was subcloned into the multiple cloning sites of the pGEX-KG vector downstream of DNA coding for the glutathione-S-transferase protein. The SEPT2 cDNA was subcloned into the vector at the EcoRI and XhoI sites.

GENERATION OF MONOCLONAL ANTIBODIES

Three female BLB/c mice (8 week-old) were boosted with 10 μg each of recombinant glutathione-S-transferase (GST)-septin proteins prepared using typical GST-fusion protein production protocol (Guan and Dickson, 1991). Purified recombinant septin proteins were emulsified with Freund’s or Ribi's adjuvant, with injections preformed every 3 weeks. After a series of three injections, sera from tail bleeds were tested by
Western blotting. Two mice with the best titers were selected for fusion. Five days before the fusion, mice were boosted by peritoneal injection of 20 μg recombinant septin proteins. On the day of fusion, mouse spleens were removed and fused with NS-1 myeloma cells in a 3:1 ratio using polyethylene glycol 1500 as the fusion agent. The treated cells were resuspended in PHMII media (from Gibco) containing 20% fetal calf serum (Sigma) and hypoxanthine/aminopterin/thymidine (HAT), and plated into 15 96-well plates per spleen. After 10-14 days hybridoma colonies were visible by eye and were tested by ELISA and Western blotting. Positive colonies were subcloned twice and grown up to larger volumes for further testing. The cells were adapted into PFHMII medium (Gibco BRL) containing 4% fetal calf serum for antibody production. Supernatants containing antibodies secreted from individual hybridoma clones were harvested and used for subsequent Western blotting, indirect immunofluorescence and immunoprecipitation studies.

**IN VITRO PROTEIN BINDING AND IMMUNOPRECIPITATION ASSAYS**

For *in vitro* protein binding studies, GST-fusion proteins of exocyst subunits or septin proteins were bound to glutathione beads at a concentration of 2 μg protein/μl beads via GST/glutathione interaction. These beads were then incubated with soluble recombinant proteins (1μg protein/μl bead concentration) or brain lysate (10mg protein/ml concentration) for at least 4 hours at 4 °C. The binding buffer for all *in vitro* studies contain 50 mM Tris, pH 8.0, 150 mM NaCl and 1 mM dithiothreitol. Following the incubation, beads were washed with the binding buffer and bound proteins were subjected to western blotting analyses. For immunoprecipitation studies, monoclonal antibodies were cross-linked onto protein A beads at a concentration of 2mg antibody/ml beads with
dimethylpimelimidate. The prepared beads were then incubated with recombinant proteins for at least 4 hours at 4 °C. Following the incubation, the beads were washed intensively and proteins bound to beads were solubilized in protein sample buffer. The proteins samples were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting to detect bound proteins.

**Fig 4.** A schematic diagram illustrating the *in vitro* protein binding assay strategy. GST-fusion proteins or antibodies bound to beads are incubated with either purified recombinant proteins or brain lysates. Following incubation, beads were washed and proteins bound to the fusion protein or antibody “baits” are analyzed by western blotting.

**WESTERN BLOTTING**

Rat brain lysates were prepared by homogenizing whole rat brain or dissected rat brain parts in seven volumes of lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl and 2 mM EDTA). The lysates were centrifuged twice at 3000 x g to remove intact cells and nuclei. Recombinant septin proteins were produced as described above. Protein concentrations were determined by BioRad Comassie Blue Protein assay.
Protein samples were resolved on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes for Western blot analysis. The nitrocellulose membranes were blocked by incubation with 5% milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) for 1 hr at room temperature. Membranes were then incubated with hybridoma supernatants containing monoclonal antibodies overnight at 4 °C. Next day the membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse antibodies (1:5000 dilution in 5% milk/TBST) for 1 hr at room temperature. Following incubation, membranes were washed with TBST and labeled protein bands were visualized by chemiluminescence (ECL reagent, NEN).

TUBULIN AND SEPTIN POLYMERIZATION ASSAY

To assess the ability of purified recombinant septin proteins in forming filaments, mixed septin proteins (SEPT2, 4, 6, 7, and 9; septins) or individual septins SEPT2, SEPT4, SEPT6 and SEPT9 were incubated at 1 mg/ml concentration to allow polymerization in the presence of 1 mM GTP in a final buffer condition containing 50 mM MOPS, 1.5 mM Hepes, 50 mM NaCl, 1 mM EGTA, 0.5 mM MgSO4, and 10% glycerol, pH 7.0. As a control, 25 μM purified tubulin (Cytoskeleton Inc. ) was incubated for 1 h in a same buffer. 1 mM of GTP and 2 μM taxol were then added to the mixture to initiate tubulin polymerization at 25 °C.

NEGATIVE STAINING TRANSMISSION ELECTRON MICROSCOPY

To visualize microtubules following exocyst activity assay, the tubulin or septin
mixture was adsorbed onto formvar/carbon-coated 200-mesh copper grids (EMS, Fort Washington, PA) for 1 min. The grids were then stained with 1% uranyl acetate for another minute and air-dried for 30 min before visualization at Rutgers electron microscopy facility. All procedures were carried out at room temperature.
RESULTS

A. PRODUCTION OF RECOMBINANT SEPTIN PROTEINS

Since five septin proteins, SEPT 2, 4, 6, 7 and 9 have been previously shown to be present in brain and co-purify with the exocyst complex, these proteins were produced individually from *E. coli* bacteria and purified via glutathione affinity chromatography. Recombinant septin proteins are notoriously insoluble. We have systematically tested bacterial growth temperature (18 °C, 25 °C, 30 °C and 37 °C), protein production time (4 hours, 12 hours and 24 hours), and fusion protein purification buffer (20 mM Tris, pH 8.0, 50 mM Tris, pH 8.0, 50 mM Hepes, pH 7.4) and salt concentration (NaCl concentrations of 50 mM, 150 mM, 300 mM and 500 mM). We found that growing bacteria at 25 °C, allowing protein production to proceed for 12 hours and purifying septin proteins in a buffer composition of 50 mM Tris, pH 8.0 and 300 mM NaCl produced most soluble septin proteins. However, even under these conditions, we could only produce 20-100 µg recombinant proteins per liter of bacteria. Fig. 5 shows a Coomassie blue-stained gel of purified septin proteins. The purified septin proteins were subsequently used for monoclonal antibody generation and *in vitro* protein binding studies.

B. GENERATION OF ANTI-SEPTIN MONOCLONAL ANTIBODIES

For the first few immunization attempts, mice were injected with purified rat brain septin complex composed of SEPT 2, 4, 6, 7 and 9. However, not a single mouse produced detectable response against any septin protein. In our later immunization attempts, we injected mice with purified soluble recombinant septin proteins (Fig. 5). These proteins
were produced in *E. coli* bacteria as GST-fusion proteins and purified by glutathione-agarose chromatography. Their GST moieties were subsequently removed by cleavage with the enzyme thrombin. The purified proteins were injected into mice with either Freund's or Ribi's adjuvant. Only mice immunized with Ribi's adjuvant produced immune response to the injected proteins. Two mice with best response from each immunization trial were selected for cell fusion. From four fusion experiments (8 mice), monoclonal antibodies against the immunized five septin proteins were generated. The generated antibodies were tested for their usefulness in immunoprecipitation, immunocytochemistry and western blotting. Table 2 below summarizes all the generated septin monoclonal antibodies and their experimental usefulness. All antibodies were identified by their ability to recognize their corresponding recombinant septin proteins and endogenous septin proteins in rat brain lysate (Fig. 6). The recombinant septin proteins produced in *E. coli* and endogenous septin proteins seem to have very similar, if not identical, molecular weights. Anti-SEPT7 antibody recognized two protein bands for very similar molecular weights in the brain lysates. This detected SEPT7 double bands could be due to a slight degradation of a population of SEPT7 in brain, a result of differential post-translational modification of SEPT7 or a splicing variant of SEPT7. However, no splicing variants have been described for SEPT7 so far. Anti-SEPT9 and –SEPT2 antibodies also recognized a doublet (for SEPT9) and multiple (for SEPT2) protein bands in the recombinant septin protein lanes. These multiple bands are likely a result of degradation or incomplete synthesis of recombinant proteins. Each antibody was also tested for its ability to immunoprecipitate its corresponding antigen from rat brain lysate. To qualify as a good immunoprecipitating antibody, the immunoprecipitated proteins must
be detectable by Coomassie blue staining of SDS-polyacrylamide gels. Only two antibodies, anti-SEPT2 NH17G10 and anti-SEPT4 H8B1 antibodies were found to satisfy this criterion. Other anti-septin antibodies may precipitate septins with much weaker affinity or not at all. The criterion for a good immunocytochemistry antibody is that the antibody can reproducibly detect endogenous septins, preferably in the form of some organized cellular structures. Antibodies that produce a diffuse staining pattern throughout the cells, including the nucleus, were not used for subsequent immunocytochemistry experiments. Two antibodies against SEPT2 satisfied this criterion. These two antibodies, along with a polyclonal antibody generated against SEPT2, revealed SEPT2 enrichment near the microtubule-organizing center of neuroendocrine PC12 cells (Data not shown). This localization will be described in more detail in Chapter 2.
Table 2. Septin monoclonal antibodies available for immunoprecipitation (IP), immunocytochemistry (IC) and western blotting (WB) studies.

<table>
<thead>
<tr>
<th></th>
<th>Anti-SEPT2</th>
<th>Anti-SEPT4</th>
<th>Anti-SEPT6</th>
<th>Anti-SEPT7</th>
<th>Anti-SEPT9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IP</strong></td>
<td>NH17G10</td>
<td>H8B1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>IC</strong></td>
<td>NH17G10</td>
<td>N510A3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>WB</strong></td>
<td>NH17G10</td>
<td>H7A8</td>
<td>KH4F8</td>
<td>C10D5</td>
<td>ES4C10</td>
</tr>
<tr>
<td></td>
<td>N510A3</td>
<td>H8B1</td>
<td>KH4F9</td>
<td>ES4C10</td>
<td>E4A5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>KH6E4</td>
<td></td>
<td>E8B5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>K12A2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>K16B6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>K9F5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>KH10B3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
C. CHARACTERIZATION OF SEPTIN PROTEIN ASSOCIATIONS AND FILAMENT FORMATION

Since endogenous septin proteins have been purified from various sources, including the rat brain, as filaments, it brings up the question as to whether septins can form homopolymeric and/or heteropolymeric filaments in the cell. Understanding the composition and the dynamics of septin filament formation may provide important insights into the structure/function relationship between septin filament composition and septin function.

To date, one of the problems in assessing the composition of septin filaments is the difficulty in producing soluble recombinant septin proteins for in vitro septin binding and filament formation studies. Using different fusion protein production protocols described above, small amounts of recombinant septin proteins were produced. These proteins seemed to denature and precipitate out of resuspension within 24 hours after purification. Therefore, all in vitro experiments were carried out immediately after protein purification.

Since five septin proteins, SEPT2, 4, 6, 7 and 9 have been shown to co-purify and coimmunoprecipitate from rat brain, it brings up the possibility that these septins may interact with each other. To test this hypothesis, individual septins, with their GST moiety, were bound to glutathione beads. The bound septins were then incubated with soluble septin proteins with their GST moiety removed by thrombin cleavage. Soluble septins that bind to the immobilized septin “baits” were detected by western blotting following SDS-PAGE. Fig. 7 shows that no significant cross-interactions among different septin proteins were detected. These results may suggest that there is no interaction among different septin proteins. Alternatively, it is also possible that the recombinant proteins
were not folded properly for protein binding studies. It is interesting to note that there seems to be significant homophilic interactions for each septin. These observations may suggest that there might be a significant homophilic interaction for each septin, thus allowing them to form homophilic septin filaments. Different homophilic septin filaments can then interact with each other, forming a large heterophilic septin filaments composed of multiple septins. Alternatively, the detected septin proteins could also be due to “sticky” septin proteins that bound to the glutathione beads nonspecifically or to the immobilized GST-septin fusion proteins in the absence of GST-moiety. In this regard, septin proteins have been found to lose their GST-moiety due to non-specific protein degradation process and still remain bound to the glutathione beads due to their “stickiness” or aggregation with GST-septins on the beads.

To confirm whether septin proteins can form homophilic filaments or the observed binding data are results of non-specific protein binding to the glutathione beads, soluble recombinant septin proteins without their GST moieties were incubated at room temperature in the presence of 1 mM GTP for one hour to promote septin filament formation, as previously described for yeast septins. The resulting filaments were detected by electron microscopy (Fig. 8). Microtubules formed from tubulin monomers in the presence of GTP were used as controls. Under this condition, abundant microtubules could be seen forming from tubulin (mt). However, no discernible septin filaments were observed when all five septins, SEPT2, 4, 6, 7, and 9 (septins) or individual septins, SEPT2, 4, 6 and 9 were subjected to the same treatment. Even after a prolonged incubation up to 4 hours, no septin filaments were observed. These results suggest that recombinant proteins under the conditions we have optimized so far are not competent for filament studies. It is
possible that some post-translational modifications are required for proper septin folding and interactions. In the future, the production of functional mammalian septin proteins may be carried out in insect cell lines, which are devoid of endogenous mammalian septins, for proper post-translational modifications.

D. TISSUE DISTRIBUTION AND NEURONAL DEVELOPMENTAL EXPRESSION OF SEPTIN PROTEINS

To gain more insights into the function of septin proteins, the tissue distribution of SEPT2, 4, 6, 7 and 9 was studied. Fig. 9 shows that all five septin proteins have broad tissue distribution. Not surprisingly all five septins are present in brain. These septins, however, do not have the same tissue distribution pattern. It is likely that different tissues have different septin composition. It is also interesting to note that SEPT2, unlike other septins, has identical tissue distribution as the exocyst subunit sec15. This observation is in agreement with previous findings that SEPT2 co-purify and co-immunoprecipitate with exocyst subunits. These results bring up the possibility that SEPT2 may have a functional relationship with the exocyst complex. This possibility will be investigated in Chapter 2.

Although the overexpression of a SEPT2 mutant has been shown to perturb neurite outgrowth in neuroendocrine PC12 cells, it is not clear what physiological roles it may play in neurons. To begin answering this question, the expression level of SEPT2 in brain during development was investigated. SEPT2 showed a slight increase in protein level from embryo to adulthood (Fig. 10). The exocyst subunit sec15 also exhibited a similar slight increase in its expression level during development. Interestingly, the expression level of SEPT4 transiently increased during development, with peak level occurring
between E18 to P21. In contrast, SEPT6 and 7 did not show detectable changes in their expression level during development. Two protein bands of a higher molecular weight, however, showed up for SEPT6 at E18 and P5 stages. It is not clear whether these protein bands are unrelated cross-reacting bands or represent alternatively spliced or post-translationally modified forms of SEPT6.
DISCUSSIONS

Septins are members of a growing family with increasing number of functions ascribed to them. Although many members of this family were originally discovered for their requirement in cytokinesis, it is now believed that septins participate in many other cellular processes. In yeast *Saccharomyces cerevisiae* and fruitfly *Drosophila melanogaster*, various septins have now been found to serve as a scaffold to transport protein factors to the plasma membrane or as a barrier to compartmentalize proteins during various cellular events such as cell division and cell wall formation (reviews: Longtine *et al.*, 1996; Field and Kellog, 1999; Kartmann and Roth, 2001; Mitchison and Field, 2002). In nematode *Caenorhabditis elegans*, septins unc-59 and unc-61 have been implicated to play a role in axonal migration and guidance (Finger *et al.*, 2003). In mammals, the best-studied neuronal septin, SEPT5, has been implicated in neurodegeneration and in regulating neurotransmission. SEPT5 knock-out has been found to promote, rather than to inhibit, exocytosis (Beites *et al.*, 1999; Dent *et al.*, 2002). This diverse array of functions associated with septins, along with our findings that various regions of brain and different neuronal cell types have different septin composition, suggests that the septin composition within each neuronal cell type may play a role in regulating neuronal development and function.

Currently, the majority of studies in the septin field are focused on identifying the molecular associations of septins. The structure/function analysis of septin proteins, due to difficulty in obtaining soluble septin proteins for *in vitro* studies, is quite limited. Furthermore, due to the lack of septin antibodies, the majority of septin studies in mammalian tissues were carried out using exogenous septin proteins overexpressed in cell
In this chapter, we have produced five soluble recombinant septin proteins: SEPT2, 4, 6, 7 and 9. These proteins have similar molecular weights as their endogenous counterparts in brain. However, \textit{in vitro} protein binding and filament polymerization assays revealed that these proteins are incompetent for protein interaction and filament formation observed for endogenous septins. Results from these studies suggest that septin proteins produced from \textit{E. coli} are probably not folded correctly or modified properly for protein interaction and filament formation. These hypotheses are supported by the observation that the recombinant septin proteins precipitate out of solutions within 24 hours after purification. To overcome this difficulty, it would be necessary to produce the septin proteins in a cellular system closer to mammalian cells. A choice system would be the insect cell line sf21. In this system, mammalian septins could be purified away from insect septins and the exogenously produced mammalian septins are more likely to fold correctly with the aid of cytosolic protein chaperones.

To study endogenous septins, we have generated monoclonal antibodies against five septins: SEPT2, 4, 6, 7, and 9. These antibodies are useful for detecting endogenous as well as recombinant septins in western blot assays. In addition, antibodies against SEPT2 are also useful for immunoprecipitation and immunofluorescence studies. Using these antibodies we found that septin proteins have a wide tissue distribution and do not show drastic changes in protein levels during brain development. Interestingly, SEPT2 was found to have a similar tissue distribution and developmental protein expression profile as that of sec15, a subunit of a protein complex, exocyst, that is essential for vesicle trafficking underlying many biological processes including neurite outgrowth. These observations, along with findings that SEPT2 and exocyst subunits have a similar subcellular
localization (Chapter 2), bring up the possibility that a part of SEPT2 function in the nervous system may involve its association with the exocyst complex. To test this hypothesis, we proceeded to study a functional relationship between SEPT2 and the exocyst complex.
Fig. 5. Recombinant septin proteins used for immunization.
Coomassie-blue stained sodium dodecyl polyacrylamide gel showing recombinant SEPT6, SEPT7, SEPT9, SEPT2, and SEPT4 proteins purified from *E. coli* bacteria. The purified recombinant proteins have the same molecular weights as their corresponding brain proteins: SEPT6 at 52kDa, SEPT7 at 50kDa, SEPT9 at 47kDa, SEPT2 at 46kDa, and SEPT4 at 44kDa.
Fig. 6. Anti-septin antibodies recognized septin proteins in brain lysates and recombinant septin proteins.

Anti-septin monoclonal antibodies were subjected to Western blot analysis using rat brain lysates (b) and purified recombinant septin proteins from *E. coli* (f). The monoclonal antibodies recognized protein bands of similar molecular weights in recombinant protein and total brain protein lanes, suggesting that the recombinant septin proteins have similar molecular weights as their endogenous counterpart. Antibodies used in this blot are: KH4F9 against SEPT6, C10D5 against SEPT7, ES4C10 for SEPT9, NH17G10 for SEPT2 and H8B1 for SEPT4.
**Fig. 7. Intermolecular association of septin proteins.**

To assess the interactions among SEPT2, 4, 6, 7 and 9, individual GST-septin proteins were bound to glutathione beads and incubated with soluble recombinant septin proteins. Recombinant septin proteins bound to the GST-septin protein “baits” were analyzed by western blotting using anti-septin antibodies. SEPT6 was detected by antibody KH4F9, SEPT7 was detected by C10D5, SEPT9 was detected ES4C10, SEPT2 was detected by NH17G10 and SEPT4 was detected by H8B1.
Fig. 8. Electron microscopic analysis of septin filament formation
To assess the ability of purified recombinant septin proteins in forming filaments, septin proteins were incubated at 1 mg/ml concentration to allow polymerization in the presence of 1mM GTP. Mixed septin proteins (SEPT2, 4, 6, 7, and 9; septins) or individual septins SEPT2, SEPT4, SEPT6 and SEPT9 were used for the polymerization trials. Microtubule formation from 1mg/ml tubulin was used as a control (mt). While significant microtubule formation was observed, no discernible septin filament formation was detected. Bar = 10 μm
Fig 9. Tissue distribution of septins.

The tissue distribution of SEPT2, 4, 6 and 7 was analyzed by western blotting. For comparison, the tissue distribution of the exocyst complex subunit sec 15 was also assessed by western blotting. The septins have a broad and overlapping tissue distribution. However, they do not show similar tissue distribution pattern. While SEPT2 and exocyst subunit sec15 showed a similar tissue distribution pattern, SEPT6 and 7 also shared a similar tissue distribution pattern, with enrichment in brain.
Fig 10. Septin protein levels in brain during development
The levels of septin protein in brain during development were assessed by western blotting. For comparison, the level of exocyst subunit sec15 was also monitored. The expression level of SEPT2 is similar to that of sec15, with a slight increase from embryo to adulthood. Septins 6 and 7 did not show detectable change in their protein level during development. SEPT4 exhibited a transient increase in protein level from E18 to P5.
Chapter 2  A member of the septin family, SEPT2, plays a role in neurite outgrowth
INTRODUCTION

Historically, septins were first identified for their role in cytokinesis in yeast *Saccharomyces cerevisiae* (Hartwell, 1971). Mutations in these proteins resulted in defective cell division, leading to the production of multinucleated cells. Subsequently, additional septins were identified in other organisms including mammals (reviews: Longtine *et al*., 1996; Field and Kellog, 1999; Kartmann and Roth, 2001; Mitchison and Field, 2002; Kinoshita, 2003; for septin nomenclature see Macara *et al*., 2002). To date, septins have been found to be broadly expressed in mammals and virtually all mammalian tissues. As in yeast, perturbation of mammalian septin function leads to defective cytokinesis, suggesting that they are likewise important for cell division. However, the discovery of multiple septins in adult brain and post-mitotic neurons brings up the possibility that septins may also be required for additional cellular functions in neurons.

Western blot, cell biological and co-immunoprecipitation experiments using brain lysates showed that at least six septins, SEPT2, SEPT4, SEPT5, SEPT7, SEPT9 and SEPT6, are present in brain (Hsu *et al*., 1998; Beites *et al*., 1999; Kinoshita *et al*., 2000). Since septins have been found to be capable of forming heteropolymeric complexes, the differential and yet overlapping distribution of these septins in brain brings up the possibility that different neuron types may have different septin composition, possibly giving rise to different septin function in these cells (Kinoshita *et al*., 2000). In neurons, SEPT5 has been implicated to play a role in attenuating neurotransmission by inhibiting exocytosis (Beites *et al*., 1999). SEPT2, 4, 6, 7 and 9, on the other hand, have been found to co-immunoprecipitate and co-purify with the exocyst complex (Hsu *et al*., 1998). The
exocyst is a multisubunit complex essential for exocytosis (reviews: Hsu et al., 1999; Lipschutz and Mostov, 2002; Novick and Guo, 2002). Perturbation of exocyst function inhibited the exocytic pathway and decreased protein targeting to various plasma membrane domains in yeast, Madine-Darby canine kidney (MDCK), acinar and normal rat kidney (NRK) cells (Novick et al., 1980; Grindstaff et al., 1998; Shin et al., 2000; Yeaman et al., 2001). Likewise, in neurons and neuroendocrine pheochromocytoma (PC12) cells, disruption of the exocyst function by knock-out or by dominant negative construct overexpression inhibited neurite outgrowth but did not affect neurotransmission (Murthy et al., 2003; Vega and Hsu, 2001). This in vitro co-immunoprecipitation and co-purification of SEPT2, 4, 6, 7 and 9 with exocyst suggest that these septins, unlike SEPT5, may play a role in promoting exocytosis underlying neurite outgrowth. In addition, similarity in tissue distribution and brain developmental expression between SEPT2 and the exocyst subunit sec15 brings up the possibility that there may be functional relationship between SEPT 2 and the exocyst complex. To test this hypothesis, we studied the in vivo association of exocyst subunits with SEPT2 and investigated the molecular mechanisms underlying its function in neurite outgrowth.
MATERIALS AND METHODS

CELL CULTURE

PC12 cells were grown on polylysine/laminin-coated 30mm plates (NUNC; Rochester NY) or 4-well permanox chamber slides (NUNC; Rochester NY) and cultured in Dulbecco’s Modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 7.5% fetal calf serum, 7.5% horse serum, 10μg/ml streptomycin and 10U/ml penicillin. For cell transfection studies, PC12 cells were first plated on polylysine-coated 24-well plates (Corning; Corning, NY) at a cell density of 60,000/cm². At 48 hrs following cell transfection, PC12 cells were replated back to polylysine/laminin-coated 30mm plates for neuronal differentiation. To induce neuronal differentiation, cells were cultured in the presence of 50ng/ml nerve growth factor (NGF; Invitrogen, Carlsbad, CA) for 5 days before they were used for immunocytochemistry and electrophysiological recording.

SEC8 AND SEPT2 KNOCKDOWN

Exocyst sec8 and SEPT2 knock-downs were performed using shRNAs as described by Paddison et al. (2002). ShRNA constructs for sec8 and SEPT2 were generated by subcloning synthetic oligonucleotides containing 29 nucleotide sequences corresponding to 419-448 nt and 534-563 nt of sec8 and SEPT2 primary sequence, respectively, into the BseRI and BamHI sites of pShag-1 vector (Cold Spring Harbor Laboratory; Cold Spring Harbor, NY). For knock-down studies in PC12 cells, the shRNA constructs were co-transfected with either EGFP-pBI or nucEGFP-pBI (Clontech; Palo Alto, CA) vector at a 1:1 ratio. The constructs were introduced into PC12 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as previously described (Vega and Hsu,
2001). The exocyst and SEPT2 protein levels in the transfected cells as well as the surrounding non-transfected cells were detected by immunostaining using anti-exocyst subunit and anti-SEPT2 monoclonal antibodies described below. The extent of exocyst and SEPT2 knock-down was assessed by quantitating the immunostaining using Photoshop 5.5.

ELECTROPHYSIOLOGICAL RECORDING

**Patch-clamp recording:** Whole-cell recordings were obtained with standard techniques (Hamill *et al.*, 1981). The currents were recorded with an Axoclamp 200A amplifier, digitized at 5-20 kHz with an INDEC IDA 15125 interface, filtered at 5 kHz and stored. Recording parameters and stimulus protocols were controlled by custom software written with Borland C++ that utilized device driver libraries supplied by INDEC. Data analysis programs were written with Microsoft Visual Basic. The composition of the external bath solution for voltage clamp recordings was 1.67 mM Ca, 1 mM Mg, 5.36 mM K, 137 mM Na, 17 mM glucose, 10 mM HEPES, and 50 mM sucrose. Ca\(^{2+}\)-channel currents were isolated from other voltage-dependent currents by ionic substitution and addition of sodium and potassium channel blockers as detailed below. The external bath solution for recording Ca\(^{2+}\)-channel currents contained 20 mM Ba-acetate, 135 mM tetraethylammonium-aspartate, 10 mM HEPES, and 0.001 mM tetrodotoxin (pH 7.5). The pipette solution contained 123 mM Cs-aspartate, 10 mM Cs-EGTA, 5 mM MgCl\(_2\), and 4 mM ATP (pH 7.5). All recordings were made at room temperature.

**Data Analysis:** Cell capacitance was computed by integrating current during a capacitative transient and dividing by pulse voltage. Peak current measurements were
made with a standard 60 ms depolarizing pulse from a holding potential of -80 mV to a test potential of 0 mV. Linear leak and capacitative currents (measured with hyperpolarizing pulses of 1/10 the test potential) were subtracted prior to analysis of peak current. Calcium current density was obtained by dividing peak current by cell capacitance. Current densities were calculated for each of the recordings, and the values from a single set of cells were averaged. The mean current densities from the matched sets were compared statistically using Student’s $t$ test.

**ANTIBODIES AND IMMUNOCYTOCHEMISTRY**

Monoclonal antibodies against SEPT2 and exocyst subunits exo84 and exo70, NH17G10, 84X10A1 and 70X13F3 respectively, were generated using soluble recombinant septin or exocyst proteins as described and were used for both immunocytochemistry and Western blot studies (Vega and Hsu, 2001, Wang and Hsu, 2003). Monoclonal antibody against sec8, 8S15E12, was generated as reported (Hsu et al., 1996) and was only suitable for Western blot study. Polyclonal antibodies against tubulin were purchased from Cytoskeleton Inc. (Denver, CO). To monitor exocyst and SEPT2 subcellular localization, PC12 cells were fixed with 2% paraformaldehyde for 3 min, followed by a 1 to 2-min incubation with cold methanol. The fixed cells were rehydrated in phosphate-buffered saline (PBS) for 30 min and permeabilized with DMEM containing 2% fetal calf serum and 0.05% Triton X-100. Cells were washed five times with PBS and incubated with primary antibodies for 2 hours at room temperature or for overnight at 4°C. Fluorescein-conjugated goat anti-mouse or rhodamine-conjugated goat anti-rabbit (Jackson ImmunoResearch Labs; West Grove, PA) antibodies were then used as secondary antibodies. Labeled cells were
visualized by inverted fluorescence microscope (Axiovert 200; Zeiss, Thornwood NY). To study exocyst and SEPT2 colocalization, the anti-SEPT2 monoclonal antibody was labeled with Cy5 fluorophore using the Zenon IgG labeling kit according to the manufacturer’s instructions (Molecular Probes; Eugene OR). Cells were first labeled with anti-exo84 antibody and FITC-conjugated anti-mouse secondary antibodies. After five washes with PBS, the cells were then incubated with Cy5 labeled anti-SEPT2 antibody for 4 hours at room temperature or overnight at 4°C. The incubation was done in the presence of excess non-specific mouse immunoglobulin to minimize the exchange of fluorophore-conjugated secondary antibodies between anti-exo84 and anti-SEPT2 mouse monoclonal antibodies.

WESTERN BLOT

Transfected PC12 cells were lysed and scraped off culture plates in protein sample buffer containing 5% sodium dodecyl sulphate, 30% glycerol and 20 mM Tris, pH 8. The resulting PC12 cell lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes for Western blot analysis as described (Vega and Hsu, 2001). The amount of total protein loaded per lane was assessed by quantitating the total Ponceau protein staining per lane on the blot using Kodak 1D Image Analysis software. Western signals per lane were also quantitated by the same program. The amount of exocyst subunits, SEPT2 and tubulin per lane was normalized by dividing the western signal by the total Ponceau signal in the same lane.
RESULTS

A. ENDOGENOUS SEPT2 AND EXOCYST SHOW IN VIVO CO-LOCALIZATION IN PC12 CELLS

As a first step to investigate a functional relationship between exocyst and SEPT2, we examined whether the previously observed in vitro association between exocyst and SEPT2 can occur in vivo. Neuroendocrine PC12 cells, a cell line that has been widely used to study neuronal differentiation and neurite outgrowth, were used to monitor the exocyst and SEPT2 localization upon neuronal differentiation. Typically, PC12 cells were treated with NGF to induce neuronal differentiation. The distribution of endogenous exocyst subunits and SEPT2 was then assessed by immunostaining using respective monoclonal antibodies. After a 5-day treatment with NGF, PC12 cells at different stages of neuronal differentiation and neurite outgrowth could be observed. Both exocyst subunit exo84 and SEPT2 colocalized in all cells regardless of their differentiation state. Three major types of localization were observed for exo84 and SEPT2. In the first type of localization, both exo84 and SEPT2 were found as a compact perinuclear enrichment with no discernible internal structure visible even by confocal microscopy (Fig. 11E-G). Occasionally however, short filament-like structures could be seen coming out of this compact perinuclear enrichment. PC12 cells with this type of exo84 and SEPT2 localization had not yet developed neurites and were often round and small. In the second type of localization, exo84 and SEPT2 appeared as a tangle of filaments in the perinuclear region. Some filaments could be seen to distribute from this perinuclear enrichment toward the plasma membrane. PC12 cells with this type of exo84/SEPT2 localization were often larger and
flatter than the first type of cells but they still did not have neurites. Finally in PC12 cells which developed neurites, both exo84 and SEPT2 were observed to come out from their perinuclear enrichment and enter into the neurite and growth cone as a filament-like structure (Fig. 11B-D, arrowheads). Interestingly, when neurites originated from plasma membrane domains at the opposite side of the exocyst/SEPT2 perinuclear enrichment, exo84/SEPT2-containing filaments could be seen going around the nucleus before entering into the neurite (Fig. 11B-D, arrows). Once they were in the growth cone, exo84 and SEPT2 often formed a circle or a compact concentration (Fig. 11B, E).

The observed colocalization was not likely due to fluorophore bleed-through since co-staining using monoclonal antibodies against SEPT2 and of another protein with different subcellular distribution in PC12 cells, such as actin, did not show significant co-localization (data not shown). In addition, the specificity of anti-exo84 and anti-SEPT2 monoclonal antibodies against their respective antigen had been tested by enzyme-linked immunoadsorbent assay (ELISA) and western blotting and the antibodies showed no cross-reactivity (Vega and Hsu, 2003; Wang and Hsu, 2003). Finally, exo84 and three other exocyst subunits, exo70, sec15 and sec6, have also been found to have similar if not identical subcellular localization (Vega and Hsu, 2001; Wang and Hsu, 2003). These results strongly suggest that SEPT2 co-localizes with multiple exocyst subunits, and possibly the intact exocyst complex, in PC12 cells.

**B. BOTH EXOCYST AND SEPT2 ARE UPREGULATED UPON NGF-INDUCED NEURONAL DIFFERENTIATION**
The *in vivo* co-distribution of exocyst and SEPT2 from their perinuclear enrichment toward the growth cone suggests a function for these two macromolecules in the neurite outgrowth process. In addition, a previous tissue distribution study has shown that although the exocyst complex is ubiquitously expressed, it is most enriched in brain and kidney, tissues with highly polarized cells (Ting *et al.*, 1995). Taken together, these results suggest that exocyst and SEPT2 may be upregulated upon NGF-induced polarized growth. To test this hypothesis, we examined the protein levels of exocyst subunits and SEPT2 before and after NGF-induced neuronal differentiation. PC12 cells were cultured in the presence and absence of NGF for five days and the amounts of SEPT2 and of exocyst subunits sec8, exo84 and exo70 proteins in undifferentiated and NGF-differentiated PC12 cells were compared by Western blotting (Fig. 12). Individual western signals (Fig. 12A) were normalized against total PC12 cell protein in the same lane (Fig. 12B) as described in Materials and Methods. Exocyst subunits sec8, exo70 and exo84 showed approximately 1.7-fold increase in protein levels in differentiated compared to undifferentiated PC12 cells (Fig. 12C). The SEPT2 protein level also showed a 2.5-fold increase in differentiated PC12 cells. For comparison, the upregulation of βIII tubulin, a neuronal marker, showed a 3-fold increase. These western results are consistent with our cell biological observations in which we found that exocyst and SEPT2 perinuclear enrichment was always larger and brighter in NGF-differentiated than in undifferentiated PC12 cells. In fact, we have noticed that exocyst and septin immunostaining became noticeably brighter within 24 hours after the addition of NGF, well before neurite outgrowth took place. Thus these results, along with the findings that exocyst and SEPT2 co-immunoprecipitate/co-purify *in*
vitro and co-localize in vivo, suggest these two macromolecules may coordinate with each other in initiating and/or promoting neurite outgrowth upon neuronal differentiation.

C. EXOCYST SUBUNIT SEC8 KNOCK-DOWN DOES NOT AFFECT SEPT2 LOCALIZATION AND PROTEIN LEVEL BUT SEPT2 KNOCK-DOWN ABOLISHES EXOCYST PERINUCLEAR ENRICHMENT AND DECREASES EXOCYST PROTEIN LEVEL

The exocyst complex has been previously shown to be required for neurite outgrowth, possibly by promoting membrane addition at the growth cone (Vega and Hsu, 2001; Murthy et al., 2003). Overexpression of a SEPT2 GTPase mutant, on the other hand, has been shown to promote aberrant neurite outgrowth (Vega and Hsu, 2003). The molecular mechanisms of SEPT2 in neurite outgrowth, however, remain unclear. The colocalization and co-upregulation of exocyst and SEPT2 bring up the possibility that SEPT2 may play a role in neurite outgrowth by regulating the localization of the exocyst complex in the cell. To test this hypothesis, we knocked down SEPT2 protein level by shRNA (Paddison et al., 2002). As a control, we also knocked down exocyst for comparison. If the exocyst/SEPT2 localization were dependent on exocyst, decreased exocyst level should affect SEPT2 localization. Alternatively, if the exocyst localization were dependent on SEPT2, knock-down of SEPT2 should disrupt exocyst localization.

To knock down SEPT2 or exocyst protein levels in PC12 cells, we generated shRNA constructs specific for SEPT2 and various exocyst subunits using the shRNA vector pShag-1 (Paddison et al., 2002). PC12 cells were co-transfected with individual shRNA constructs and the nucEGFP-pBI vector. This cotransfection resulted in the
coexpression of two constructs, allowing the identification of cells with reduced SEPT2 or exocyst level by the expression of nuclear enhanced green fluorescent protein (EGFP). In addition, since nuclear EGFP remains in the cell after immunostaining processing, the localization of SEPT2 and exocyst subunits could be assessed in individual transfected cells. The efficiency of each shRNA construct in decreasing protein level was then assessed by quantitating the immunostaining in transfected cells using respective anti-exocyst and SEPT2 monoclonal antibodies. In this study, immunostaining, rather than western blotting, was used to assess the efficiency of individual shRNA constructs for two major reasons. First, the maximal transfection efficiency in PC12 cells was only 25%. Under this condition, western blotting was not sensitive enough to reliably detect the decreased protein levels even if there was a 100% knock-down of exocyst and SEPT2 in all transfected cells. Second, immunostaining allowed us to monitor changes in exocyst and septin localization in addition to their protein levels.

For exocyst knock-down, eight sec8, four exo84, four exo70 and two sec5 shRNA constructs were generated. Based on previous observations in which temperature-sensitive mutations of individual yeast exocyst subunits can perturb the formation of the exocyst complex (TerBush et al., 1996) and that mammalian exocyst subunits co-immunoprecipitated/co-purified in vitro and have similar if not identical localization in vivo (Hsu et al., 1996, 1998; Vega and Hsu, 2001; Wang and Hsu, 2003), we expect that the knock-down of one exocyst subunit would affect the assembly and localization of the entire complex. Indeed, we found one shRNA construct against sec8, 8-419, that was effective in decreasing exocyst subunit protein levels (Fig. 13E-G). When PC12 cells were transfected with this construct, approximately 70% of nuclear EGFP-positive cells had
approximately 80% decrease in exo70 and exo84 protein levels compared to neighboring untransfected cells (Fig. 13H). The SEPT2 perinuclear localization and protein level in transfected cells, however, remained unaffected (Figs. 13G, H). These results confirm that the knock-down of one exocyst subunit can affect the protein level of other exocyst subunits, possibly by destabilizing the exocyst complex and thus causing the degradation of other subunits. Furthermore, these results also reveal that SEPT2 localization is not dependent on exocyst localization.

When PC12 cells were transfected with six sept2 shRNA constructs, one construct, sep2-534, decreased the SEPT2 protein level by 90% in approximately 90% of nuclear EGFP-positive cells (Fig. 13I-K). Interestingly, the exo70 and exo84 perinuclear enrichment was also absent in the transfected cells. In addition, the exo70 and exo84 protein levels were also decreased by approximately 80% (Fig. 13L). These results suggest that the mislocalization of exocyst due to decreased SEPT2 protein level can result in decreased exocyst protein level. In addition, these observations also indicate that while the exocyst localization is dependent on SEPT2, the SEPT2 localization is not dependent on the exocyst complex. Thus the apparent filamentous exocyst distribution observed in PC12 cells may be due to its association with SEPT2-containing filaments. Consistent with this idea, SEPT2, but not exocyst complex, has been found to be capable of forming filaments under both in vitro and in vivo conditions (Hsu et al., 1998; Kinoshita et al., 2002).

The observed decrease in exocyst and SEPT2 protein levels was not likely a side-effect of the cell transfection procedure. When PC12 cells were transfected with the shRNA pShag-1 vector, the exo70, exo84 and SEPT2 perinuclear enrichment was not
diminished in over 90% of nuclear EGFP-positive cells (Fig. 13A-C). The protein levels of exocyst and SEPT2 also remained relatively unchanged (Fig. 13D). In addition, the decrease in exocyst and septin protein levels was not due to non-specific shRNA expression since sec8 and SEPT2 shRNAs exerted differential effects on exocyst and SEPT2 localization and protein level. Furthermore, the concerted decrease in exo70 and exo84 protein levels in sec8 and SEPT2 knock-downs was not due to antibody cross-reactivity. The specificity of these monoclonal antibodies has been tested as described (Vega and Hsu, 2001; Wang and Hsu, 2003).

D. BOTH SEPT2 AND EXOCYST KNOCK-DOWNS INHIBIT NEURITE OUTGROWTH

We have previously shown that the overexpression of an exocyst sec10 subunit c-terminal deletion construct inhibited neurite outgrowth in PC12 cell, suggesting that exocyst plays a role in promoting neurite outgrowth. The dependence of exocyst localization on SEPT2 brings up the possibility that SEPT2 knock-down may disrupt exocyst function and thus abolish neurite outgrowth. To test this hypothesis, we co-transfected the pShag-1 vector and shRNA constructs with the reporter vector EGFP-pBI and counted transfected cells with EGFP-visible neurites at five days after NGF-induced neuronal differentiation (Fig. 14). When PC12 cells were transfected with the pShag-1 vector, almost 40% of GFP-positive cells showed well-developed neurites (Figs. 14A and B, pShag). When sec8 shRNA vector 8-419 were introduced into PC12 cells, only 5% of transfected cells developed neurites (Figs. 14A and B, 8-419). The introduction of SEPT2 shRNA construct 2-534, interestingly, also drastically decreased
neurite outgrowth with only 2.5% of transfected cells developing neurites (Figs. 14A and B, 2-534). The transfected cells were not undergoing imminent cell death since they were alive for at least 7 days after NGF addition and were healthy for electrophysiological recording (Fig. 15). These results indicate that both exocyst and SEPT2 function is required for neurite outgrowth upon neuronal differentiation and bring up the possibility that SEPT2, like the exocyst complex, may be involved in plasma membrane protein targeting during neuronal differentiation.

E. SEPT2 AND EXOCYST KNOCK-DOWNS DECREASE NGF-INDUCED CALCIUM CHANNEL INSERTION INTO THE PLASMA MEMBRANE

To investigate the role of SEPT2 in plasma membrane protein trafficking upon NGF-induced neuronal differentiation, we monitored the incorporation of an endogenous plasma membrane protein, the voltage-gated calcium channel, into the plasma membrane of PC12 cells with exocyst and septin knock-downs. It has been previously shown that there is an increased plasma membrane calcium channel density upon NGF-induced neuronal differentiation (Striet and Lux, 1987; Plummer et al., 1989). Therefore, if exocyst and SEPT2 function is required for plasma membrane protein addition during neuronal differentiation, we should expect a lack of increase in the plasma membrane calcium channel density upon the addition of NGF in the presence of exocyst and SEPT2 knock-downs. To carry out this study, PC12 cells were transfected with shRNA constructs and the reporter vector EGFP-pBI at a 1:1 ratio and allowed to differentiate for five days in the presence of NGF. The transfected cells, which could be identified by correlating EGFP
expression from the reporter vector, were then subjected to whole-cell patch clamp recording (Fig. 15).

Examples of recorded cells were shown in Fig. 15A. Because not all PC12 cells developed neurites upon NGF exposure, recordings were carried out on EGFP-positive cells, both with and without neurites. The population of active voltage-gated calcium channels that was present in the plasma membrane was estimated by measuring the peak inward barium current which occurred typically at a transmembrane potential of 0 mV (Fig. 15B). Calcium current magnitude was converted to calcium current density by dividing by membrane surface area. The total cell body surface area was measured by stepping the intracellular potential to –70 mV from a holding potential of –80 mV. With the recording conditions used, this voltage elicited minimal activation of voltage-gated ionic current and thus the current recorded reflects charging of the whole-cell membrane capacitance. Because capacitance is proportional to membrane surface area, integration of the capacitative current can be used to calculate membrane surface area using a value of 0.9 μF/cm² for specific capacitance (Gentet et al., 2000).

Comparison of pShag-1 vector-transfected PC12 cells in the presence and absence of NGF treatment shows that NGF induced an expected increase in plasma membrane calcium current density (Fig. 15B, C), as previously reported by other investigators (Streit and Lux, 1987; Plummer et al., 1989). When PC12 cells were transfected with the sec8 (8-419) and SEPT2 (2-534) shRNA constructs, there was a corresponding 70% and 77% decrease of calcium current density respectively compared to cells transfected with the pShag-1 vector (pShag(+)). With 8-419 transfection, we observed two distinct populations of EGFP-positive cells in terms of their calcium current density levels. About 30% of
EGFP-positive cells had calcium current density which was at least 2-fold over that found in the remaining 70% of recorded EGFP-positive cells. The calcium current density of these cells was similar to that observed with pShag-1 transfection. We suspect that these cells contained only the reporter vector. This is consistent with our immunostaining results in which we found that only about 70% of EGFP-positive cells had decreased exocyst protein level. Therefore, we did not include the data from these 30% cells in our calculation. Even if we had included these data in our calcium current density assessment, we would still observe a 50% decrease in the calcium current density of 8-419 transfected cells. Overall, the calcium current density in exocyst and SEPT2 knock-down cells was similar to that seen for undifferentiated PC12 cells transfected with the pShag-1 vector (Fig. 15C, pShag (-)), which was about 20% of that in NGF-differentiated cells (Fig. 15C, pShag (+)).

In order not to bias our results, we have recorded from PC12 cells with and without neurites in the presence of NGF for each construct. It is interesting to note that the majority of cells transfected with the same construct showed similar electrophysiological properties, regardless of their neurite outgrowth morphology. For pShag-1 vector-transfected cells, we consistently observed an NGF-induced increase in calcium channel density in cells without neurites as well as with neurites. These observations suggest that calcium channel addition to the plasma membrane during NGF-induced neuronal differentiation is not dependent on neurite outgrowth. The lack of neurite development in these cells may be a side-effect of transfection by Lipofectamine 2000. We consistently observed fewer PC12 cells developing neurites following Lipofectamine 2000 transfection for all of our constructs. Similarly, in exocyst- and SEPT2-knockdown cells, the majority of transfected
cells, with or without neurites, exhibited decreased calcium channel density compared to pShag-1-transfected cells. The 2.5-5% of exocyst- and SEPT2-knockdown cells that developed neurites (Fig. 15) often had unusually short and thin neurites. It is possible that neurite outgrowth had initiated in these few cells before the exocyst or SEPT2 was knocked down to inhibit plasma membrane addition. The addition of calcium channel to the plasma membrane may have occurred after the onset of neurite outgrowth and was thus still inhibited by the exocyst and SEPT2 knock-downs. Presently, it is not clear what happened to calcium channels that were not incorporated into the plasma membrane in exocyst- and SEPT2-knock-down cells. It is possible that they may still be present in secretory vesicles accumulating within these cells as previously observed for secretory vesicles in yeast exocyst mutants (Novick et al., 1980).

It is also interesting to note that while the knockdown of SEPT2 and of exocyst subunit sec8 greatly inhibited calcium channel insertion into the plasma membrane of PC12 cells and neurite outgrowth, the overall membrane capacitance of PC12 cells did not change significantly. These results suggest that there was no detectable decrease in plasma membrane surface area in the presence of decreased exocyst and SEPT2 function. There are at least two explanations for these contradictory observations. First, plasma membrane addition in PC12 cells is mediated by an exocyst-independent exocytotic pathway. Second, capacitance measurement in these experiments could not accurately measure the plasma membrane surface area covering, at least, the distal ends of neurites. The second alternative is a more reasonable explanation since the capacitance measurement seems to be similar in PC12 cells with or without neurite outgrowth, suggesting that it may not detect the small increment of plasma membrane surface area arisen from extended neurites.
Furthermore, since exocyst is found to be required for exocytosis in all cell types studied so far, it is likely to be a core component of the exocytic machinery in all cells.
DISCUSSIONS

In this chapter we investigate a potential new function for SEPT2 in promoting plasma membrane protein addition underlying neurite outgrowth. Three lines of evidence support a role for SEPT2 in promoting neurite outgrowth. First, SEPT2 exhibited a similar tissue distribution and developmental expression profile as the exocyst subunits sec15, bringing up the possibility of a functional relationship between SEPT and the exocyst complex. Second, both SEPT2 and exocyst are upregulated upon neuronal differentiation in PC12 cells. In undifferentiated PC12 cells, SEPT2 and exocyst subunits exhibit perinuclear enrichment near or at the microtubule-organizing center. Upon neuronal differentiation, both SEPT2 and exocyst subunits showed distribution from their perinuclear enrichment to the neurites and the growth cones. This coordinated upregulation and recruitment of SEPT2 with exocyst to the growth cone are consistent with a role for SEPT2 in promoting neurite outgrowth. Third, SEPT2 knock-down causes the mislocalization of exocyst as well as a decrease in exocyst protein level in neuroendocrine PC12 cells. These molecular perturbations are accompanied by the inhibition of plasma membrane protein trafficking and neurite outgrowth. Taken together, these findings suggest that SEPT2 is required for membrane addition underlying neurite outgrowth and that its function in exocytosis may be due to, at least in part, its regulation of exocyst function. It is interesting to note that the overexpression of a SEPT2 GTPase mutant has been previously found to result in excessive and aberrant neurite outgrowth (Vega and Hsu, 2003). It is possible that the overexpressed SEPT2 mutant caused the mislocalization of
exocyst without reducing the exocyst protein level in the cell, thus resulting in plasma membrane addition outside the growth cone and ultimately aberrant neurite outgrowth.

Currently, despite the growing importance of septins in neuronal function and degeneration, the molecular mechanisms of their function in neurons remain largely unknown. Several models of the mechanisms underlying septin function have been proposed. One model in particular, based on SEPT5 findings, proposes that septins may exert their function by interacting with syntaxin, a plasma membrane t-SNARE protein required for vesicle docking and/or fusion with the plasma membrane. This association allows septins to inhibit regulated exocytosis in neurons such as neurotransmission. Based on the results from this paper, we propose that septins, such as SEPT2, may promote exocytosis through their association with exocyst, a protein complex essential for exocytosis. Upon NGF-induced neuronal differentiation, exocyst and SEPT2 are both upregulated in preparation for plasma membrane addition underlying neurite growth. The upregulated exocyst, in association with SEPT2, is specifically recruited from its perinuclear enrichment near or at the microtubule-organizing center to the growth cone where a high level of plasma membrane addition occurs. The concentration of exocyst in the growth cone subsequently mediates and/or facilitates Golgi-derived vesicle transfer/docking/fusion to the plasma membrane. The specific targeting of SEPT2 and exocyst toward exocytic sites is likely to be temporally and spatially regulated by cellular factors such as the cytoskeletal elements and their associated proteins, signaling molecules and other associating septins. In this regard, SEPT2 has been reported to associate with microtubules and actin filaments (Kinoshita et al., 1997, 2002; Vega and Hsu, 2003; Nagata et al., 2003). Disruption of these two cytoskeletal systems by drug treatment
perturbed SEPT2 distribution in the cell. In addition various septins, such as SEPT4 and SEPT7, that co-immunoprecipitated and co-purified with SEPT2 from brain (Hsu et al., 1998), have been shown to interact with phospholipids (Xie et al., 1999) and the small GTPase Cdc42-Borg system (Joberty et al., 2001). Elucidation of the regulation of SEPT2-containing filament assembly, composition and function by these factors should provide important insights into the coordination between the cytoskeletal system and the protein targeting process underlying neurite outgrowth during neuronal differentiation, axonal pathfinding and synapse formation.
Fig. 11. **SEPT2 and exocyst subunits co-localize in PC12 cells and show specific distribution, via a filament-like structure, from their perinuclear enrichment to the growth cone.**

The subcellular localization of SEPT2 and exocyst subunit exo84 in NGF-differentiated PC12 cells was examined by immunofluorescence microscopy. (A) Enlarged view of insets showing a PC12 cell body and growth cone. Septin filaments could be seen coming out of their perinuclear enrichment and going into neurites that originate either at the same side as the enrichment (arrowheads) or at the opposite side from the enrichment (arrows). The plasma membrane of the cell is outlined by dotted lines in inset as small arrows. (B, E) Exo84 localization visualized by FITC-conjugated anti-mouse antibody bound to anti-exo84 monoclonal antibody. (C, F) SEPT2 immunostaining with TRITC-labeled anti-septin monoclonal antibody. (D, G) The overlays of exo84/SEPT2 staining from B/E and C/F respectively. In the growth cones, exo84 and SEPT2 appeared as a circle (E) or a compact concentration (B). N = nucleus. GC = growth cone. Bar = 20 μm.
Fig. 12. Both exocyst and septin2 are upregulated upon NGF-induced neuronal differentiation.
The upregulation of SEPT2 (SEPT2), exocyst subunits sec8 (8), exo70 (70), exo84 (84) and the NGF-inducible neuronal marker βIII tubulin (Btub) was assessed by western blotting. The western signal in each lane (A) was normalized against the total PC12 protein detected by the Ponceau protein stain in that lane. An example of the Ponceau staining of PC12 cell lysates from undifferentiated (-) and differentiated (+) PC12 cells is shown in B (PC). A graph comparing exocyst subunit, SEPT2 and beta III tubulin protein levels in undifferentiated (white bars) and differentiated (grey bars) PC12 cells was shown in C. The graph represents the mean, with standard deviation, from four western blot analyses.
Fig. 13. Exocyst subunit sec8 knock-down does not affect SEPT2 perinuclear localization and protein level but septin2 knock-down abolishes exocyst perinuclear enrichment and decreases exocyst protein level.

PC12 cells were transfected with pShag-1 vector (pShag; A-C), exocyst sec8 shRNA construct (8-419; E-G), and SEPT2 shRNA construct (2-453, I-K). NucEGFP-pBI vector was co-transfected with these shRNA constructs to allow the identification and immunostaining of transfected cells. Forty-eight hours after transfection, NGF was added to the cells to induce neuronal differentiation. The differentiated cells were immunostained with antibodies against exocyst subunits exo70 (exo70; A, E, I) and exo84 (B, F, J), and SEPT2 (C, G, K) at five days after NGF addition. Transfected cells were identified by nuclear EGFP fluorescence (green nuclei). The lack or presence of perinuclear enrichments in transfected cells were denoted by arrowheads and arrows respectively. All nuclei were visualized by DAPI (blue). Bar = 50 μm. Exocyst and SEPT2 protein levels in transfected cells were assessed by quantitating immunostaining in transfected cells. Graphs summarizing the mean (with standard deviation) of immunostaining quantitation in pshag-1 vector-, sec8 shRNA-, and SEPT2 shRNA-transfected cells compared to surrounding untransfected cells were shown in D, H, and L respectively. Cont denotes exo70, exo84 and SEPT2 protein levels in untransfected cells in the vicinity of transfected cells (n = 60 for each construct). 70, 84 and SEPT2 denote protein levels of exocyst subunits exo70 and exo84 and SEPT2 in transfected cells respectively (n = 20 for each immunostaining quantitation per construct).
Fig. 14. Both SEPT2 and exocyst knock-downs by shRNA inhibit NGF-induced neurite outgrowth in PC12 cells.

PC12 cells were transfected with shRNA pShag-1 (pShag) vector, sec8 shRNA 8-419 (8-419) and SEPT2 shRNA (2-534) constructs. EGFP-pBI vector was co-transfected with these constructs to allow the identification of transfected cells by EGFP fluorescence. Forty-eight hours after transfection, NGF was added to the cells to induce neuronal differentiation. The neurite outgrowth phenotype of transfected cells was assessed by EGFP fluorescence (A). The mean (with standard deviation) of cell count from at least five different microscopy fields (n = 200-300 for each construct) was plotted in B. Bar = 50 μm.
Fig. 15. SEPT2 and exocyst knock-downs decrease NGF-induced calcium channel insertion into the plasma membrane.

PC12 cells were transfected with pShag-1(pShag), sec8 shRNA (8-419) and SEPT2 shRNA (2-534) constructs along with the EGFP reporter vector EGFP-pBI. Transfected cells were identified by correlating EGFP expression from the EGFP-pBI vector. Only cells with EGFP fluorescence were recorded. (A) Examples of EGFP fluorescence (GFP) and EGFP fluorescence/bright field overlay (composite) images of typical cells used for electrophysiological recordings. For each construct transfection, cells with different morphology (cells with long or short neurites and cells with or without neurites) were selected for recording. Two recorded cells (denoted by arrowheads) from each construct transfection are shown. Bar = 50 μm. (B) Example traces of whole-cell recordings from four PC12 cell transfected with constructs as indicated. Calcium currents were measured with barium as the charge carrier to eliminate calcium-induced inactivation of calcium channels. The voltage pulse used is indicated above the traces. Maximal current was seen at a test potential of 0 mV. The largest currents were obtained from NGF-treated cells transfected with the pShag-1 vector. NGF-treated cells transfected with 2-534 or 8-149 constructs had much smaller currents, comparable to those seen in undifferentiated cells transfected with pShag-1. Plots of calcium current density are shown in C respectively. N = 7 for pShag (-); n = 9 for pShag (+); n = 10 for 8-419; n = 8 for 2-534. Student’s t test was used for statistical analysis within each data set.

[Diagrams and images are shown in the original text.]
CONCLUSION AND FUTURE DIRECTIONS

Septin proteins were first discovered for their requirement in cell division. It has been postulated that septins form a ring structure at the cleavage furrow between two dividing cells and facilitate the separation of the mother/daughter cells. However, the discovery of multiple septins in postmitotic neurons raises the possibility that septins may perform other functions in the cell. In this regard, various septin proteins have been found to associate with cytoskeletal proteins and signaling molecules including microtubules and actin. Findings from this thesis project suggest a role for SEPT2 in exocytosis through its direct or indirect association with the exocyst complex. In addition, findings from showed that SEPT2 was required for the proper recruitment of mitotic motors to the kinetochore of segregating chromosomes (Spiliotis et al., 2005) suggests that SEPT2 may have a functional relationship with mitotic motors such as kinesin. Based on these observations, we propose a working model for the function of SEPT2 on exocytosis (Fig. 16). Upon budding from Golgi, secretory vesicles associate with kinesin motors to be transported to the vicinity of the plasma membrane. SEPT2 may play a role in recruiting mitotic motors and the exocyst complex to microtubules for binding to secretory vesicles. SEPT2 and the exocyst complex travel down the microtubules with the kinesin motors to the vicinity of the plasma membrane where exocyst, and possibly SEPT2, may mediate the dissociation of vesicles from microtubules to the actin network for subsequent vesicle docking and fusion with the plasma membrane. According to this model, SEPT2 function may be required at three stages in the exocytotic pathway. At the earliest stage, SEPT2 may be involved in the loading of secretory vesicles and kinesin motors to microtubules near the
microtubule-organizing center. Disruption of SEPT2 function would be expected to result in the inability of secretory vesicles leaving from the vicinity of Golgi. At the next stage, SEPT2 may be involved in dissociating the kinesin motors from microtubules, thus regulating the transfer of secretory vesicles from microtubules to actin at the vicinity of the plasma membrane. Perturbation of SEPT2 function at this stage would be expected to ultimately result in the lack of vesicle docking and fusion at the plasma membrane. At the last stage, SEPT2 may function in the release of secretory vesicles from the actin network to the plasma membrane. Perturbation of SEPT2 function at this stage would also be expected to prevent vesicle docking and fusion at the plasma membrane.

An ideal system to assess SEPT2 function at these three stages in the exocytotic pathway is cultured hippocampal neurons. Currently, perturbation of vesicle transfer from microtubules to actin could not be distinguished from problem of vesicle transfer from actin to the plasma membrane since both events would lead to the lack of vesicle docking and fusion at the plasma membrane. These events could not be studied using in vitro assays because of the requirement of an intact cytoskeletal network for the vesicle transfer process. Likewise, due to the proximity of microtubule ends to the actin network and the plasma membrane, it is not possible to distinguish whether the lack of vesicle fusion upon perturbation of SEPT2 function is due to the inhibition of vesicle transfer from microtubules to actin, from actin to plasma membrane or vesicle docking at the plasma membrane by either light or electron microscopy. One way to bypass this technical problem is to study vesicle trafficking in neurons. Neurons are ideal for studying vesicle transfer from microtubules to actin and from actin to the plasma membrane because the synaptic terminals are largely devoid of stable microtubules. Therefore, membrane protein
delivery from the neurite shaft to synaptic terminals involves vesicle transfer from microtubules in the shaft to the actin network in the synaptic terminal. Similarly, protein insertion from the synaptic terminal domain to the plasma membrane of the terminal would allow assessment of vesicle trafficking from actin to the plasma membrane. Using cultured hippocampal neurons as a model system, we would expect the following phenotypes for the disruption of each proposed SEPT2 function (Fig. 17). If SEPT2 function were required for vesicle transfer from Golgi to microtubules, no synaptic protein would be observed to travel down the neurite shaft upon disruption of SEPT2 function by SEPT2 shRNA knockdown. Newly synthesized synaptic proteins would accumulate in the cell body only. Likewise, if perturbation of SEPT2 function disrupted vesicle transfer from microtubules to actin, synaptic protein transfer from neurite shaft to synaptic terminals would be inhibited. In this scenario, synaptic proteins would be observed in the neurite shaft but not in the terminals. Finally, if SEPT2 were required for vesicle transfer from actin to the plasma membrane, knockdown of SEPT2 protein level would inhibit the insertion of synaptic plasma membrane protein into the plasma membrane.
Fig. 16. A working model proposed for potential function of SEPT2 in exocytosis.  
The association of SEPT2 with microtubule motors, microtubules and the exocyst complex bring up the possibility that this protein may be involved in at least three stages in the exocytotic pathway.  At the first stage (1), SEPT2 may be required for the recruitment of microtubule-associated motors and the exocyst complex to the secretory vesicles and microtubules.  At the second stage, SEPT2 may play a role, along with the exocyst complex, in transferring secretory vesicles from microtubules to the cortical actin network.  At the third stage, SEPT2 may be involved in transferring secretory vesicles from the cortical actin network to the plasma membrane.
Fig. 17. A model of how perturbation of SEPT2 function may affect synaptic protein trafficking in neurons.

Stage 1: if SEPT2 function were perturbed at stage 1, synaptic protein accumulation near or at Golgi and microtubule-organizing center may be observed.

Stage 2: if SEPT2 function were perturbed at stage 2, synaptic protein accumulation may be found in the cell body and the neurite shaft.

Stage 3: if SEPT2 function were perturbed at stage 3, synaptic protein will be found in the synaptic terminal. However, no protein insertion into the plasma membrane will be observed.
I have begun to set up a hippocampal neuron culture system to test this working model for SEPT2 function. First, I looked for the presence of SEPT2 protein in cultured hippocampal neurons. I found that SEPT2 is present in hippocampal neurons, but at a lower level than in glial cells (Fig. 18). Second, I generated a shRNA construct for SEPT2 that has a GFP marker on the same vector (pSIH-H1-copGFP). Due to the low transfection efficiency in neurons, the availability of this vector will enable a definitive identification of transfected neurons. In addition, I have also generated an overexpression construct (pAdTrack-SEPT2) that allows for the expression of SEPT2 and GFP from the same vector as two separate proteins. I have previously expressed GFP-tagged SEPT2 in mammalian cells and found that this protein aggregates at the perinuclear region of the cell and result in the eventual death of the transfected cells. To assess the efficiency of SEPT2 knockdown and overexpression, I transfected these two constructs into NRK cells. Preliminary results revealed that shRNA construct 2-129 for SEPT2 could knock-down the SEPT2 protein level in NRK cells by about 75% in 48 hrs. Likewise, the overexpression construct resulted in an approximately 6 fold increase in SEPT protein level in transfected NRK cells (Fig 19.). The availability of these reagents will allow the setup of a cell-based assay system to investigate the function of SEPT function in vesicle trafficking underlying neuronal development and function. Furthermore, elucidation of molecular mechanisms underlying SEPT2 function may provide important insights into various neurodegeneration states that correlate with perturbation of septin protein levels and possibly function.
**Fig. 18. SEPT2 is expressed in hippocampal neurons.**
The expression of SEPT2 in 2div and 5div hippocampal neurons was examined by immunofluorescence microscopy. (A, C) βIII tubulin staining of hippocampal neurons in 2div and 5div neurons. (B, D) SEPT2 staining of hippocampal neurons and glia cells in the neuronal culture. Bar = 10 μm
Fig. 19. Manipulation of SEPT2 protein levels by shRNA knockdown and overexpression constructs

(A, B, C, D) Examples of NRK cells transfected with vector control and SEPT2 overexpression constructs. (A) pAdTrack-CMV vector-transfected cells do not show SEPT2 overexpression (B). In contrast, cells transfected with pAdTrack-SEPT2 overexpression construct (C) show upregulated SEPT2 expression (D). A graph summarizing the mean (with standard deviation) immunostaining quantitation results in NRK cells. Control(-) denotes SEPT2 protein levels in untransfected cells (n = 34 for each construct).

(F, G, H, I) Examples of NRK cells transfected with nonspecific shRNA construct control and SEPT2 shRNA constructs. (F, G) Control vector (70-11)-transfected cells do not show detectable decrease in SEPT2 protein level compared to untransfected cells. (H, I) Cells transfected with SEPT2 shRNA construct (2-129) show decreased SEPT2 protein immunostaining level. (J) A graphs summarizing the mean (with standard deviation) immunostaining quantitation of SEPT2 in SEPT2 shRNA construct- and control construct-transfected cells in comparison to untransfected cells. Control(-) denotes SEPT2 protein levels in untransfected cells (n = 20 for each construct) Bar = 10 μm
REFERENCES


Howe, A. G., Fairn, G. D., MacDonald, K., Bankaitis, V. A. and McMaster, C. R. (2007). "Regulation of phosphoinositide levels by the phospholipid transfer protein


a human septin gene to a region on chromosome 17q, commonly deleted in sporadic epithelial ovarian tumors. " Cancer Res 60(17): 4729-34.


Curriculum Vitae

Hyun Jong Kim

Academic Background

1991-1994 Yonsei University (Seoul, Korea)
Bachelor of Science in Biology

1995-1997 Yonsei University (Seoul, Korea)
Master of Science in Biology

2000- Present Rutgers University (New Brunswick, New Jersey)
Ph. D Program in Cell and Developmental Biology

Academic Awards
Recipient of Yonsei University Scholarship (1991)
Recipient of Yonsei University Scholarship (1992)
Recipient of the Graduate Research Assistant Fellowship (2002)

Experience

June 2001- January 2009 Graduate Assistant Rutgers University
PhD Thesis: The role of SEPT2 on neuronal development

February 2009- Present Teaching Assistant Rutgers University
Advanced Neuroscience Lab

Publications