Characterization of the Intersectin Family of Proteins: Endocytic Links to Signaling & the Cytoskeleton

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dedicated to Punti Chopra Wiggler

Nothing in the world can take the persistence. Talent will not; nothing is more common than unsuccessful men with talent. Genius will not; unrewarded genius is almost a proverb. Education will not; the world is full of educated derelicts. Persistence and determination alone are omnipotent. Great minds have purposes, little minds have wishes.

--Washington Irving

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ABSTRACT

Membrane trafficking via clathrin-mediated endocytosis is an essential process for maintaining cellular homeostasis. Within the synapse, clathrin-mediated endocytosis is critically involved coordinating the efficacy of neurotransmission with the rapid replenishment of synaptic vesicles in the nerve terminal. The orchestration of each step in synaptic vesicle recycling is critically dependent upon the precise localization of synaptic proteins. In this thesis, we have characterized intersectin-s and intersectin-l, proteins implicated in regulating the localization of components of clathrin-mediated endocytosis. We have demonstrated that intersectin-s and –l are modular domain-containing proteins that function as molecular scaffolds to form endocytic complexes in nerve terminals. Through their various modular domains, including Eps15 homology (EH) and Src homology 3 (SH3) domains, intersectins target elemental components of clathrin-mediated endocytosis, such as epsin and dynamin, to endocytic structures.

Epsins are a family of endocytic proteins that encode an Epsin N-terminal homology (ENTH) domain, which is a module that has been found in a variety of proteins with apparently disparate functions. We have characterized the ENTH domain as an evolutionarily conserved domain that mediates protein-protein interactions with tubulin and microtubules. We propose epsin proteins may functionally link the microtubule cytoskeleton with components of the endocytic machinery, including the intersectins.

In the final two studies included in this thesis, we demonstrate that intersectin-s and intersectin-l are additionally associated with signal transduction machineries. We found that intersectin-l associates with and regulates Cdc42-mediated signaling cascades, while intersectin-s directly binds mSOS a regulator of the Ras signaling pathway. Cdc42 and Ras signal transduction pathways influence a myriad of cellular functions including differentiation, cell division, apoptosis and regulation of actin cytoskeletal architecture.

Collectively, these studies suggest that intersectin-s and -l may regulate synaptic vesicle recycling via their ability to influence the localization of proteins associated

with clathrin-mediated endocytosis. In addition intersectin proteins may play complementary roles in signal transduction. These studies do not only extend our appreciation for specific regulators of vesicular recycling, but also offer insights into their partnership with signal transduction machineries.

RÉSUMÉ

Le trafic membranaire via l'endocytose médiée par la clathrine est un processus important pour maintenir l'homéostasie cellulaire. Au niveau de la synapse, l'endocytose médiée par la clathrine est impliquée pour coordonner l'efficacité de la transmission neuronale par le réapprovisionnement rapide des vésicules synaptiques au niveau des terminaisons nerveuses. Le déroulement de chaque étape lors du recyclage des vésicules synaptiques dépend de la localisation précise des protéines synaptiques. Dans cette thèse, nous avons caractérisé deux protéines, l'intersectine-s et l'intersectine-l, qui sont impliquées dans la régulation de la localisation des composants de l'endocytose médiée par la clathrine. Nous avons démontré que l'intersectine-s et -l sont des protéines contenant des domaines modulaires qui permettent de former des complexes endocytiques dans les terminaisons nerveuses. Par leurs divers domaines modulaires, comme le domaine d'homologie à Eps15 (EH) et le domaine d'homologie à Src de type 3 (SH3), les intersectines ciblent dans les structures endocytiques des composants élémentaires de l'endocytose médiée par la clathrine, telles que l'epsine et la dynamine.

Les epsines sont une famille de protéines endocytiques qui contiennent un domaine d'homologie à la partie amino-terminale de la protéine epsine (ENTH). Ce module a été trouvé dans une variété de protéines avec des fonctions apparemment différentes. Nous avons caractérisé le domaine ENTH comme un domaine conservé au cours de l'évolution et qui médie des interactions avec des protéines tels que la tubuline et les microtubules. Nous proposons que les epsines peuvent fonctionellement lier le cytosquelette des microtubules avec des composants de la machinerie endocytique, y compris les intersectines.

Dans les deux études finales incluses dans cette thèse, nous démontrons que l'intersectine-s et l'intersectine-l sont en plus associées aux machineries de transduction de signal. Nous avons constaté que l'intersectine-l s'associe à Cdc42 et régule la cascade de signaux transmis par Cdc42, alors que l'intersectine-s lie directement mSOS, une protéine qui régule la voie de signalisation par Ras. Les voies de transduction de signaux de Cdc42 et de Ras influencent un éventail de fonctions cellulaires comprenant

la différentiation, la division de cellules, l'apoptose et la régulation de l'architecture du cytosquelette d'actine.

Collectivement, ces études suggèrent que les intersectine-s et -l peuvent réguler le recyclage des vésicules synaptiques par leur capacité à influencer la localisation des protéines impliquées dans l'endocytose médiée par la clathrine. En complément, les intersectines peuvent jouer des rôles dans la transduction des signaux. Ces études détaillent non seulement notre connaissance sur les régulateurs spécifiques du recyclage des vésicules, mais donnent également un aperçu sur leur association avec la machinerie de la transduction de signaux.

LIST OF ABBREVIATIONS

ANTH	AP180 N-terminal homology
AP	adaptor protein
CALM	clathrin assembly lymphoid myeloid leukemia protein
CCP	clathrin-coated pit
CCV	clathrin-coated vesicle
Cdc42	cell-division cycle 42
CHC	clathrin heavy chain
CLC	clathrin light chain
CNS	central nervous system
COPI / COPII	coat protein I / coat protein II
CRIB	Cdc42/Rac interactive binding domain
Dap160	dynamin-associated protein of 160 kiloDaltons
DH	Dbl homology
EGF	epidermal growth factor
EH	Eps15 homology
EM	electron microscopy
ENTH	epsin N-terminal homology
Eps15	epidermal growth factor pathway substrate 15
Epsin	Eps15 interacting protein
GAP	GTPase activating protein
GEF	guanine-nucleotide exchange factor
GFP	green fluorescent protein
GST	glutathione S-transferase
GTPase	guanosine triphosphatase
HIP1 / HIP12	Huntingtin-interacting protein1 / Huntingtin-interacting protein12
Ibp	intersectin binding protein
kDa	kiloDaltons
LPAAT	lysophosphatidic acid acyl transferase
MAP	microtubule-associated protein

MP90	mitotic phosphoprotein of 90 kilodaltons
mSos	mammalian Son of sevenless
MTs	microtubules
NPF	asperagine-proline-phenylalanine
NRS	normal rabbit serum
NSF	N-ethylmaleimide-sensitve factor
N-WASP	neuronal Wiskott Aldrich syndrome protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PH	pleckstrin homology
PRD	proline-rich domain
PSD	postsynaptic density
PtdIns(4,5)P_2	phosphatidylinositol (4,5)-bisphosphate
Rac1	Ras-related C3 botulinum toxin substrate
RER	rough endoplasmic reticulum
RhoA	Ras-homologous member A
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH	scr homology
SM	starting material
TGN	*trans*-Golgi network
WASP	Wiskott Aldrich syndrome protein

CONTRIBUTION OF AUTHORS

CHAPTER 2

The rationale for this project was devised by Dr. P. McPherson and myself in collaboration with Dr. B. Kay. Dr. P. McPherson and I wrote the manuscript in collaboration, with assistance from Drs. B. Kay, J. O'Bryan and C. Der. Dr. M. Yamabhai generated intersectin and epsin 2 (Ibp2) constructs. Excellent technical assistance was provided by J. Philie and E. de Heuvel.

Contribution of figures:	N. Hussain	Figures 2.1, 2.3, 2.4, 2.6a
	A. Ramjaun	Figure 2.2
	M. Guy & D. Baranes	Figure 2.5
	M. Yamabhai	Figure 2.6B
	P. McPherson	Figure 2.7

CHAPTER 3

I devised the rationale of this investigation in collaboration with Drs. P. McPherson and B. Kay. I wrote the manuscript with support from Dr. P. McPherson. Drs. M. Yamabhai and B. Kay generated the *Xenopus leavis* epsin (GST-MP90) construct that was critical in the initial identification of the interaction characterized in this chapter. A. Bhakar and Dr. S. Ferguson assisted with primary culture and confocal microscopy, respectively. Excellent technical assistance was provided by J. Philie and E. de Heuvel.

Contribution of figures:	N. Hussain	Figures 3.1-3.5, 3.7-3.10
	P. McPherson	Figure 3.6a-b

CHAPTER 4

I contributed significantly to the rationale, intellectual development and writing of this manuscript in collaboration with Drs. P. McPherson and X-K. Tong. In addition, I technically directed the first author on several of the experiments. Excellent technical assistance was provided by J. Philie and E. de Heuvel.

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	A. Kurakin	Figure 4.3
	E. de Heuvel	Figure 4.4
	E. Abi-Jaoude & D. Baranes	Figure 4.5a-c
	P. McPherson	Figure 4.5d-e

CHAPTER 5

I devised the rationale of this study and directed its intellectual development with some guidance from Dr. P. McPherson. Dr. P. McPherson and I wrote the manuscript in collaboration. Drs. S. Jenna and N. Lamarche-Vane assisted with microinjection assays and made significant intellectual contributions to the project. Drs. T Stossel and M. Glogauer collaborated to provide Figure 5.3. S. Wasiak, and Drs. B. Kay, M. Guipponi, and S. Antonarakis provided the human intersectin-s and -l clones used to generate each of the intersectin constructs used in this study. Excellent technical assistance was provided by J. Philie and E. de Heuvel.

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	E. de Heuvel	Figure 5.1d
	M. Glogauer & T. Stossel	Figure 5.3
	C. Quinn	Figure S3a-b

CHAPTER 1. THE EVOULUTION OF MEMBRANE TRAFFICKING

Matthias Schleiden and Theodor Schwann asserted that the definitive structural unit of all organs and tissues is the cell, a doctrine known as the cell theory. In the broadest sense, their cell theory had a monumental impact on human perception of living organisms; minimally, their theory launched a new scientific discipline: the field of cell biology.

The following decades of research based on the cell theory led to significant advances, including the identification of the nucleolus and the mitochondrion in 1898. That same year while investigating the structure of nervous tissue, Italian histologist Camillo Golgi, identified a reticular structure in cells (Golgi, 1898) that would provide the most compelling indication hitherto that all eukaryotic cells are divided into membrane-bound compartments. Paradoxically, Golgi's accomplishment predated the era in which membrane-bound organelles were accepted as functional integrals of a eukaryotic cell. The cellular ubiquity and function of Golgi's reticular structure remained wrapped in skepticism, and the subject of thousands of publications for the subsequent century (Griffiths, 2000). This reticular formation, renamed the 'Golgi apparatus', eventually prevailed to be recognized as the first identified organelle of membrane trafficking. Arguably, efforts to quell the controversy surrounding Golgi's discovery provided the foundation for several avenues of research within the field of cell biology, including membrane trafficking.

The principal conclusion Golgi drew from his seminal research proved equally as contentious as his discovery of the Golgi apparatus. Golgi argued that nervous tissue is composed of a syncitium or continuum of functionally interconnected cells. In direct contrast to his hypothesis was the 'neuron doctrine' spearheaded by Golgi's rival, Ramón y Cajal, which stated that neurons are distinct units that are structurally and functionally contiguous (James, 1998). Cajal's postulate ultimately prevailed. The neuron doctrine became a fundamental concept that has influenced all aspects of biology. From the basic direction with which we approach the study of membrane

trafficking, to the structural basis upon which we understand synaptic activity, and ultimately to our efforts in parsing brain function into components of consciousness, each of these have at their intellectual centre Cajal's neuron doctrine (James, 1998).

Ironically, Cajal appears to have forever regretted not identifying the Golgi apparatus himself. Prior to Golgi's seminal publication, Cajal noted a reticular structure in cerebral pyramidal cells but dismissed notions to publish the observation since he could not reproduce the result (Ramón y Cajal, 1996). Cajal would later record in his memoirs:

> *Had it not been for such considerations [of reproducibility], the so-called reticular apparatus, which the neurologist of Pavia discovered in 1898 (by means of a formula, indeed, which is notably uncertain) it would figure today among my assets and under my name.* (Ramón y Cajal, 1996)

Despite not identifying the Golgi apparatus, Cajal's research efforts in neurobiology would win him a Nobel Prize in 1906, awarded in conjunction with Golgi. Moreover, Cajal's name would be raised into the next century as being the greatest neuroanatomist of his time, and he continues to be attributed with launching the field of neuroscience (James, 1998).

Within this period of naissance for neuroscience, Elie Metchnikoff's discovery of cellular immunity established immunology as another novel field in cell biology. Metchnikoff demonstrated that intracellular organelles could internalize and digest extracellular particles introduced to a cell (Metchnikoff, 1887). Although Metchnikoff's finding received less notoriety than identification of the Golgi apparatus, it was no less critical in the evolution of membrane trafficking research (Silverstein, 2003). An important derivative of his immunological finding was the notion that membrane bound compartments create distinct environments within which cells undergo specialized processes critical in maintaining normal cellular function (Mellman, 1996).

In the mid-20th century, the development of subcellular fractionation techniques coupled with the invention of electron microscopy (EM) helped validate the conjecture

stemming from Metchnikoff's historic finding. Numerous groups, notably those of George Palade, Keith Porter, and Christian de Duve, used these tools to develop a principle that would become the conceptual foundation for all future research regarding membrane trafficking (Tartakoff, 2002). This tenet, outlined in 1975 by Palade, stated that a functional connection between the membrane-bound constituents of a cell and its external environment is achieved via vesicular carriers that transport material vectorially, i.e. from a 'donor' membrane fusing to a 'target/acceptor' organelle (Palade, 1975).

Numerous issues implicitly confound Palade's notion of cell communication systems. For instance, because membrane and protein constituents are variable between organelles, vesicular trafficking would continuously require molecular sorting of donor and target elements. How is it that molecular components are selectively included and excluded from nascent vesicles? In addition, how are vesicles spatially and temporally targeted to specific membranes? These and related issues have supplied the field of membrane trafficking with intellectual fodder for decades of scientific inquiry.

In effort to delineate some of the advances made regarding these issues, I will provide a brief overview of the secretory pathway in non-neuronal and neuron specific systems. However, the focus of my doctoral research has centered on characterizing neuronal regulators of clathrin-mediated endocytosis—the principal mechanism in neurons for budding/recovery of vesicular components from donor/target membrane. Thus, I will place particular emphasis on the endocytic pathway as it occurs in neurons.

A. General Principles of the Secretory Pathway

Evolving from the previous era in trafficking research which was dominated by ultra-structural characterization of the secretory pathway, contemporary cell biologists combined applications in genetics, biochemistry, and molecular biology to pursue issues in cell biology. The reductionist approach, devoted to biochemically characterizing the individual components of cell systems, marked a new era in membrane trafficking research. For example, studies conducted in lower order organisms, such as yeast, coupled with the biochemical characterization of transport

vesicles, in particular those involved in neurotransmitter release called synaptic vesicles, demonstrated that many molecular components of vesicular transport in yeast are homologous to those required for synaptic vesicle trafficking (Eakle et al., 1988; Malhotra et al., 1989; Wilson et al., 1989). These studies were the first to indicate that secretory processes within mammalian cells, including neurons, are in fact specializations of more general trafficking mechanisms developed in yeast, demonstrating evolutionary conservation of vesicle trafficking mechanisms. In general, each vesicular trafficking event is a multi-step process that includes specific targeting, docking, and fusion of a vesicle with its acceptor organelle resulting in the transfer of vesicular contents through the acceptor membrane as well as the transfer of membrane-associated proteins. Recent studies have determined that each of these steps is itself regulated by a panoply of molecules, as will be discussed below.

i. Regulated and Constitutive Exocytosis

The secretory pathway involves the release, or 'exocytosis', of intracellular material to the cell surface or the external environment, which can be accomplished via two forms of vesicular transport—the regulated and constitutive secretory pathways (Gumbiner and Kelly, 1982; Tartakoff et al., 1978).

In the regulated secretory pathway, proteins destined for transport are selectively packaged into specialized membrane bound organelles called secretory granules or vesicles. Secretory granules are assembled in the *trans*-Golgi network (TGN), are transported to the cell surface, and unlike the constitutive pathway, selectively release their cargo when the cell is provoked by an extracellular stimulus (Kelly, 1999). The precise physiological stimuli leading to exocytosis of secretory granule contents are dependent upon the type of secretory cell stimulated. Regulated transport of secretory granules is accomplished in a number of highly specialized cells, including endocrine, exocrine, mast cells, platelets, large granular lymphocytes, neutrophils, and neurons. A unique characteristic of the regulated pathway is that secretory granules can be stored within the cytoplasm of the cell for extended periods, allowing for the maintenance of significant intracellular reserves of mature secretory proteins (Burgess and Kelly, 1987; Dannies, 2001). Thus, secretory granule proteins are continuously available to the cell

for specific and rapid release upon secretagogue stimulation, even in the absence of *de novo* protein synthesis and processing.

The constitutive pathway differs from regulated secretion in a number of characteristic ways. Firstly, material transported through the constitutive pathway is non-selectively packaged into membrane bound vesicles that undergo continuous secretion. These vesicles, originating from the TGN and early endosomes, are critical factors in the maintenance of protein secretion and delivery of newly synthesized and recycled membrane components to the cell surface (Orci et al., 1987; Palade, 1975; Tooze and Burke, 1987). A second distinguishing feature is the concentration of cargo proteins transported within each pathway. For instance, in cells constitutively secreting proteins such as immunoglobulin, the concentration of immunoglobulin inside the vesicle is only two fold higher than that within the rough endoplasmic reticulum (RER), while regulated secretory granules transport extremely condensed and concentrated protein cargo (Dannies, 2001; Hearn et al., 1985). The granules of exocrine and endocrine cells, which transport peptide products and other secretory substances, typically harbour proteins concentrated ten to two hundred times that within early Golgi cisternae (Dannies, 2001; Salpeter and Farquhar, 1981).

ii. Endocytosis: A Necessity for All Eukaryotic Cells

An inherent feature common to regulated and constitutive exocytosis is the insertion of vesicular membrane and component proteins into the plasma membrane upon vesicle fusion (Breckenridge and Almers, 1987; Heuser and Reese, 1973; Steinman et al., 1983). Since the rate of membrane addition is appreciably greater than that required for cell growth, a process that removes the added proteins and lipids is required (Steinman et al., 1976).

Endocytosis is the process by which extracellular material, as well as membrane proteins and lipids are influxed to intracellular organelles (Ceccarelli et al., 1973; Heuser and Reese, 1973; Steinman et al., 1983). Endocytosis has many functions, including the compensation of exocytic events, as is exemplified by the recovery of synaptic vesicles within the nerve terminal, and in the internalization of 'cargo', such as

receptors and extracellular proteins, where cargo entry can occur in a constitutive or regulated fashion.

The classical view of endocytosis suggests that internalized vesicles are transported through the endosomal system, beginning with the early endosomes where they are either sorted to late endosomes and lysosomes for degradation of their cargo and membrane components, or are recycled back to the plasma membrane (Heuser and Reese, 1973; Kelly, 1999). An important feature of the endosomal system is that each compartment has a progressively lower luminal pH (Mellman et al., 1986). Vesicle targeting to acidic organelles within this system provides spatial and temporal control over the dissociation of ligand-receptor complexes and thus, a mechanism for receptor down-regulation (Kornfeld and Mellman, 1989). In addition, active receptors found on endosomes can create mobile platforms for the intracellular interaction of signaling molecules, called signaling endosomes, to further link this system to cell signaling regulation (for reviews see Barker et al., 2002; McPherson et al., 2001). Thus, endocytosis is not merely a mechanism of membrane recycling; it is a means by which cellular, and organismal homeostasis is maintained. Endocytosis is involved in a multitude of activities, including the acquisition of hormones and essential nutrients, antigen presentation and pathogen entry, metabolic and proliferative signal regulation, receptor regulation, and synaptic transmission (Marsh and McMahon, 1999; Mellman, 1996).

The strictest regulation of compensatory endocytic trafficking is found within the mammalian central nervous system (CNS). Efficient endocytosis is particularly required of synaptic vesicle trafficking since erroneous regulation could lead to vesicle depletion, and ultimately the failure to preserve nerve terminal integrity (Harata et al., 2001). The pressures on synaptic vesicles to maintain high fidelity recycling, their great abundance in brain, and the relative ease with which they are biochemically isolated, make synaptic vesicles ideal for studying the components and mechanisms required in membrane trafficking. I will provide a brief overview of the general architecture of a synapse before discussing some advances these studies have provided for our understanding of synaptic vesicle endocytosis.

B. The Architecture of a Synapse

As originally stated in Cajal's neuron doctrine, the CNS is composed of cells that come into close apposition for communication with one another. Synaptic communication occurs via propagation of electrical signals, which are converted into chemical signals for transmission to another neuron at a site called the chemical synapse. Ultra-structurally, chemical synapses are cell-cell adhesions between adjacent membranes from pre- and postsynaptic neurons separated by a cleft that is typically 25-50 nm wide (Heuser et al., 1979; Peters et al., 1991). Within the synaptic cleft are molecules and matrix proteins that putatively tether the pre- and postsynaptic membranes together (Ichimura and Hashimoto, 1988; Phillips et al., 2001).

Swellings or varicosities at the tips and along the length of presynaptic axons comprise presynaptic nerve terminals. Presynaptic terminals house chemical compounds, referred to as neurotransmitters, within membrane bound vesicles. Since their initial identification in nerve terminals (Palade, 1954; Robertson, 1956), electron-microscope analyses have provided the basis for designating two general classes of vesicles containing neurotransmitters: those with either electron-dense or electron-lucent cores.

Electron-dense vesicles range in diameter (approximately 70-200 nm) and typically harbour slow-releasing peptide or amine transmitters (Basbaum and Heuser, 1979). Similar to endocrine/exocrine granule cells, dense-core vesicles are synthesized in the cell body via the secretory pathway and transported to the cell periphery along microtubules (Black and Lasek, 1980; Droz and Leblond, 1962; Matsuuchi et al., 1988). These vesicles are dispersed throughout the presynaptic cytoplasm, and can exocytose their contents into cleft regions as wide as 2 μm (Burnstock and Horkfelt, 1979; Zhu et al., 1986).

In contrast to their dense-core counterparts, synaptic vesicles, which are electron-lucent, are far more abundant and of a relatively uniform 50 nm diameter (Burns and Augustine, 1995; De Camilli and Jahn, 1990; Heuser et al., 1979). Synaptic vesicles typically contain chemical neurotransmitters used for extremely rapid signaling, and as such, neurons have streamlined their trafficking to provide the utmost efficiency. While

membrane precursors of synaptic vesicles are transported from the Golgi to nerve terminals, the actual synthesis, and packaging of synaptic vesicles with neurotransmitter occurs within the terminal proper. Three distinct pools of synaptic vesicles have been described that reside within the nerve terminal: the resting, reserve, and docked pools. Following stimulation of exocytosis, these vesicle pools maintain their distinct identities when recovered (Li and Murthy, 2001; Murthy and Stevens, 1998; Pleribone et al., 1995), and appear to be hierarchical in terms of their tendency to exocytose and in their relative distances from the presynaptic membrane (Figure 1.0).

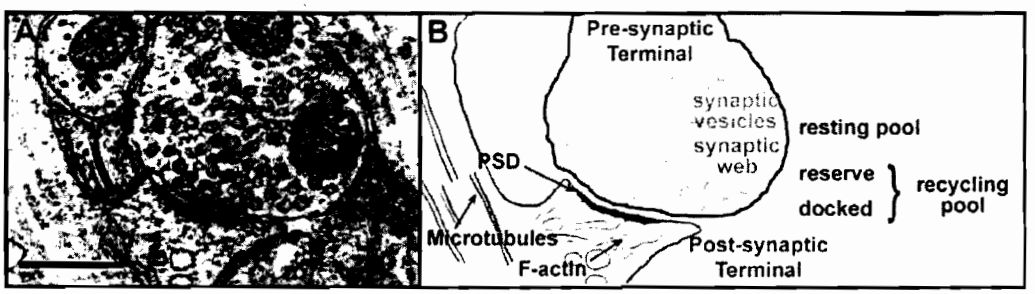

Figure 1.0 Schematic of the synapse. A. Presynaptic terminal forming a glutamatergic synapse with a dendritic spine. Scale bar 400 nm. B. Tracing of A, identifying major synaptic structures, and outlining three pools of synaptic vesicles as outlined for cultured hippocampal neurons (sizes of different vesicular pools are not to drawn to scale). PSD, post-synaptic denisty. Adapted from (Kennedy, 2000)

At the base of this hierarchy is the resting pool of vesicles, which accumulate in an area 0.2-0.5 μm from the presynaptic terminal membrane (Betz and Bewick, 1992; Murthy and Stevens, 1999; Ryan and Smith, 1995). While the number of vesicles within each pool will depend upon the specific type of neuron investigated, studies using cultured cortical and hippocampal neurons suggest that the resting pool constitutes approximately 180 vesicles filled with neurotransmitter (Betz and Bewick, 1992; Murthy and Stevens, 1999; Ryan and Smith, 1995). Although the vesicles within the resting pool do not directly participate in neurotransmitter release following stimulation under physiological conditions, they will undergo exocytosis following extensive stimulation (e.g. via application of α-latrotoxin) (Ceccarelli and Hurlbut, 1980; Murthy and Stevens, 1999). These vesicles are predicted to function as a storage supply for the

'recycling pool', which are those vesicles actively involved in exocytosis and neurotransmission (Südhof, 2000).

The 'recycling pool' collectively refers to the reserve and docked pools of synaptic vesicles. Studies conducted in cultured hippocampal neurons suggest that the reserve pool of these cells constitutes approximately 17-20 vesicles located less than 0.2 μm from the presynaptic membrane (Murthy and Stevens, 1999). These vesicles are predicted to be more rapidly recycled than the resting vesicle pool (Barker et al., 1972; Murthy and Stevens, 1998; Murthy and Stevens, 1999; Pyle et al., 2000; Schikorski and Stevens, 2001; Sun et al., 2002).

The second subset of the recycling vesicles, the docked pool (estimated to consist of approximately 5-10 vesicles in cortical and hippocampal neurons) (Murthy and Stevens, 1999; Schikorski and Stevens, 1997) clusters directly adjacent to and ready for fusion with the plasma membrane. Following electrical stimulation and membrane depolarization this 'readily-releasable pool' of vesicles exocytose neurotransmitter into the synaptic cleft (Heuser et al., 1979; Peters et al., 1991). Numerous studies provide evidence that subsequent to exocytosis the docked pool recovers the ability to re-exocytose very rapidly, without re-entering and recycling through the reserve pool (Pyle et al., 2000; Schikorski and Stevens, 2001; Sun et al., 2002). However, the precise temporal kinetics, and specific mechanisms of fusion/recovery of docked vesicles in synaptic vesicle recycling are issues that remain unclear (for reviews see Morgan et al., 2002; Valtorta et al., 2001).

The area containing docked vesicles is called the active zone. Active zones are electron dense webs of scaffolding proteins located just beneath the plasma membrane (Bloom and Aghajania, 1968). This synaptic web of proteins extends from the plasma membrane throughout the terminal and participates in targeting both the vesicles and regulators of vesicle trafficking throughout the active zone, and extracellularly in the alignment of pre- and postsynaptic membranes (Figure 1.0) (Hirokawa et al., 1989; Pfenninger et al., 1972; Phillips et al., 2001). Unlike the synapses formed for dense-core vesicles, the postsynaptic reception component of synaptic vesicles is a relatively continuous electron dense thickening, called the postsynaptic density (PSD) which also contributes to aligning pre- and postsynaptic membranes (Phillips et al., 2001). The

PSD is a specialized signal transduction structure involved in concentrating and anchoring postsynaptic receptors in the membrane directly adjacent to the synaptic cleft and the presynaptic membrane (for review see Sheng, 2001).

The regulation of synaptic vesicle trafficking, the structural specializations in pre- and postsynaptic membranes coupled with the proximity of membranes created by a tight synaptic cleft, each contributes to make synaptic vesicles exceptionally rapid vehicles for neuronal communication. Their particular abundance in brain has made biochemical analyses of the molecular machinery involved in neuronal membrane trafficking events possible. Thus, analysis of synaptic vesicle constituents has led to fundamental insights regarding the proteins and mechanisms that govern vesicular trafficking in neuronal and non-neuronal cells alike.

C. Endocytosis at the Nerve Terminal

Two opposing hypotheses regarding the speed, mechanisms, and locations of endocytosis at the synapse were developed thirty years ago under a cloud of scientific contention. Despite years of scrutiny, the latest research seems to have only renewed debate over the saliency of each model (Figure 1.1). The "kiss-and-run" hypothesis (Fesce et al., 1994), originally suggested by Ceccarelli and colleagues argues that synaptic vesicles form a fusion pore within the active zone during exocytosis, and recover by simple detachment from the plasma membrane (Ceccarelli et al., 1973). In contrast, Heuser and Reese purported that synaptic vesicles completely fuse with the plasma membrane during exocytosis, and slowly endocytose through the assistance of

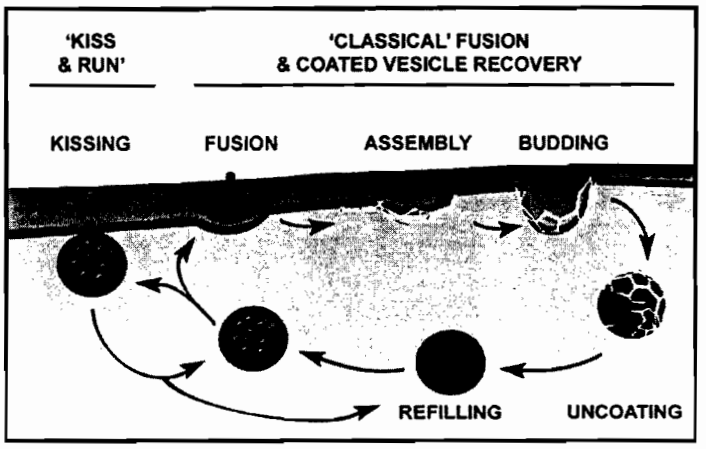

Figure 1.1 Schematic model of 'Kiss and Run' versus 'Classical' full fusion and coated vesicle retrieval of synaptic vesicles.

coat complexes that assemble distal to the active zone on the membrane (Heuser and Reese, 1973).

iii. Ceccarelli's lasting Kiss

As evidence supporting a link between coated vesicular structures and endocytosis grew, proponents of the Heuser and Reese model burgeoned until it became considered the 'classical' mode of all synaptic vesicle endocytosis events. Consequently, support for Ceccarelli's concept of the 'kiss and run' vesicle waned over time. The initial divergence between these models stemmed from opposing interpretations of freeze-fracture and thin-section electron microscopy of synaptic terminals at neuromuscular junctions. Ironically, microscopy is at the centre of a revival of Ceccarelli's hypothesis, as it has provided new evidence that 'kiss and run' is an integral element in synaptic vesicle recycling.

The strongest evidence for kiss and run endocytosis was developed outside the nervous system, through studies of dense-core granule secretion in the endocrine system. Using these cells as models for synaptic recycling, investigators identified 'capacitance flickers', which are transient increases in membrane surface area measured electrophysiologically as rapid reversals in capacitance. These flickers were interpreted as consecutive kiss and run events, that is the opening and closing of fusion pores over a period of milliseconds (Alés et al., 1999; Alvarez et al., 1993; Artalejo et al., 1998; Henkel et al., 2000; Thomas et al., 1994). Unfortunately, measuring capacitance flickers generated by synaptic vesicle fusion with the membrane proved to be more challenging than in secretory-granules because of their small size leading to diminished affects on membrane capacitance. However, recent advances in fluorescence microscopy and electrophysiology have developed imaging and capacitance measurements techniques that resolve the fusion and retrieval of single and multiple vesicles following mild physiological stimulation within central synapses (Betz and Wu, 1995; Ryan and Reuter, 2001). Using these techniques, recent studies indicate that synaptic vesicles can be immediately reused after exocytosis, arguably without ever leaving the active zone of the presynaptic plasma membrane (Figure 1.1) (Harata et al., 2001; Pyle et al., 2000; Stevens and Williams, 2000; Zenisek et al., 2000). In addition, there appear to be two

processes involved in synaptic vesicle endocytosis, a fast component predicted to represent kiss and run, and a slower mode likely accomplished through coated vesicle retrieval (Murthy and Stevens, 1998; Pyle et al., 2000; Stevens and Williams, 2000; Sun et al., 2002). Whether or not 'kiss and run' mechanisms cooperate with coated vesicle recovery, whether they are separately mediated by distinct molecular regulators, and the temporal kinetics of each are some of the questions that remain to be determined before a clear picture of synaptic vesicle endocytosis can be drawn.

iv. Coated Vesicle Retrieval of Synaptic Vesicles

The archetypal view of the vesicular endocytic pathway proposed by Heuser and Reese (Heuser and Reese, 1973) predicted that it is initiated by the recruitment and assembly of 'coat' protein complexes to the cytoplasmic face of the plasma membrane (Figure 1.1) (for reviews see Marsh and McMahon, 1999; Mellman, 1996). Coat proteins are believed to assist in the deformation of donor membrane such that an invaginated pit is formed. The eventual scission at the neck of this emergent vesicle results in the sequestering of selected membrane proteins, and the formation of a free vesicle. Once the coated vesicle is formed, disassembly of the coat releases coat proteins into the cytoplasm making them available for further cycles of vesicle formation, and permits the vesicle to fuse with its specific target compartment (for reviews see Allan and Balch, 1999; Rothman and Orci, 1992).

Since the first identification and subsequent characterization of a coat complex more than 30 years ago, multiple complexes have since been recognized and associated with endocytic or secretory processes (Kanaseki and Kadota, 1969). Three of the coat complexes that have been extensively characterized include coat protein I (COPI) and II (COPII), and clathrin (Glick and Malhotra, 1998; Schmid, 1997).

COPI complexes participate in the recovery of vesicles from the Golgi apparatus for transport to the endoplasmic reticulum (ER), as well as traffic between Golgi cisternae (Malhotra et al., 1989; Orci et al., 1989), while COPII complexes are involved in anterograde transport of ER-derived vesicles to the Golgi (Barlowe et al., 1994; Gu et al., 1997). An extensive body of literature concerning the structure and regulated assembly of COPI and COPII coat complexes has been generated (for review see

Barlowe, 2000; Haucke, 2003), however, these topics are outside the scope of this thesis and will not be discussed further.

EM analyses of endocytosis in mosquito oocytes in 1964 captured the first image of a coated structure surrounding a nascent endocytic vesicle (Roth and Porter, 1964). A decade later, this coat component was isolated and found to be an array comprised of a three legged protein that Barbara Pearse named 'clathrin' (Figure 1.2A) (Pearse, 1975). Since their initial identification clathrin coats have become the most extensively studied of the coat complexes. Clathrin coats mediate the formation of endocytic vesicles from the plasma membrane, as well as TGN-derived vesicles destined for fusion with endosomal compartments in neuronal and non-neuronal cells (for reviews see Brodsky et al., 2001; Pishvaee and Payne, 1998). As these coat complexes play a paramount role in the coordination and regulation of vesicular trafficking between membranous organelles, they continue to be a topic of intense investigation in the field of cell biology. The principal focus of my doctoral research has involved characterizing proteins and mechanisms that regulate clathrin-mediated endocytosis. Therefore, the following sections examine some of these regulatory proteins and mechanisms in detail.

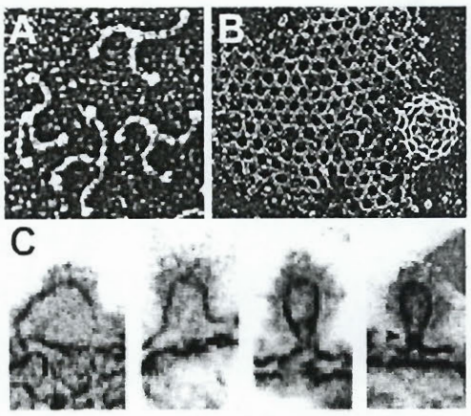

Figure 1.2 Clathrin triskelia and cages. A. Clathrin triskelia visualized by rotary platinum shadowing. B. Clathrin-coated pit emerging from a membrane lattice. C. Examples of different stages in clathrin-mediated endocytosis observed in giant reticulospinal axon of the lamprey. Images adapted from (Brodin et al., 2000; Brodsky et al., 2001; Schmid, 1997).

D. Clathrin-mediated Endocytosis: A multi-step process

Although multiple endocytic recycling pathways exist in cells (Johannes and Lamaze, 2002), several lines of evidence suggest that clathrin-mediated endocytosis plays a principal role in recycling at the nerve terminal. The first indication of this came with the demonstration that clathrin-coated vesicles (CCVs) are present at the nerve

terminal, and are selectively labeled when stimulated in the presence of fluid-phase endocytic tracers (Heuser and Reese, 1973). Supporting evidence emerged with the purification and biochemical characterization of synaptic vesicle associated proteins; these investigations revealed that several synaptic vesicle proteins are enriched in synapse-derived CCVs (Maycox et al., 1992; Pfeffer and Kelly, 1985). Furthermore, recent studies have indicated that clathrin adaptor proteins required for endocytosis display critical functions in regulating both the size and number of synaptic vesicles in the nerve terminal (Ford et al., 2002; Ye and Lafer, 1995a; Zhang et al., 1998). Finally, using a variety of experimental techniques, disruption of synaptic vesicle recycling *in vivo* has been shown to simultaneously reduce the number of synaptic vesicles in the nerve terminal and cause an accumulation of clathrin-coated structures attached to the pre-synaptic plasmalemma; these coated pits are presumed to be frustrated endocytic intermediates (Gad et al., 1998; Koenig and Ikeda, 1989; Shupliakov et al., 1997). Therefore, given the compelling evidence generated thus far the involvement of clathrin-mediated vesicle formation in synaptic vesicle recycling is seemingly irrefutable. Recent proteomics analysis of brain derived CCVs will undoubtedly further our understanding of recycling in the nerve terminal, as components of the synaptic vesicle proteome are revealed and their specific roles in endocytosis are characterized (Blondeau and McPherson, *unpublished observations*)

Based on ultra-structural analyses, clathrin-mediated endocytosis in the nerve terminal can be broken down into four distinct stages, including nucleation of coat complexes on the membrane, invagination and constriction of the coated pit, and finally uncoating of the formed vesicle (Figures 1.2C and 1.3). These steps and the numerous accessory proteins demonstrated to regulate and maintain the efficacy of endocytosis at the synapse are outlined in the following sections.

v. Nucleation of coat complexes to membrane 'hot spots'

The rate of endocytosis is limited by the time required to assemble molecular components of the endocytic machinery to specific segments of synaptic membrane. Neurons partially circumvent this temporal limitation by forming micro-domains, called

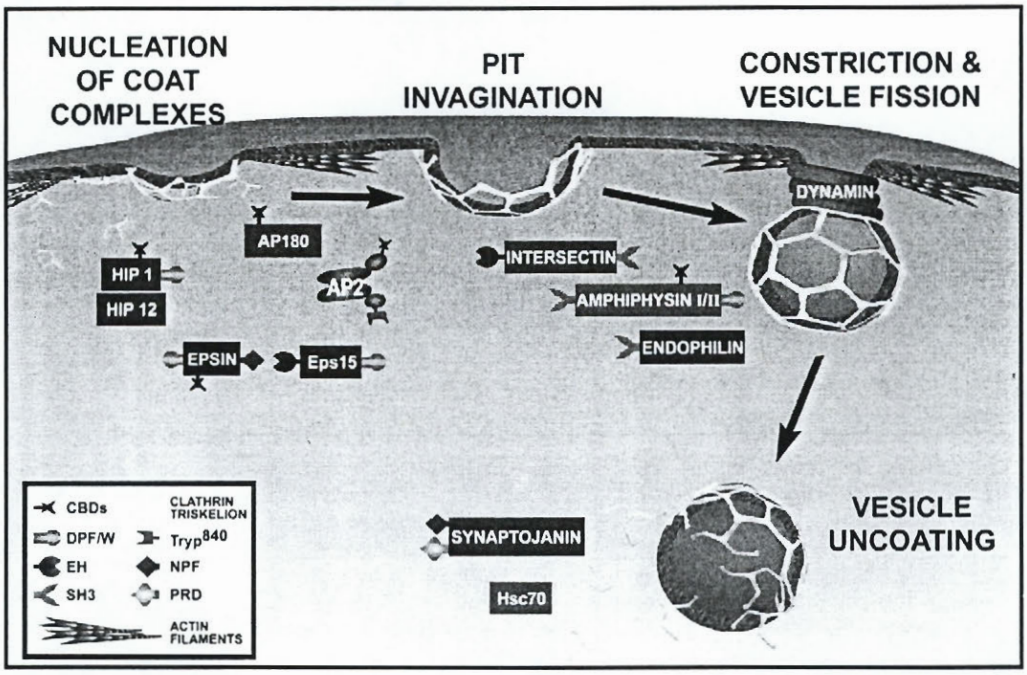

Figure 1.3 Schematic summary of the established steps in clathrin-mediated endocytosis. Putative sites of action for some of the proteins implicated in synaptic vesicle recycling are noted.

'hot spots', of preassembled clathrin and other endocytic components on the plasma membrane (Estes et al., 1996; Gaidarov et al., 1999; Gustafson et al., 1998; Heuser and Kirchhausen, 1985; Roos and Kelly, 1998; Roos and Kelly, 1999). Hot spots were first identified by Heuser and colleagues as hexagonal arrays of clathrin assembled into a honeycomb-like lattice on patches of plasma membrane, and have since been demonstrated to specifically form and extend approximately 1 micron from the edge of exocytic active zones (Figure 1.2B) (Heuser and Kirchhausen, 1985; Roos and Kelly, 1999).

It remains unclear how hot spots are restricted in their localization within nerve terminals. One possibility is that the cytoskeletal network helps segregate the membrane into sub-domains (Figure 1.3) (Jeng and Welch, 2001; McPherson, 2002; Qualmann et al., 2000; Schafer, 2002). In support of this notion, F-actin filaments have been noted within endocytic vesicle clusters by quick-freeze, deep-etch preparations of neuromuscular junctions (Hirokawa and Heuser, 1982; Hirokawa et al., 1989), and sparse actin labeling has been seen by immunoelectron microscopy of nerve terminals

in dendritic spines (Cohen et al., 1985). Although the identification of cytoskeletal structures within the active zone have been rare, it is important to note that conventional fixation methods are notoriously poor preservatives of cytoskeletal elements (Mellman and Warren, 2000). Recent studies provide evidence that actin and its associated motor protein, myosin, may influence the ability of vesicles to fuse within the nerve terminal (Bernstein and Bamburg, 1989; Mochida et al., 1994; Morales et al., 2000), and that activation of specific GTPases can cause an increase in actin polymerization within endocytic hot spots (Gustafson et al., 1998; Merrifield et al., 2002).

In the classical model of the fluid mosaic, lipids were not recognized to play a significant role in the formation of membrane domains (Singer and Nicolson, 1972). More recently, however, several lines of evidence suggest that dynamic assemblies of cholesterol and glycosphingolipids, called lipid rafts, create differences along the lateral plane of the membrane (Hurley and Meyer, 2001). Thus, another possible mechanism for defining endocytic hot spots is the formation of lipid-specific micro-domains within the membrane bilayer (Cremona and De Camilli, 2001; Martin, 2001). The fact that depletion of cholesterol, a component of lipid rafts, from membranes is sufficient to disrupt clathrin-mediated endocytosis highlights the importance of lipid constitution for this process (Martin, 1998; Rodal et al., 1999; Saiardi et al., 2002; Subtil et al., 1999). While it is increasingly clear that hot spots act as nucleation sites for the repetitive generation of clathrin-coated vesicles, there is no consensus as to the precise mechanisms, nor whether combinations of mechanisms dictate their restricted localization on the membrane.

vi. Invagination

Cytoplasmic clathrin is organized into three-legged trimers called triskelia, consisting of three heavy (180 kDa) and three light chain proteins (25-30 kDa) (Figure 1.2A) (Brodsky et al., 2001; Pearse and Crowther, 1987). Soon after their identification, it was demonstrated that clathrin triskelia self-assemble into circular, honeycomb-like cages of uniform size when placed in alkali conditions *in vitro* (Kirchhausen and Harrison, 1981; Pearse, 1975; Schmid et al., 1982). In search for factors that could stimulate the assembly of clathrin-coats under physiological pH, a heterotetrameric

protein complex of was identified, and ultimately named clathrin adaptor protein-2 (AP2) (Keen et al., 1979). Multiple adaptor proteins have since been identified for their ability to nucleate and assemble clathrin triskelia on membranes. These adaptors are broadly classified as being either heterotetrameric proteins, consisting of the adaptor proteins-1 to 4 (AP1 to AP4), or monomeric adaptors, which include AP180, members of the β-arrestin family, and GGAs (Golgi-localized, γ-ear-containing, ARF-binding proteins) (for review see Kirchhausen, 2002).

Each of the adaptors bind varied integral membrane proteins and membrane inositol lipids; a property which provides their relatively distinct intracellular localizations, and underlies their ability to mediate different pathways of protein sorting and vesicular transport (Kirchhausen, 2002; Schmid, 1997). In particular, the μ-subunit of the heterotetrameric AP2 adaptor is used to bind sequence motifs within the cytoplasmic domains of signaling receptor molecules in order to help sequester receptors into invaginating CCVs, and thereby regulate signal transduction (Ohno et al., 1995). Adaptors contain additional peptide motifs called 'clathrin boxes' that mediate their direct interaction with clathrin (Dell'Angelica et al., 1998; Doray and Kornfeld, 2001; Krupnick et al., 1997; Morgan et al., 2000; Puertollano et al., 2001; Ramjaun and McPherson, 1998; ter Haar et al., 2000). In terms of function, adaptor proteins are critical for the invagination stage of clathrin-mediated endocytosis because they recruit triskelia to pre-assembled hexagonal clathrin lattices (hot spots) and rearrange them to incorporate pentagonal clathrin arrays (Figure 1.3) (Heuser and Anderson, 1989; Heuser and Kirchhausen, 1985; Kirchhausen, 2000; Nossal, 2001). As a consequence of lattice reformation, bound membrane is curved inward, leading to the invagination and eventual formation of a clathrin-coated pit with a diameter of approximately 50 nm (Heuser and Anderson, 1989; Heuser and Evans, 1980; Kirchhausen, 2000; Nossal, 2001).

Although a great deal is known about the molecular components of endocytosis, the precise mechanisms inducing membrane invagination are still poorly understood. Undoubtedly, several factors will be uncovered that act cooperatively to induce membrane curvature. Recent discoveries directly linking phospholipids to membrane invagination events have led researchers to cast considerable attention in this direction

substantiated *in vivo* (Honing et al., 1994; Newmyer and Schmid, 2001). The uncoating activity of Hsc70 requires the presence of auxilin (Ungewickell et al., 1995), a protein which binds to AP2 binding protein and recruits Hsc70 to CCVs. Although the precise mechanism leading to vesicle uncoating has not been entirely elucidated, it is clear that once at CCVs, auxilin stimulates Hsc70 ATPase activity (Barouch et al., 1997; Holstein et al., 1996) which results in Hsc70 mediated dissociation of individual clathrin triskelia from the clathrin coat (Ma et al., 2002).

Another enzyme implicated in the uncoating process is synaptojanin. Synaptojanin is an inositol-5-phosphatase demonstrated to form two separate and stable complexes in brain tissue—one with endophilin I, and the other with amphiphysin I and II (de Heuvel et al., 1997; McPherson et al., 1996; Micheva et al., 1997a; Ringstad et al., 1999). Synaptojanin has also been shown to co-localize precisely with dynamin in the nerve terminal, and concomitant with dynamin, undergoes dephosphorylation in response to nerve terminal depolarization (McPherson et al., 1994b). The association and disassociation of cytosolic endocytic components are directly regulated by cycles of dephosphorylation and phosphorylation in the nerve terminal (Slepnev et al., 1998). For example, in brain extracts, the phosphorylation of dynamin and synaptojanin inhibits their interaction with amphiphysin I and II (Slepnev et al., 1998; Wigge et al., 1997b). It is conceivable that specific phosphorylation events could provide a cellular mechanism for temporally regulating various stages of clathrin-mediated endocytosis. The regulation of phosphoinositide levels in brain was suggested to function in synaptic vesicle uncoating following the discovery that synaptojanin knockout mice display a significant accumulation of clathrin coated vesicles throughout the nerve terminal compared to wild-type animals (Cremona et al., 1999). Based on this study, synaptojanin is predicted to negatively regulate interactions between the membrane and components of the clathrin-coat.

RESEARCH RATIONALE

Extensive research concerning CCV recycling has led to the discovery of several nerve terminal-enriched proteins implicated in synaptic vesicle recycling (Kelly, 1999).

Three main objectives were outlined for my doctoral studies aimed at contributing to our understanding of the molecular mechanisms underlying vesicular recycling:

1) Numerous proteins have been identified that function in clathrin-mediated endocytosis, and many of these contain Eps15 homology (EH) or Src homology 3 (SH3) modular protein-interaction domains. Given the propensity of these domains within intersectin, our first objective was to determine whether intersectin may be involved in clathrin-mediated endocytosis.

2) We predicted the specific interactions mediated by the various modular domains within intersectin would be elemental in dictating its function(s). Thus, we sought to identify proteins that form complexes with each of the modular domains in intersectin. Upon identifying various protein complexes mediated by intersectin, our objective was to characterize these proteins further in effort to elucidate their respective effects on intersectin function.

3) We determined that intersectin-l expression is restricted to neurons, while intersectin-s is ubiquitously expressed. This distrubution is indicative of specialized neuronal functions particular to intersectin-l, with potentially general cellular functions being attributable to intersectin-s. Given the fact that intersectin-l is distinguished from intersectin-s by the presence of a carboxy terminal extension encoding three additional modular domains we sought to identify protein interactions mediated by this region with the overall objective being to identify functions of intersectin-l that may be of particular relevance to neurons.

PREFACE TO CHAPTER 2: The identification of intersectin-s & intersectin-l as molecular components of endocytosis

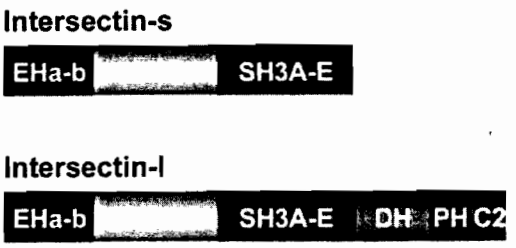

Figure 2.0 Schematic of intersectin-s (short) and intersectin-l (long) modular domain structure.

Src homology 3 (SH3) and Eps15 homology (EH) domains are modular domains found in multiple proteins, and mediate protein-protein interactions in a variety of cellular processes including endocytosis. In search for novel proteins containing these modules, Montarop Yamabhai and Brian Kay screened a cDNA expression library with a proline-rich peptide that interacted with a broad spectrum of SH3 domains. In this screen they identified a protein called intersectin-short (intersectin-s) (Yamabhai et al., 1998). Intersectin-s contains two EH domains, a central helix-forming region, and five SH3 domains, while a longer form of the protein (intersectin-l) includes additional Dbl homology (DH), pleckstrin homology (PH), and C2 domains (Guipponi et al., 1998; Roos and Kelly, 1998; Sengar et al., 1999).

Based on the propensity of modular domains within intersectin, this protein was predicted to form macromolecular endocytic complexes (Yamabhai et al., 1998). The foundation of my doctoral research lay in testing the validity of the above prediction. In addition, we suggested that if intersectin-s and –l are endocytic-scaffolding proteins, examination of these molecules, and their specific ligand partners, should undoubtedly advance our overall understanding of vesicular recycling processes. The first component of my doctoral research was to determine if intersectin-s and –l are indeed components of clathrin-mediated endocytosis.

CHAPTER 2. SPLICE VARIANTS OF INTERSECTIN ARE COMPONENTS OF THE ENDOCYTIC MACHINERY IN NEURONS AND NON-NEURONAL CELLS

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Keywords: clathrin, Dap160, dynamin, EH domain, endocytosis, Eps15, *Rattus norvegicus*, SH3 domain, *Xenopus laevis*

Running title: intersectin as a component of endocytosis

ABSTRACT

We recently identified and cloned intersectin, a protein containing two Eps15 homology (EH) domains and five Src homology 3 (SH3) domains. Using a newly developed intersectin antibody, we demonstrate that endogenous COS-7 cell intersectin localizes to clathrin-coated pits, and transfection studies suggest that the EH domains may direct this localization. Through alternative splicing in a stop codon, a long form of intersectin is generated with a C-terminal extension containing Dbl homology (DH), pleckstrin homology (PH), and C2 domains. Western blots reveal that the long form of intersectin is expressed specifically in neurons, whereas the short isoform is expressed at lower levels in glia and other nonneuronal cells. Immunofluorescence analysis of cultured hippocampal neurons reveals that intersectin is found at the plasma membrane where it is co-localized with clathrin. Ibp2, a protein identified based on its interactions with the EH domains of intersectin, binds to clathrin through the N terminus of the heavy chain, suggesting a mechanism for the localization of intersectin at clathrin-coated pits. Ibp2 also binds to the clathrin adaptor AP2, and antibodies against intersectin co-immunoprecipitate clathrin, AP2, and dynamin from brain extracts. These data suggest that the long and short forms of intersectin are components of the endocytic machinery in neurons and nonneuronal cells.

INTRODUCTION

The Eps15 homology (EH) domain is an important protein-protein interaction module functioning in endocytosis. The core of the EH domain-binding motif is composed of the amino acids asparagine-proline-phenylalanine (NPF) (Paoluzi et al., 1998; Salcini et al., 1997; Yamabhai et al., 1998). This sequence is often found at the C terminus of EH domain-binding proteins where the free carboxylate can contribute to binding (Yamabhai et al., 1998). The EH domain was originally identified in the epidermal growth factor receptor phosphorylation substrate Eps15 (Wong et al., 1995). Through its EH domains, Eps15 binds to epsin, a recently identified protein implicated in endocytosis (Chen et al., 1998). Eps15 is localized to the rim of clathrin-coated pits (Tebar et al., 1996), likely through its interactions with AP2 (Benmerah et al., 1996; Benmerah et al., 1995; Cupers et al., 1998; Iannolo et al., 1997) and/or with epsin (Chen et al., 1998). EH domains are also found in the yeast proteins Pan1p and End3p, which are required for endocytosis and normal organization of the actin cytoskeleton (Benedetti et al., 1994; Tang and Cai, 1996; Tang et al., 1997; Wendland and Emr, 1998; Wendland et al., 1996).

The Src homology 3 (SH3) domain, a 50-70-amino acid motif that binds to proline-rich ligands (Pawson and Scott, 1997; Ren et al., 1993) has also been implicated in endocytosis (McPherson, 1999). For example, amphiphysins I and II are nerve terminal-enriched proteins that demonstrate SH3 domain-dependent binding to proline-rich sequences in dynamin and synaptojanin (David et al., 1996; Leprince et al., 1997; McPherson et al., 1996; Ramjaun et al., 1997; Wigge et al., 1997b), enzymes which function in the endocytosis of clathrin-coated vesicles (McPherson et al., 1996; Sweitzer and Hinshaw, 1998a; Takei et al., 1998). In fact, over-expression of the SH3 domains of amphiphysins I and II leads to a functional block in endocytosis in a number of different systems (Owen et al., 1998; Shupliakov et al., 1997; Volchuk et al., 1998; Wigge et al., 1997b).

A link between EH and SH3 domain-mediated protein-protein interactions has been revealed with the identification and cloning of *Xenopus laevis* intersectin, a protein containing two N-terminal EH domains, a central helix forming region that has a high

probability of forming coiled-coil interactions, and five C-terminal SH3 domains (Yamabhai et al., 1998). Intersectin is homologous to Dap160, a *Drosophila* protein with two EH domains and four SH3 domains that was identified based on its affinity for dynamin (Roos and Kelly, 1998). Through this unique combination of protein-protein interaction modules, intersectin has the potential to form macromolecular complexes between EH domain- and SH3 domain-binding proteins. A human form of intersectin has also been recently identified through genomic analysis of chromosome 21 (Guipponi et al., 1998). Interestingly, those authors determined that intersectin undergoes alternative splicing in the stop codon leading to a short form (intersectin-s), which has the same domain structure as the *Xenopus* protein, and a long form (intersectin-l) that contains a C-terminal extension with Dbl Homology (DH), Pleckstrin Homology (PH), and C2 domains (Guipponi et al., 1998).

In this manuscript, we have generated an antibody against the EH domains of frog intersectin which we have used to characterize the expression and localization of the antigenically related mammalian protein. Western blots reveal that intersectin-s is expressed in a wide variety of tissues and cell lines, including in COS-7 cells, where it localizes to clathrin-coated pits on the plasma membrane. Transfection studies suggest that the EH domains may mediate this localization. Intersectin-l is expressed predominately in neurons where it is also co-localized with clathrin. We previously identified two related proteins that bind to the EH domains of intersectin: intersectin-binding protein (Ibp)1, the mouse homologue of epsin (Chen et al., 1998), and Ibp2 (Yamabhai, et al., 1998). Here, we demonstrate that Ibp2 binds to clathrin and AP2, suggesting that it may be involved in the localization of intersectin at clathrin-coated pits. In fact, clathrin, AP2, and dynamin were found to co-immunoprecipitate with intersectin. Taken together, these data suggest that both the short and long alternatively spliced forms of intersectin are components of the molecular machinery for endocytosis in non-neuronal cells and neurons.

EXPERIMENTAL PROCEDURES

Antibodies--To generate an intersectin antibody, a full-length intersectin cDNA clone from *Xenopus laevis* (Yamabhai et al., 1998) was used as a template in PCR reactions with Pfu DNA polymerase (Stratagene) with the forward primer 5'-CTGTGCGGATCCAATTTGGACATCTGGGCCATAACG and the reverse primer 5'-CTGTGGAATTCAAGATGGGGGAATATACTCTGGAGG. The PCR product, encoding amino acids 11 to 306 of intersectin, including both EH domains (Yamabhai et al., 1998), was cloned into the BamHI-EcoRI sites of pGEX-2T (Amersham Pharmacia Biotech) and pTrcHisA (Invitrogen). The resulting GST (GST-EHa/b) and His_6 (His_6-EHa/b) fusion proteins were expressed and purified as described (David et al., 1996; McPherson et al., 1994a). Two rabbits (2173, 2174) were injected with approximately 50 μg of GST-EHa/b using Titermax adjuvant (CytRx Corp.) with standard protocols. Antibody production was monitored by Western blots against His_6-EHa/b, and antibodies were affinity purified using strips of polyvinylidene difluoride membrane containing the same fusion protein (Sharp et al., 1993). For preabsorption experiments, 2173 serum was diluted in phosphate-buffered saline (20 mM NaH_2PO_4, 0.9% NaCl, pH 7.4) containing 1% bovine serum albumin and incubated overnight at 4°C with either GST or His_6-EHa/b immobilized on polyvinylidene difluoride membranes. A polyclonal antibody against clathrin (Simpson et al., 1996) and monoclonal antibody AC1-M11 against α-adaptin (Robinson, 1987) were generous gifts of Dr. Margaret Robinson (Cambridge University). A monoclonal antibody against clathrin was produced from the hybridoma X22 (ATCC) and was also kindly supplied by Dr. Mark McNiven (Mayo Clinic). A monoclonal antibody against dynamin (HUDY-1) was purchased from Upstate Biotechnology Inc. (Albany, NY).

Transfections of COS-7 Cells with Intersectin Constructs--A cDNA construct encoding hemaglutinin-tagged full-length intersectin was prepared by PCR using an intersectin cDNA template with Pfu DNA polymerase and the following primers (F stands for forward and R stands for reverse):

intersectinFa (5'-GATCCATGGCTCAGTTTGGAACTCCG);
intersectinFb (5'-CATGGCTCAGTTTGGAACTCCG);
intersectinRa (5'-GATCCCAGTTGTTAAAGCTGTAGGGT); and
intersectinRb (5'-CCAGTTGTTAAAGCTGTAGGGT). One PCR reaction was performed with the primer pair intersectinFa with intersectinRb, and a second reaction used intersectinFb with intersectinRa. The two reaction products were mixed, and the double-stranded DNA was denatured and allowed to re-anneal; one-fourth of the re-annealed molecules contain sticky ends that are compatible for ligation to a BamHI cleaved vector. The re-annealed mixtures were ligated into the pCGN-Hygro mammalian expression vector which carries a BamHI site in-frame with the epitope tag. Constructs encoding the EH domains (amino acids 11-306) were prepared in an identical manner using the following primers:
EHFa (5'-GATCCAATTTGGACATCTGGGCCATA);
EHFb (5'-CAATTTGGACATCTGGGCCATA);
EHRa (5'-GATCCAGATGGGGGAATATACTCTGG); and
EHRb (5'-CAGATGGGGGAATATACTCTGG). The constructs were verified by sequence analysis and transfected into COS-7 using the calcium phosphate precipitation method (Sambrook et al., 1989).

Preparation and Examination of Plasma Membranes--COS-7 cells were plated into tissue culture wells containing 22-mm coverslips previously coated with poly-L-lysine. To coat the coverslips, poly-L-lysine stock (Sigma) was diluted 1:100 in 0.15 M boric acid, pH 8.4, filter sterilized, and incubated over coverslips for 30 min. After incubation, coverslips were washed extensively with sterile water and once with COS-7 cell medium. Plasma membranes were prepared from the cells (either transfected or nontransfected) essentially as described (Shpetner et al., 1996). Briefly, the cells were maintained for 1 h at 4°C and were then washed and sonicated in 12 ml of buffer A (25 mM HEPES, pH 7.0, containing 25 mM KCl, 2.5 mM magnesium acetate, and 0.2 mM dithiothreitol) for 2 s using a 1/2-inch tapered horn 1 cm above the coverslip at setting 5.0 (Sonics Materials Vibra Cell). The cells were then washed three times in buffer A and fixed in buffer B (20 mM HEPES, pH 6.8, 100 mM KCl, 5 mM $MgCl_2$, 3 mM

EGTA, and 3% paraformaldehyde). Following fixation, coverslips were washed in phosphate-buffered saline before being processed for immunofluorescence analysis.

Analysis of Tissue and Subcellular Distribution--Postnuclear supernatants from different tissues and cell lines were prepared and processed for Western blots as described (Ramjaun et al., 1997). Rat medial septal neurons were prepared as described (Mazzonia and Kenigsberg, 1996) as were glial cells from rat hippocampus (Gosslin and Banker, 1990). To examine the subcellular distribution of intersectin in neurons, dissociated cell cultures were prepared from the CA3 and dentate regions of hippocampi from P1 rat pups as described (Baranes et al., 1998). Cells were maintained in culture from 1-7 days before processing for immunofluorescence analysis. Images were captured with a Zeiss scanning laser confocal microscope 410.

Binding Assays--A GST fusion protein, encoding the C-terminal 511 amino acids of Ibp2 (GST-Ibp2), was prepared by PCR from an Ibp2 cDNA template (Yamabhai et al., 1998) using Pfu DNA polymerase (Stratagene) with the forward primer 5'-CTGTGCGGATCCTCAAGCAAGGCACTGACACTG and the reverse primer 5'-CTGATGAATTCCTCACTAGAGAAGGAAAGGGTT. The resulting PCR product was subcloned into the BamHI-EcoRI sites of pGEX-2T (Amersham Pharmacia Biotech). GST-Ibp2 was expressed and purified as described previously (McPherson et al., 1994a), except that the plasmid was transformed into BL21 Escherichia coli, and the cells were grown and induced at 30°C. GST-Ibp2 was used in binding assays with a Triton X-100 soluble rat brain extract as described (Ramjaun et al., 1997). For immunoprecipitation analysis, rat brain synaptosomes were resuspended to a protein concentration of approximately 10 mg/ml in buffer C (20 mM HEPES, pH 7.4, 128 mM NaCl, 3 mM KCl, 1.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 11 mM glucose) and incubated for 1 h at 37°C. Synaptosomal membranes were then pelleted, resuspended in buffer D (20 mM HEPES, pH 7.4, 50 mM $NaPO_4$, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 50 mM Na-pyrophosphate, 50 mM Na-fluoride, 10 µg/ml PMSF), and disrupted by sonication. The samples were spun in a microcentrifuge at maximal velocity, and the supernatants were incubated with anti-intersectin antisera

(2173, 2174), pre-coupled to protein A-Sepharose. Following an overnight incubation at 4 °C, the beads were washed in buffer D, and proteins were recovered with SDS gel sample buffer.

Binding of Biotinylated Ibp2 and MP90 to Clathrin Terminal Domain--cDNA clones encoding Ibp2 (Yamabhai et al., 1998), MP90 (Stukenberg et al., 1997) (generous gift of Dr. Todd Stukenberg, Harvard Medical School), and Luciferase (Promega Co.) were *in vitro* transcribed and translated in the presence of biotinylated lysine tRNA using the Transend™ nonradioactive translation detection system (Promega Co.) according to the manufacturer instructions. The biotinylated proteins were diluted in phosphate-buffered saline and incubated overnight at 4 °C with GST fusion proteins, encoding the amino-terminal 579 amino acids of the clathrin heavy chain (Goodman Jr. et al., 1997) (generous gift of James Keen, Kimmel Cancer Institute) or the amino acids NPFL (Yamabhai et al., 1998), pre-bound to glutathione-Sepharose. The beads were subsequently pelleted by microcentrifugation and washed three times in 1 ml of phosphate-buffered saline, and the bound proteins were eluted with SDS gel sample buffer and prepared for Western blot analysis using streptavidin conjugated to alkaline phosphatase.

RESULTS

Intersectin Localizes to Clathrin-coated Pits--We have recently identified and cloned *X. laevis* intersectin, a novel protein composed of multiple EH and SH3 domains, protein modules implicated in endocytosis (Yamabhai et al., 1998). To explore the subcellular localization of mammalian intersectin, we raised two rabbit polyclonal antisera (2173, 2174) against the tandem EH domains of the frog protein because the primary structures of this region are highly related (*i.e.* 87% identical). We then used the 2173 antibody for immunofluorescence analysis of plasma membranes prepared from COS-7 cells using a procedure that leads to membranes rich in clathrin-coated pits (Moore et al., 1987; Shpetner et al., 1996; van Delft et al., 1997). In double-labeling experiments, antiserum 2173 yielded a bright, punctate staining pattern that was virtually identical to the pattern seen for clathrin (Fig. 2.1A). The specificity of the staining was confirmed by pre-absorption experiments. Both untreated and GST pre-absorbed antisera yielded bright, punctate staining, whereas staining was virtually undetectable using antiserum pre-absorbed against the EH domains (Fig. 2.1B). Affinity purified antibodies demonstrated an identical immunofluorescence pattern as untreated antiserum (data not shown).

To begin to explore the mechanism of intersectin targeting, we performed immunofluorescence analysis on plasma membranes prepared from COS-7 cells transfected with recombinant full-length intersectin. At the level of exposure used for Fig. 2.2A (~300 msec integration using a Sony CCD video camera), membranes from transfected cells, which represent approximately 10% of the clathrin-positive membranes, were strongly positive with antibody 2173. Endogenous intersectin, which is weakly detectable in the nontransfected cells at this level of exposure (Fig. 2.2A), is readily detectable upon longer exposures (~3 s integration; see Fig. 2.1). Interestingly, a similar staining pattern was observed in COS-7 cells transfected with a construct encoding the two EH domains of intersectin (amino acids 11 to 306) (Fig. 2.2A). By Western blot, both constructs were seen to be highly overexpressed (data not shown). At higher magnification, double-labeling immunofluorescence revealed nearly complete co-localization of both full-length intersectin and its EH domains with clathrin-coated

pits on the plasma membrane (Fig. 2.2B). Taken together, the data in Figs. 2.1 and 2.2 suggest that intersectin is localized to clathrin-coated pits, possibly through its EH domains, although a role for the SH3 domains in membrane targeting cannot be ruled out.

Expression of Intersectin Isoforms in Mammalian Cells--To further explore the expression of intersectin, we used affinity purified antibody 2173 for Western blots of a crude rat brain extract. Two bands of immunoreactivity were evident (Fig. 2.3A), 190 kDa (intersectin-l) and 145 kDa (intersectin-s) in size. The molecular masses of these bands are in good agreement with the predicted molecular masses of the long (195, 576 Da) and short (137, 711 Da) isoforms of human intersectin, alternatively spliced variants identified through genomic analysis of chromosome 21 (Guipponi et al., 1998). To confirm the identity of these bands, we performed *in vitro* binding assays using a fusion protein encoding a region of the intersectin-binding protein, Ibp2, which contains the NPF repeats that interact with the EH domains of intersectin (Yamabhai et al., 1998). Both the 190 and the 145 kDa species bind to the fusion protein, suggesting that they are intersectin-l and intersectin-s, respectively (Fig. 2.3B). This result is further strengthened by the observation that both species also bind specifically to a GST fusion protein encoding the peptide sequence NPFL (data not shown). Western blot analysis of extracts from a wide variety of tissues and cell lines demonstrates that intersectin-l is expressed predominately in the brain, whereas intersectin-s is ubiquitously expressed (Fig. 2.4). Interestingly, intersectin-l is expressed in neurons and is not detected in glia (Fig. 2.3A). Intersectin-s is expressed in glia, and the low levels of intersectin-s seen in neuronal cultures is likely because of glia contamination of the neurons (Fig. 2.3A) (Mazzonia and Kenigsberg, 1996). Also of interest, the pheochromocytoma cell line, PC12, expresses intersectin-s (Fig. 2.4), whereas intersectin-l was only weakly detectable, even after NGF-induced differentiation (data not shown).

Localization of Intersectin in Neurons--To explore the subcellular localization of intersectin, we performed immunofluorescence analysis of hippocampal neurons in

culture using confocal microscopy (Baranes et al., 1996). Intersectin immunoreactivity appeared as small spots, 0.5-1.0 μm in diameter, which were located within cell bodies in Golgi-like structures, at the plasma membrane and throughout the length of axons and dendrites (Fig. 2.5, *upper left* and *bottom panels*). These intersectin punctae corresponded to regions enriched in clathrin (Fig. 2.5), although not all intersectin positive punctae were positive for clathrin (note the *red* staining in the *top right panel*).

Intersectin Interacts Indirectly with Components of Clathrin-coated Pits--Through its EH domains, intersectin binds through the NPF tripeptide with Ibp1 and Ibp2 (Yamabhai et al., 1998). Interestingly, Ibp1 and Ibp2 also contain the peptide sequences LVDLD and LVNLD, respectively, which resemble clathrin-binding motifs (Dell'Angelica et al., 1998; Krupnick et al., 1997; McPherson, 1999; Ramjaun and McPherson, 1998). To determine whether the Ibps interact with clathrin, we generated a GST fusion protein encoding the C-terminal 511 amino acids of Ibp2 (GST-Ibp2) that includes the three NPF repeats and the LVNLD sequence (despite repeated attempts, we were unable to generate soluble GST fusion proteins encoding Ibp1). When incubated with rat brain extracts, GST-Ibp2 bound specifically to clathrin (Fig. 2.6A). Consistent with the previous observation that epsin binds to AP2 (Chen et al., 1998), Ibp2 also bound to AP2, as assessed by Western blots with an antibody to the αa- and αc-isoforms of adaptin (Fig. 2.6A).

Several clathrin-binding proteins, including arrestin3 (Goodman Jr. et al., 1997), interact with clathrin through the N terminus of its heavy chain, a region known as the terminal domain (Kirchhausen and Harrison, 1981). To determine whether the terminal domain is responsible for the binding of the Ibps, we tested the binding of Ibp2 and MP90, a related mitotic phosphoprotein (Stukenberg et al., 1997), prepared *in vitro* by coupled transcription and translation, to a GST fusion protein encoding the amino-terminal 579 amino acids of the clathrin heavy chain. Both Ibp2 and MP90 bound to the N-terminal fusion protein, whereas neither protein bound to a control GST fusion to the peptide NPFL (Fig. 2.6B). Further, the negative control protein, luciferase, bound to neither GST fusion protein (Fig. 2.6B). Thus, both Ibp2 and MP90 bind to the terminal domain of clathrin.

To further characterize the interaction of intersectin with clathrin-coated pit components, we performed immunoprecipitation analysis from rat brain synaptosomes using antibodies against intersectin. Anti-intersectin antisera from two different rabbits (2173 and 2174) both immunoprecipitated intersectin-l and intersectin-s (Fig. 2.7). Both clathrin and AP2 were co-immunoprecipitated with intersectin as assessed with specific antibodies (Fig. 2.7). Further, dynamin was also observed to co-immunoprecipitate with the intersectin antibodies, but synaptojanin did not under these conditions (Fig. 2.7). Thus, intersectin interacts *in vivo* with distinct components of the endocytic machinery.

DISCUSSION

We have recently identified and cloned *X. laevis* intersectin, a protein containing two EH and five SH3 domains (Yamabhai et al., 1998). Intersectin is related to a *Drosophila* protein, Dap160 (Roos and Kelly, 1998), and is highly similar (81% identical) to human intersectin that was cloned based on genomic analysis of chromosome 21 (Guipponi et al., 1998). Of interest, human intersectin undergoes alternative splicing in the stop codon, leading to a short form with the same domain structure as the frog protein and a long form with a C-terminal extension containing DH, PH, and C2 domains (Guipponi et al., 1998).

The presence of EH and SH3 domains in proteins involved in endocytosis (McPherson, 1999; Wong et al., 1995) provided the first suggestion that intersectin may function in the endocytic process. Support for this idea then came from the observation that frog intersectin and fruit fly Dap160 display SH3 domain-dependent binding to dynamin (Roos and Kelly, 1998; Yamabhai et al., 1998), an enzyme that functions in the formation of clathrin-coated vesicles (Sweitzer and Hinshaw, 1998a; Takei et al., 1998). To begin to study the role of intersectin in mammalian cells, we generated a polyclonal antibody against the EH domains of frog intersectin. Analysis of intact cells in culture revealed a punctate, surface staining for intersectin that was reminiscent of clathrin-coated pits (data not shown). To explore this further, we prepared plasma membranes from COS-7 cells using a procedure that leads to plasma membranes rich in clathrin-coated pits (Moore et al., 1987; Shpetner et al., 1996; van Delft et al., 1997). Immunostaining of these preparations revealed that intersectin is highly co-localized with clathrin. As COS-7 cells only express intersectin-s, protein domains contained within this splice variant (*i.e.* EH and SH3 domains, coiled-coil region) must be sufficient to direct targeting of the molecule to clathrin-coated pits. To begin to address this, we transfected COS-7 cells with full-length recombinant intersectin as well as with a construct encoding just the tandem EH domains. Both constructs localize to clathrin-coated pits, suggesting that the EH domains are sufficient to mediate the subcellular localization of intersectin-s. However, we cannot yet rule out a role for the SH3 domains in this process. For example, SH3 domains can function in directing protein

localization (Bar-Sagi et al., 1993; McPherson, 1999), and intersectin-s could potentially target to clathrin-coated pits through SH3 domain-dependent interactions with dynamin (Yamabhai et al., 1998).

Western blot analysis reveals that intersectin-s is ubiquitously expressed, whereas intersectin-l is expressed predominately in brain. This agrees with Northern blots demonstrating two transcripts for human intersectin, a 5.3-kilobase ubiquitously expressed transcript and an approximately 15-kilobase brain-specific transcript (Guipponi et al., 1998). Although we do not currently have an intersectin-l specific antibody, Western blots suggest that intersectin-l is the major variant expressed in neurons. Using confocal microscopy, we determined that intersectin is expressed at the plasma membrane of rat hippocampal neurons in culture where it is co-localized with clathrin. Thus, like intersectin-s, intersectin-l is likely a component of clathrin-coated pits. Intersectin was found over the entire surface of the neuron including the dendrites and axons. Exo-endocytic recycling of synaptotagmin-positive synaptic vesicles occurs throughout all processes of the neuron at this stage in culture (Matteoli et al., 1992). These results raise the interesting possibility that intersectin-l and intersectin-s may function in the endocytosis of synaptic vesicles and general endocytosis, respectively. Many other proteins involved in endocytosis appear to have a similar specialization including synaptojanin, which has a 145-kDa isoform that is highly expressed in neurons, and a 170-kDa isoform, produced by alternative splicing of an exon encoding a stop codon, which is widely distributed (McPherson et al., 1994b; Ramjaun and McPherson, 1996). In fact, AP2, clathrin, dynamin, and amphiphysin I and II are all expressed at higher levels in neuronal *versus* nonneuronal cells, and many of these proteins have neuron-specific isoforms.

The nature of the specialized function of intersectin-l in synaptic vesicle endocytosis is difficult to predict. Intersectin-l contains DH, PH, and C2 domains. DH domains promote guanine-nucleotide exchange on Rho and, as is the case in intersectin-l, the DH domain is followed by a PH domain in all guanine-nucleotide exchange factors (Whitehead et al., 1997). Thus, although not tested, it is possible that intersectin-l has guanine-nucleotide exchange factor activity that may control Rho-dependent processes within neurons (Hall, 1998). PH domains mediate interactions with inositol

phospholipids (Harlan et al., 1995) and C2 domains can mediate Ca^{2+}-dependent phospholipid binding (Rizo and Südhof, 1998). Phospholipid metabolism, and in particular, the metabolism of inositol phospholipids, has been strongly implicated in the endocytosis of synaptic vesicles (De Camilli et al., 1996). Further, both PH and C2 domains are found in a wide variety of proteins that function in synaptic vesicle endocytosis including dynamin and synaptotagmin. The exact functional role in neurons of the intersectin-l-specific C terminus remains to be explored.

The identification of intersectin-s and intersectin-l as components of clathrin-coated pits is supported by the biochemical characterization of intersectin protein interactions. We have previously demonstrated that through its EH domains, intersectin interacts with mouse Ibp1 and Ibp2 (Yamabhai et al., 1998). We now demonstrate that Ibp2 binds to clathrin and AP2 *in vitro*. The importance of the interaction of Ibp2 with clathrin/AP2 and intersectin is underscored by the observation that both clathrin and AP2 co-immunoprecipitate with intersectin from rat brain synaptosomes. As the isolated EH domains of intersectin appear to be sufficient to target the protein to clathrin-coated pits, it is tempting to speculate that Ibp2, and possibly Ibp1, mediate this subcellular localization. Indeed, Chen *et al.* have suggested that epsin, the rat homologue of mouse Ibp1, may function as a linker between the EH domains of Eps15 and clathrin-coated pit components (Chen et al., 1998). The potential targeting role of the Ibps/epsins are reminiscent of the functional role of the amphiphysins in synaptic vesicle endocytosis as these proteins, through interactions with both AP2 (David et al., 1996; Wang et al., 1995a) and clathrin (McMahon et al., 1997; Ramjaun and McPherson, 1998; Ramjaun et al., 1997), appear to target synaptojanin and dynamin to endocytic sites (Slepnev et al., 1998). The Ibps/epsins appear to represent a growing protein family as the sequence of the rat homologue of mouse Ibp2 (epsin 2) has been recently deposited in GenBank™ (accession number AAC79495) by H. Chen and P. De Camilli. The same is true for intersectin, as two genes have been observed for mouse (Sengar et al., 1999).

Taken together, the data presented in this manuscript suggest a role for intersectin in endocytosis. The protein is localized to clathrin-coated pits in the different cell types we have examined, where through SH3 domains, it could regulate the function of dynamin.

Owen *et al.* have recently demonstrated that the SH3 domain of amphiphysin II prevents dynamin self-assembly into rings, thereby blocking dynamin function (Owen et al., 1998). In our own hands, the SH3 domains of intersectin inhibit transferrin receptor endocytosis in a cell-permeabilized assay (Simpson et al., 1999). Thus, it is possible that intersectin functions by binding to dynamin at clathrin-coated pits and inhibiting its access to constricted vesicular necks, its assembly into rings, or its enzymatic activity. In neurons, intersectin-l may have additional roles through promotion of guanine-nucleotide exchange on Rho. Regardless of the precise mechanisms of intersectin function, the data reported here implicate the intersectin isoforms in clathrin-mediated endocytosis, both in neurons and in nonneuronal cells.

ACKNOWLEDGEMENTS

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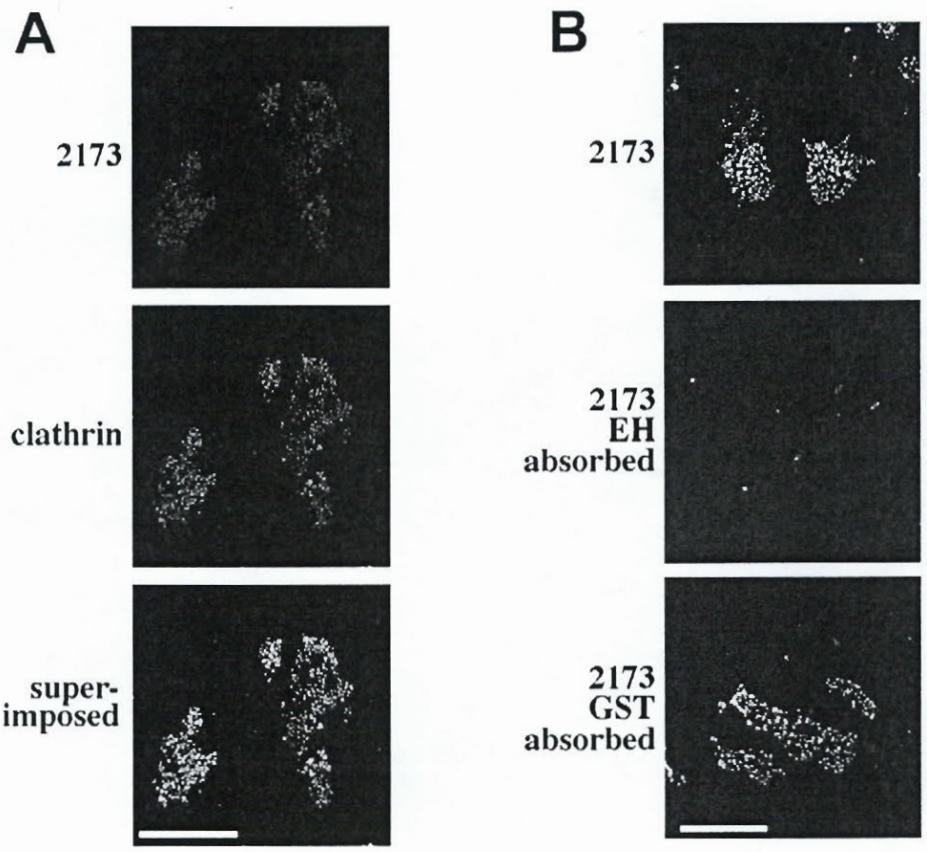

Figure 2.1 Intersectin-s localizes to clathrin-coated pits. *A*, isolated plasma membranes, prepared by sonication of COS-7 cell monolayers, were stained with an anti-clathrin mouse monoclonal antibody (*clathrin*), revealing a punctate fluorescence (Shpetner et al., 1996; van Delft et al., 1997). Endogenous COS-7 cell intersectin-s displays a similar pattern as revealed with antiserum 2173. Superimposition of the images reveals a high degree of co-localization. *B*, COS-7 cell plasma membranes were stained with antiserum 2173, 2173 serum pre-absorbed against the intersectin EH domains, or 2173 serum pre-absorbed against GST. All images were captured with a Sony CCD video camera with a time integration of ~3 s. The *scale bar* represents 20 μm.

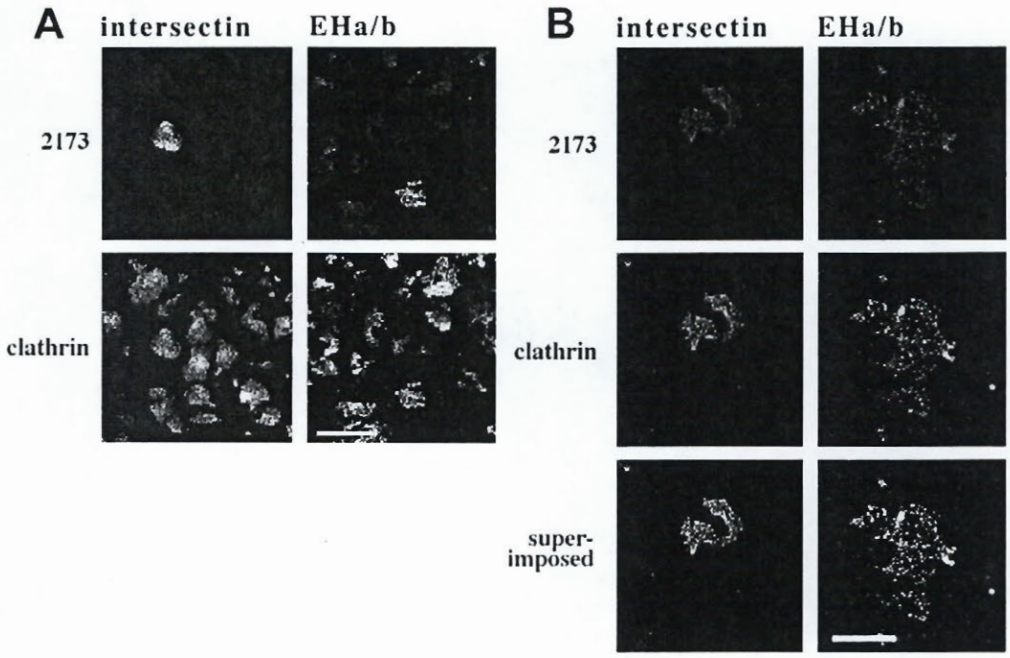

Figure 2.2 Recombinant intersectin and intersectin EH domains target to clathrin-coated pits. *A*, plasma membranes, isolated from COS-7 cell monolayers overexpressing recombinant intersectin or its two EH domains (*EHa/b*), were processed for immunocytochemistry with rabbit polyclonal antibody 2173 and a mouse monoclonal antibody against clathrin. Both full-length intersectin and the intersectin EH domains demonstrate a similar staining pattern. Only a fraction of the membranes that are positive for endogenous clathrin are positive for transfected full-length intersectin or its EH domains. The images were captured with a Sony CCD video camera with a time integration of ~300 ms. The *scale bar* represents 40 μm. *B*, at higher magnification, double labeling immunofluorescence of plasma membranes from transfected cells demonstrates a high degree of co-localization between EH domain-containing constructs and clathrin. The *scale bar* represents 20 μm.

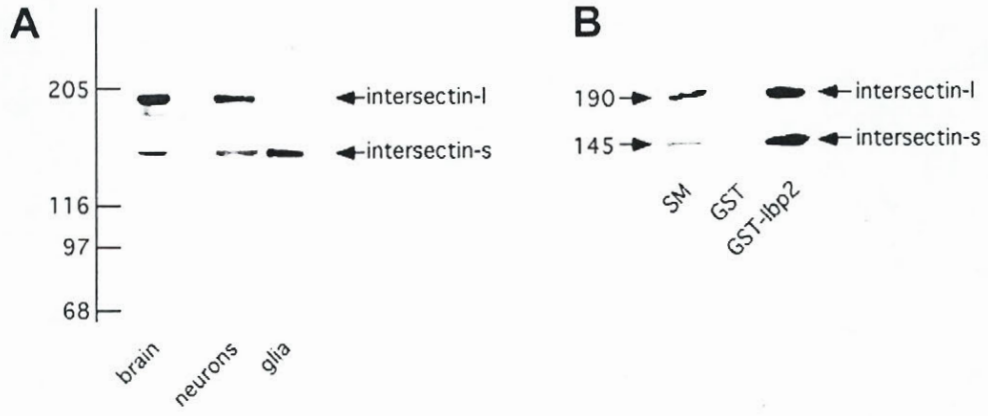

Figure 2.3 Identification of long and short intersectin proteins. *A*, a rat brain extract, along with extracts from purified glial cultures and enriched neuronal cultures, were processed for Western blots with affinity purified antibody 2173, revealing intersectin-l (~190 kDa) and intersectin-s (~145 kDa). The weak band of ~180 kDa seen in the brain extracts is presumed to be a proteolytic fragment of intersectin-l. *B*, a GST fusion protein encoding the C-terminal 511 amino acids of Ibp2 (*GST-Ibp2*), encoding the EH domain-binding sites, and GST alone (*GST*), were incubated with glutathione-Sepharose. The washed beads were then incubated with soluble extracts from rat brain (*SM*), and proteins specifically bound to the beads were processed for Western blots with antibody 2173.

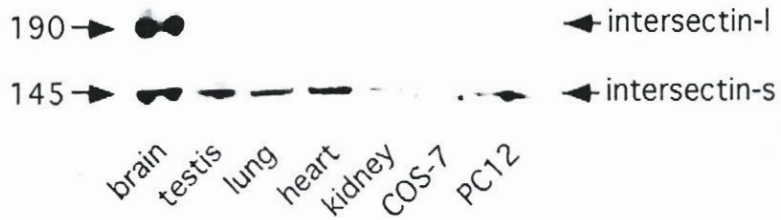

Figure 2.4 Tissue distribution of the intersectin isoforms. Crude postnuclear supernatants were prepared from a variety of tissues and cell lines and processed for Western blots with antibody 2173.

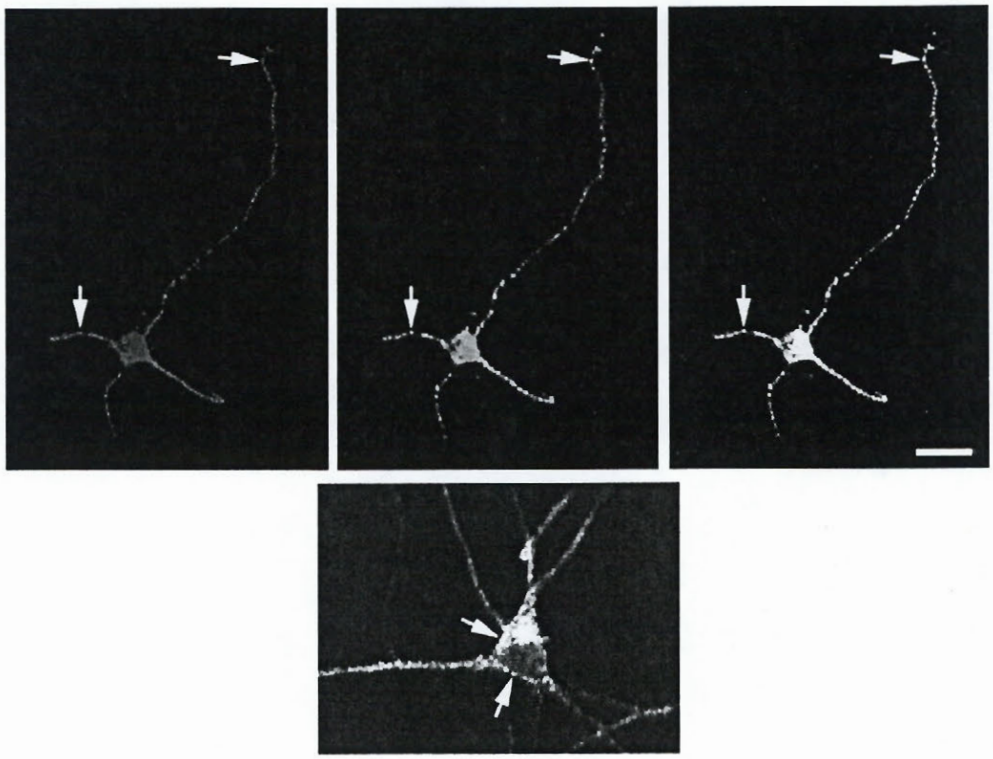

Figure 2.5 Subcellular distribution of intersectin in hippocampal neurons. A confocal section of intersectin staining in a 1-day old hippocampal neuron is demonstrated in *red*. The distribution of clathrin in the same field as the intersectin staining is shown in *green*. Superimposition of the images (*upper right panel*) demonstrates a high degree of co-localization (*yellow*) of intersectin with clathrin (two examples are indicated by the *arrows*). The distribution of intersectin immunoreactivity in a CA3 pyramidal neuron cultured for 7 days reveals intersectin in Golgi-like structures as well as at the plasma membrane (*arrows, bottom panel*). *Scale bar* equals 10 μm.

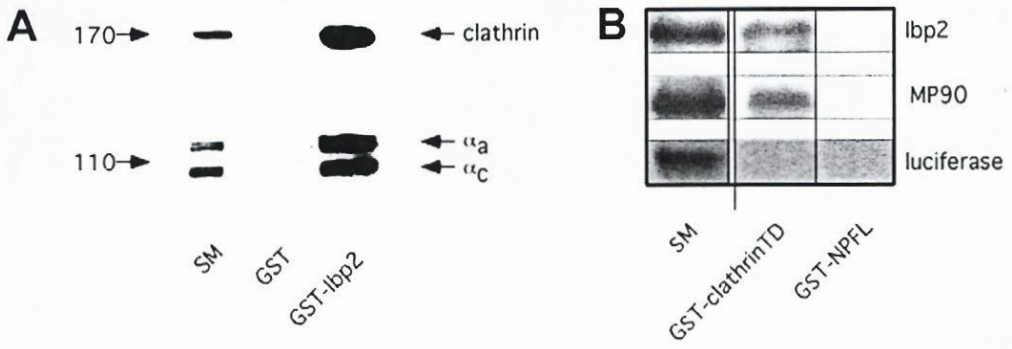

Figure 2.6 Ibp2 binding to clathrin and AP2 *in vitro*. *A*, a GST fusion protein encoding the C-terminal 511 amino acids of Ibp2 (*GST-Ibp2*), including potential EH- and clathrin-binding domains, and GST alone (*GST*) were incubated with glutathione-Sepharose. The washed beads were then incubated with soluble extracts from rat brain (*SM*), and proteins specifically bound to the beads were resolved by SDS-polyacrylamide gel electrophoresis. Western blots were reacted with an antibody against the clathrin heavy chain or the α_a and α_c subunits of AP2. *B*, to determine whether the terminal domain of clathrin was involved in Ibp2 binding, biotinylated forms of Ibp2, MP90, and luciferase were prepared *in vitro* by coupled transcription and translation and incubated with agarose beads prebound to two different GST fusion proteins, encoding either the N-terminal 579 amino acids of the clathrin heavy chain (*GST-clathrinTD*) or the peptide NPFL (*GST-NPFL*). Proteins specifically bound to the beads were eluted with SDS-polyacrylamide gel electrophoresis sample buffer and processed for Western blots, along with an aliquot of the biotinylated proteins (*SM*), using streptavidin-alkaline phosphatase.

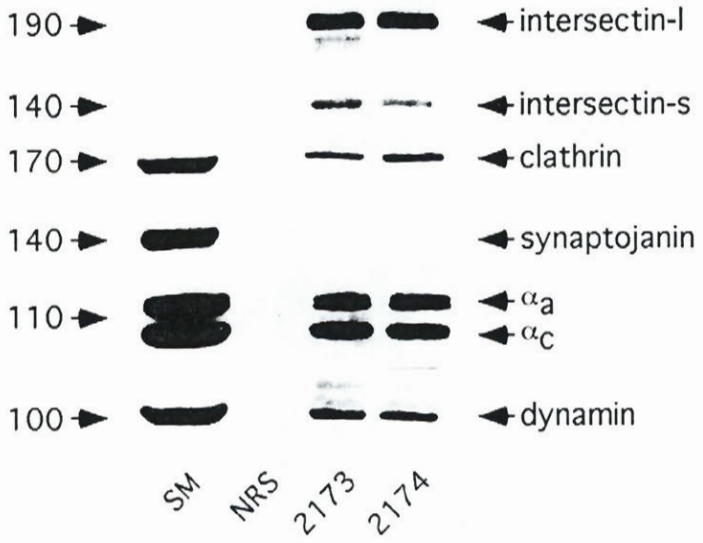

Figure 2.7 Co-immunoprecipitation of clathrin, AP2, and dynamin with intersectin. Aliquots of antisera 2173 and 2174, as well as pre-immune 2173 antisera (*NRS*), were pre-coupled to protein A-Sepharose beads for 1 h at 4 °C. Pre-coupled beads were washed and incubated overnight at 4 °C with 1 mg of a soluble extract prepared from rat brain synaptosomes (Ramjaun et al., 1997) and extensively washed the next day. Twenty µg samples of starting material (*SM*) and material bound to the beads were eluted and processed for Western blot analysis with polyclonal antibodies against intersectin (2173) and synaptojanin, and monoclonal antibodies against dynamin, clathrin, and the α_a and α_c subunits of AP2. Immune complexes were detected on separate filter strips by enhanced chemiluminescence (NEN Life Sciences). The antigens and their approximate molecular masses (kDa) are denoted with *arrows* on the *right* and *left sides* of the figure, respectively.

PREFACE TO CHAPTER 3: Characterizing partners of intersectin amino-terminal EH domains

The characterization of intersectin-s and intersectin-l as components of clathrin-coated pits is supported by the biochemical analyses of intersectin protein interactions. Through its EH domains, intersectin interacts with mouse proteins Ibp1 and Ibp2 (Yamabhai et al., 1998). Rat homologues of mouse Ibp1 and Ibp2, referred to as epsin 1 and epsin 2, respectively (for Eps15-interacting proteins) (Chen et al., 1998; Rosenthal et al., 1999) share significant homology with *Xenopus* mitotic phosphoprotein MP90 (Stukenberg et al., 1997), multiple human (Morinaka et al., 1999; Nakashima et al., 1999), and yeast proteins (Ent1p/2p) (Duncan et al., 2003; Wendland et al., 1999). These proteins represent a family of proteins collectively referred to as epsins (for review see De Camilli et al., 2002; Wendland, 2002). Epsin proteins are required for clathrin-mediated endocytosis to occur in mammalian and yeast cells (Chen et al., 1998; Rosenthal et al., 1999; Wendland et al., 1999). The amino-terminus of epsin proteins contains an evolutionarily conserved module called the ENTH (epsin N-terminal homology) domain (Kay et al., 1999). These modules have been identified in a number of other proteins implicated in clathrin-mediated endocytosis (Kay et al., 1999; Wendland, 2002) and can bind lipids to

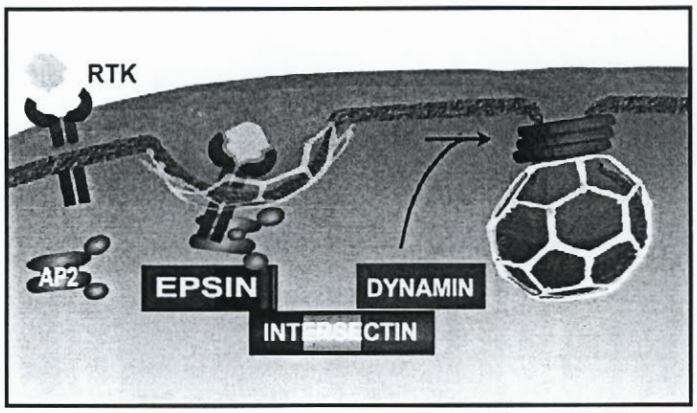

Figure 3.0 Schematic diagram of intersectin function as a scaffolding molecule at sites of endocytosis.

regulate the invagination of clathrin lattices on plasma membrane (Ford et al., 2002; Ford et al., 2001; Itoh et al., 2001).

In Chapter 2, we demonstrated that mouse epsin 2 binds to clathrin and AP2 via ligand binding motifs located within its carboxy terminus (Hussain et al., 1999). The isolated EH domains of intersectin are sufficient to target the protein to clathrin-coated pits (Hussain et al., 1999). Since epsins interact with intersectin EH domains, it is tempting to speculate that epsins mediate intersectin localization to sites of clathrin-mediated endocytosis, and may thereby play a significant role in regulating intersectin function(s).

Since identifying that intersectin forms an endocytic complex via its interactions with epsin, we wanted to analyse further the function of this critical partner. Studies of yeast epsins, Ent1p and Ent2p, reveal that their respective ENTH domains are essential for normal endocytic function and actin cytoskeleton structure (Wendland et al., 1999). Intrigued by the identification of this evolutionarily conserved protein module, we next investigated the possibility that the ENTH domain functions as a protein-protein interaction interface, mediating binding with other endocytic proteins to form complexes of critical function in endocytosis.

CHAPTER 3. A ROLE FOR ENTH/ANTH DOMAINS IN TUBULIN BINDING AND NEURITE OUTGROWTH

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Running title: ENTH domains bind tubulin

ABSTRACT

The epsin N-terminal homology (ENTH) domain is a protein module of approximately 150 amino acids found at the N-terminus of a variety of proteins identified in yeast, plants, nematode, frog, and mammals. ENTH domains are comprised of multiple alpha-helices folded upon each other to form a compact globular structure that has been implicated in interactions with lipids and proteins. In characterizing this evolutionarily conserved domain, we isolated and identified tubulin as a novel ENTH domain-binding partner. The interaction, which is direct and has a dissociation constant of approximately 1μM, was observed with ENTH domains of proteins present in various species. This protein-protein interaction is enhanced via phosphorylation of tubulin with casein kinase II, a clathrin-coated vesicle associated enzyme that regulates endocytosis. Additional support for the ENTH domain-tubulin interaction is provided by the observations that tubulin can be co-immunoprecipitated from rat brain extracts with the ENTH domain containing proteins epsin 1 and 2, and punctate epsin staining was observed along the microtubule cytoskeleton of dissociated cortical neurons. Consistent with a role in microtubule processes, the over-expression of AP180 or epsin ENTH domain in PC12 cells was found to stimulate neurite outgrowth. These data demonstrate an evolutionarily conserved property of ENTH domains to bind tubulin and microtubules, and suggest a potential functional role for ENTH domain-containing proteins in regulating neurite outgrowth.

INTRODUCTION

The epsin N-terminal homology (ENTH) domain is an evolutionarily conserved globular module of approximately 150 amino acids that occurs at the amino-terminus of a variety of proteins (Chen et al., 1998; Kay et al., 1999). Originally noted in the plant protein, Af10 (Jones et al., 1998), the ENTH domain has subsequently been characterized in epsins (Chen et al., 1998), enthoprotin (Wasiak et al., 2002) (also termed EpsinR (Hirst et al., 2002; Mills et al., 2003) or Clint (Kalthoff et al., 2002)), and in the yeast proteins Ent1p/Ent2p (Wendland and Emr, 1998; Wendland et al., 1999) and Ent3p/Ent5p (Duncan et al., 2003). Adaptor protein 180 (AP180), clathrin assembly lymphoid myeloid leukemia protein (CALM), Huntingtin-interacting proteins 1 (HIP1) and 12 (HIP12), and their yeast homologues yAP180 and Sla2p contain a structure that is so similar in primary structure to the epsin ENTH domain they were initially denoted ENTH-bearing proteins (Ford et al., 2001; Hyman et al., 2000; Itoh et al., 2001). However, recent studies have refined our understanding of this structure such that ENTH-like domains from these proteins have been re-designated ANTH domain-containing proteins in accordance with their higher structural similarity to AP180 rather than epsin (Ford et al., 2002). In effort to simplify the nomenclature applied in this study, we refer to these homologous structures as E/ANTH domains when collectively discussing proteins bearing either domain, but maintain the ENTH or ANTH nomenclature when discussing individual proteins.

A common feature among many E/ANTH domain-bearing proteins is that their C-termini contain peptide motifs indicative of a functional role in clathrin-mediated membrane budding including clathrin and clathrin adaptor protein binding elements (De Camilli et al., 2002; Kay et al., 1999). In addition to their interactions with multiple endocytic components, epsins, AP180, and HIP1/12 are localized to clathrin-coated pits and over-expression of truncated fragments of epsins or HIP1/12 mislocalize components of the clathrin-coat and inhibit receptor-mediated endocytosis (Chen et al., 1998; Chen et al., 1999; Engqvist-Goldstein et al., 2001; Legendre-Guillemin et al., 2002; Metzler et al., 2001; Mishra et al., 2001; Rosenthal et al., 1999; Ye and Lafer, 1995a). While all of the currently characterized E/ANTH containing-proteins co-

localize with clathrin and drive clathrin-coat assembly (Drake et al., 2000; Legendre-Guillemin et al., 2002; Lindner and Ungewickell, 1992; Metzler et al., 2001; Mishra et al., 2001; Rosenthal et al., 1999; Wasiak et al., 2002; Ye and Lafer, 1995b), enthoprotin is unique in this group since it is predominantly localized to the trans-Golgi network (TGN), rather than the plasma membrane, and as such likely regulates clathrin-mediated budding events specifically occurring along the TGN and endosomal pathway (Hirst et al., 2002; Kalthoff et al., 2002; Mills et al., 2003; Wasiak et al., 2002).

Recent studies have demonstrated that the E/ANTH domains of epsin and AP180 can mediate lipid binding, particularly to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2), and that this interaction is required for efficient clathrin-mediated endocytosis in COS-7 cells (Ford et al., 2001; Itoh et al., 2001). Furthermore, upon PtdIns(4,5)P_2 binding epsin's ENTH domain was shown to drive the curvature of clathrin-coated pits on the plasma membrane, while AP180's ANTH domain appears to be more involved in regulating the diameter of emergent vesicles (Ford et al., 2002). This functional difference in ENTH and ANTH domains is likely imparted by the structural variances between them. In the AP180 ANTH domain, the specific residues involved in lipid binding reside within α-helices α1, α2 and in the loop between them (Ford et al., 2001; Itoh et al., 2001). However, the epsin ENTH domain lipid-ligand pocket is coordinated by residues in α1, the α1-2 loop, α3, α4 and is dependent on the formation and binding of α0, a helix which is not present or generated in the ANTH domain (Ford et al., 2002; Ford et al., 2001; Itoh et al., 2001). The importance of helix α0 for epsin ENTH domain function is demonstrated by the fact that deletion or mutation of residues within this helix is sufficient to abrogate lipid interactions (Itoh et al., 2001) and abolish mammalian epsin ENTH's ability to induce curvature of clathrin-coated membrane lattices (Ford et al., 2002).

E/ANTH domains function in clathrin-mediated endocytosis not only in higher order organisms but also in budding yeast. Genetic experiments demonstrate that the ENTH domains of yeast epsin homologues, Ent1 and Ent2, are essential for normal endocytic function, actin cytoskeletal structure, and expression of at least one ENTH domain is required to maintain viability in Ent1 and Ent2 double mutant strains (Ent1ΔEnt2Δ) (Wendland and Emr, 1998; Wendland et al., 1999). However, the

essential function of yeast epsin ENTH domains in Ent1ΔEnt2Δ mutants appears to be independent of their ability to bind lipids (Aguilar et al. 2002). Specifically, Aguilar and colleagues have identified an ENTH domain mutant that fails to rescue Ent1ΔEnt2Δ cells despite the fact that this mutant continues to bind PtdIns(4,5)P_2 (Aguilar et al. 2002). Based on these findings, the authors suggest that at least in yeast, another critical protein-binding partner(s) must exist and assist ENTH domain functions (Aguilar et al. 2002). In fact, previous studies into the function of this module have revealed that ENTH domains can mediate interaction with proteins: the ENTH domain of mammalian epsin 1 binds weakly to the vesicular coat protein coatomer, and more robustly to human promyelocytic leukemia Zn2+ finger protein (PLZF), a transcriptional repressor (Hyman et al., 2000). Together these studies suggest multiple roles for the ENTH domain as both a lipid and protein-binding module.

As part of our functional analyses of the ENTH domain, we sought to identify novel protein targets for this module. Through affinity selection assays, we identified tubulin as a binding partner for E/ANTH domains isolated from several different species. Moreover, we determined that over-expression of the E/ANTH domains of epsin or AP180 stimulates neurite outgrowth. We propose that interaction with tubulin is an evolutionarily conserved property of E/ANTH domains and that this interaction may play a functional role in regulating neurite outgrowth and neuronal architecture *in vivo*.

EXPERIMENTAL PROCEDURES

Antibodies--Polyclonal antibodies for epsin 1 and 2 were prepared by injection of two rabbits (denoted 2345 and 2346) with 50 μg of GST fusion protein encoding the C-terminal 511 amino acids of mouse epsin 2 (formerly referred to as Intersectin binding protein 2) (Yamabhai et al., 1998) as described (Hussain et al., 1999). Sera were monitored for antibody production by Western blotting, and epsin specific antibodies were affinity purified as described (Hussain et al., 1999). Polyclonal synaptojanin antibody was prepared as described (McPherson et al., 1994b), and a monoclonal antibody (AC1M11) against α-adaptin (Robinson, 1989) was the generous gift of Dr. Margaret Robinson (Cambridge University). Monoclonal α- and βIII-tubulin antibodies were purchased from Sigma.

Generation of recombinant constructs--GST fusion protein constructs encoding the C-terminal pentapeptide sequence TNPFL of epsin 2 (GST-NPFL) and intersectin SH3A (Yamabhai et al., 1998), the ENTH domain of enthoprotin (Wasiak et al., 2002), amphiphysin II SH3 (Ramjaun et al., 1997), intersectin C2, DH-PH, and DH (Hussain et al., 2001) domains were generated as described. GST-ENTH MP90 (amino acids 1-137) (Stukenberg et al., 1997) and GST-ENTH Af10 (amino acids 1-153) (Jones et al., 1998) were amplified by PCR using full-length cDNA templates and cloned between the EcoRI and BamHI sites of pGEX-2TK (Pharmacia). GST-HIP1-ANTH (amino acids 1-125) and GST-HIP12-ANTH (amino acids 1-150) expression constructs were generated by PCR amplification from their respective full-length cDNAs (Chopra et al., 2000; Metzler et al., 2001), followed by cloning into the pGEX-6P vector (Pharmacia Biotech). The 3'-end of each HIP construct was tagged with codons for three glycines followed by six histidine residues and a stop codon. cDNAs encoding wildtype rat epsin 1, the K76A mutant (Itoh et al., 2001) and human AP180 (KIAA0656, identified by the Kazusa DNA Research Institute) (Ishikawa et al., 1998) were used as templates for PCR amplification of all epsin and AP180 constructs. PCR products were digested with EcoRI and XhoI and subcloned into either the corresponding sites of pGEX-4T1 to

generate GST-Epsin ENTH (amino acids 1-146), and GST-AP180 ANTH (amino acids 2-288), or the EcoRI and SalI sites of pEGFP-C2 for generation of GFP-Epsin (amino acids 1-553) GFP-Epsin ΔENTH (amino acids 147-553) and GFP-AP180 ANTH (amino acids 2-288) constructs. A GFP-Epsin ENTH (amino acids 1-208) construct was generated by PCR amplification and cloning into EcoRI and BamHI sites of pEGFP-C2. Rat epsin 1 α-helices α1-2 (amino acids 19-47), α2-3 (amino acids 37-65), α3-4 (amino acids 50-86), and α7 (amino acids 112-129) were generated by PCR amplification and cloning into BamHI and EcoRI sites of pGEX-4T1 or into BglII and EcoRI sites of pEGFP-C1 to generate GST and GFP tagged constructs, respectively. AP180 α7 (amino acids 115-141) was generated by PCR amplification and cloning into BamHI and XhoI sites of pGEX-4T1 or into BglII and SalI sites of pEGFP-C2. All expression constructs were verified by DNA sequencing. GST fusion proteins were over-expressed in bacteria and purified according to the manufacturer's instructions (Pharmacia).

Affinity selection assays--Rat brains were homogenized in buffer A (10 mM HEPES-OH, pH 7.4; 0.83 mM benzamidine; 0.23 mM phenylmethylsulfonylfluoride (PMSF); 0.5 μg/mL aprotinin; 0.5 μg/mL leupeptin) and centrifuged at 750 x g for 5 min. The supernatant was centrifuged a second time at 205,000 x g for 30 min to recover the soluble fraction. After adding Triton X-100 (1% final concentration) to the supernatant, aliquots (2 mg protein) were incubated overnight at 4°C with glutathione-Sepharose bound to ~25 μg of the GST fusion protein constructs. Beads were extensively washed in buffer A with 1% Triton X-100, and bound proteins were resolved by SDS-PAGE and detected by Coomassie Brilliant Blue staining or by Western blotting with monoclonal antibodies to α- or βIII-tubulin. In other experiments purified tubulin, made free of microtubule associated proteins (MAP's) by phosphocellulose chromatography (ICN Biomedicals), was diluted to 200 nM in 4°C buffer B (0.1 M MES, pH 6.4; 1 mM EGTA; 0.5 mM MgCl2; 1 mM GTP; 10% glycerol; 1% Triton X-100; 0.83 mM benzamidine; 0.23 mM PMSF; 0.5 μg/mL aprotinin; 0.5 μg/mL leupeptin) and centrifuged for 1 hr at 4°C at 100,000 x g to remove insoluble aggregates. Aliquots (1 mL) of the supernatant were incubated for 4 hr at 4°C with ~25 μg of GST fusion proteins (pre-coupled to glutathione-Sepharose), the samples were washed in buffer B

and material bound to the beads was resolved by SDS-PAGE and processed for Western blot analysis.

Tubulin binding affinity measurements--Purified tubulin was diluted to 10 μM in binding buffer (80 mM PIPES, pH 6.9; 0.5 mM EGTA; 2 mM MgCl2; 1% Triton X-100; 1 mM GTP; 10% glycerol) and incubated 30 min on ice. The sample was centrifuged for 10 min at 10,000 x g to remove insoluble aggregates. Aliquots of the supernatant, diluted in binding buffer to final concentrations ranging from 0.015 μM to 4 μM, were added to 10 μg of GST fusion protein constructs pre-coupled to glutathione-Sepharose, in final reaction volume of 250 μl for 4 hr at 4°C. The samples were washed in binding buffer, and material bound to the beads was resolved by SDS-PAGE and processed for Western blot analysis along with a standard curve of purified tubulin. Following Western blot with anti-α-tubulin or βIII-tubulin antibodies, the amount of either tubulin retained by the beads was determined by scanning the blots and comparing the optical density to that of the standard curve using NIH Image computer software.

Tubulin phosphorylation--Purified tubulin was diluted to 20 μM in phosphorylation buffer (10 mM PIPES; 100 mM Tris, pH 7.5; 10 mM MgCl2; 2 mM EGTA) and incubated 30 min on ice, followed by centrifugation for 10 min at 10,000 x g to remove insoluble aggregates. A 50 μl aliquot of the supernatant was then incubated with 25 μCi of γ32P-ATP and 14 units of purified casein kinase II (Sigma) at 37°C for 20 min, followed by the addition of 220 μl of ice cold dilution buffer (100 mM PIPES, pH 6.9; 0.25 mM EGTA; 12.5% glycerol; 1.25% Triton X-100). An aliquot of the sample was retained as a starting material and the remaining 250 μl sample was added to ~10 μg GST fusion protein constructs pre-coupled to glutathione-Sepharose beads and incubated 4 hr at 4°C. Samples were washed in binding buffer, and material bound to the beads was processed for SDS-PAGE along with an aliquot of the starting material. Proteins transferred to nitrocellulose membranes were then exposed to film. These

membranes were subsequently blotted with an antibody against α-tubulin, stripped, and re-blotted with an antibody against βIII-tubulin.

ENTH domain binding to taxol stabilized microtubules--The binding of ENTH domain to microtubules was assessed using a microtubule associated protein spin-down assay kit (Cytoskeleton Inc.) according to the manufacturer's instructions. In brief, highly purified tubulin (50 μM) in PEM buffer (80 mM PIPES, pH 6.9; 0.5 mM EGTA; 2 mM MgCl2) was incubated for 20 min at 35°C in the presence of 1 mM GTP and 2% sucrose to allow for microtubule formation. The sample was then diluted 1:10 in PEM buffer with 1 mM GTP and 40 μM taxol. Aliquots of the taxol-stabilized microtubules (20 μl) were added to purified MAP-2, bovine serum albumin (both supplied by the manufacturer) or purified GST fusion protein constructs (pre-spun for 40 min at 100,000 x g to remove insoluble aggregates) each diluted to 30 μl in PEM buffer with 1 mM GTP and 40 μM taxol. Triton X-100 was added to each 50 μl sample to 1% final, and the samples were then incubated for 20 min at room temperature before being loaded onto 50 μl of cushion buffer (PEM, 20% sucrose, 20 μM taxol). The samples were spun at 25°C for 40 min 100,000 x g, the upper 50 μl sample was retained, the cushion was carefully removed, and the pellet was resuspended in SDS-PAGE sample buffer.

Immunofluorescence analysis--Cortical neurons dissected from embryonic day (E) 15-16 mice were titurated, plated on poly-L-lysine-coated coverslips and maintained in Neurobasal media supplemented with 1x B27 (Gibco), 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin as described (Bhakar et al., 2002). PC12 cells plated on rat tail collagen-coated coverslips were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions and maintained in DMEM containing 10% bovine calf serum, 10% horse serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin. Prior to fixation with 3% parafomaldehyde, cells were rinsed twice with room temperature phosphate-buffered saline (PBS: 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na2HPO4·7H2O; 1.4 mM KH2PO4, pH 7.4). In the case of cortical neurons, cells were fixed after 3 days of *in vitro* culture (DIV3), while PC12

cells were fixed five days following transfection, and in both cases cells were maintained in their particular media throughout their respective culturing periods. Fixed cells were processed for indirect immunofluorescence with various antibodies and for direct immunofluorescence with TRITC-conjugated phalloidin as described (Lamarche-Vane and Hall, 1998). Images of cortical neurons and PC12 cells were captured using a Zeiss 510 laser scanning confocal microscope. Transfected PC12 cells bearing neurites were quantified in four experiments as the ratio of transfected cells with processes greater than one cell body relative to the total population of transfected cells.

Immunoprecipitation analysis--A soluble rat brain extract, prepared as described above, was pre-cleared by incubation with protein A-Sepharose (Sigma) for 1 hr at 4°C, and then incubated with rabbit anti-epsin 2 antisera (2345 and 2346), coupled to protein A-Sepharose, overnight at 4°C. Beads were washed with buffer A containing 1% Triton X-100, and proteins were resolved by SDS-PAGE, and processed for Western blot analysis.

RESULTS

Identification of tubulin as an ENTH domain-interacting protein--In effort to identify novel protein interactors of the evolutionarily conserved ENTH domain (Kay et al., 1999) we performed affinity chromatography on rat brain extracts using GST fusion constructs encoding the ENTH domains of Af10 and MP90. Af10, isolated from Avena fatua, is a protein of unknown function (Jones et al., 1998) and MP90, originally identified as a mitotic phosphoprotein present in Xenopus laevis embryos (Stukenberg et al., 1997), is a member of the epsin family of proteins. Pull-down experiments with these two fusion proteins, followed by detection of bound proteins with Coomassie Blue staining, revealed the selective purification of a 55 kDa protein species (Fig. 3.1). Based on the size and abundance of the affinity-selected protein, we predicted that it might be tubulin. To explore this hypothesis, we performed pull-down experiments with GST fusions to the ENTH domain of rat epsin 1, a variety of modular domains including Src-homology 3 (SH3), Dbl homology (DH), Pleckstrin homology (PH), C2 domains, and a protein fragment encoding the pentapeptide TNPFL. Affinity-selected proteins were resolved by SDS-PAGE and then Western blotted with a monoclonal antibody that recognizes the α-isoform of αβ-tubulin heterodimers. Only the ENTH domain-containing GST fusion protein bound α-tubulin (Fig. 3.2). These data confirm that α-tubulin binds the ENTH domain of epsin and demonstrate the specificity of the interaction compared to other modular domains and peptides. The GST-Af10-ENTH domain also selectively recovered α-tubulin from soluble extracts prepared from rat testis, liver, and heart, and from COS-7 and A431 cell lines (data not shown), demonstrating that ENTH domains can interact with α-tubulin present in a broad range of tissues.

Tubulin binding is direct and conserved among E/ANTH domains--As the soluble brain extracts used in Figures 3.1 and 3.2 include microtubule-associated proteins (MAPs), there was the possibility that observed ENTH/tubulin interactions are indirect. To address this issue, GST fusion proteins encoding ANTH domains isolated from AP180, HIP1, HIP12, and ENTH domains from Af10, MP90, enthoprotin, and epsin were

immobilized on glutathione-Sepharose and incubated with MAP-free, purified αβ-tubulin heterodimers. Purified tubulin bound each of the E/ANTH domain constructs, but not to the control GST fusion protein, as determined by Western blot analyses with antibodies specific for either α-tubulin (Fig. 3.3) or βIII-tubulin (data not shown). Therefore, we conclude that E/ANTH domains are capable of direct interactions with soluble tubulin. Importantly, tubulin bound equally well to wild-type epsin ENTH and a mutated form of epsin's ENTH domain (K76A) that abrogates lipid interactions (Itoh et al., 2001) (Fig. 3.3), indicating that lipid interactions are not required to mediate the observed E/ANTH binding to tubulin.

Localization of tubulin binding to specific E/ANTH domain helices--In AP180, the lipid binding pocket of the ANTH domain is comprised of multiple residues within α1, α2, and the loop between, while the epsin ENTH lipid pocket is contributed to by residues in α0, α1, α1-2 loop, α3 and α4. To determine further if the elements within the E/ANTH domain responsible for tubulin binding are distinguished from lipid binding residues, we generated GST fusion proteins encoding various helical segments from epsin ENTH and AP180 ANTH. Not all of the GST fusion proteins encoding isolated helices could be tested for tubulin binding due to insolubility and/or misfolding. Soluble GST fusion proteins encoding helical segments α1-2, α2-3, α3-4 and α7 of epsin ENTH domain and α7 of AP180 ANTH domain were used in affinity chromatography assays with purified tubulin (Fig. 3.4). Western blot analysis with α-tubulin antibody revealed that the isolated helical regions of α1-2 and α7 of epsin ENTH, and α7 of AP180 ANTH were independently sufficient to mediate this direct interaction *in vitro* (Fig. 3.4). Therefore, at least for epsin, multiple regions of the ENTH domain could be cooperatively involved in mediating tubulin interactions within the intact module. While helix α7 in epsin ENTH or AP180 ANTH has not been implicated in lipid binding (Ford et al., 2002; Ford et al., 2001; Itoh et al., 2001), it is predicted to mediate protein-interactions based on a high degree of conservation of solvent accessible residues therein (Hyman et al., 2000).

Characterization of the affinity of the ENTH domain for tubulin--To measure the affinity of the tubulin/ENTH domain interaction, we performed saturation-binding studies. As seen in Figure 3.5, addition of increasing concentrations of purified tubulin to a constant amount of GST-MP90 ENTH domain led to increasing binding which saturated at ~2.5 μM for α-tubulin. Western blot analyses with antibodies to the neuronal-specific βIII-tubulin isoform revealed a similar saturation profile (Fig. 3.5). Comparison of the blot signals for the affinity-selected α-tubulin with known amounts of tubulin, resolved on adjacent lanes, permitted a quantitative analysis of binding (Fig. 3.5). Scatchard plot analyses revealed a dissociation constant of 1.05 ± 0.18 μM, n=3 (Fig. 3.5 is a representative experiment). This value compares favorably with the dissociation constants of well-established tubulin-binding proteins such as stathmin (Curmi et al., 1997) and Tau (Gustke et al., 1994).

ENTH domains bind assembled microtubules--Many of the specific functions of microtubules, including their roles in intracellular membrane trafficking, depend upon their dynamic ability to cycle between soluble αβ-tubulin heterodimers, and insoluble microtubules (Mandelkow and Mandelkow, 1992). To determine if ENTH domains interact with microtubules in addition to tubulin heterodimers, purified tubulin was assembled into microtubules that were stabilized with the addition of taxol. Upon incubation with microtubules followed by separation of soluble and insoluble fractions, a GST fusion protein to the ENTH domain of MP90 (GST-MP90 ENTH) sedimented only in the presence of microtubules, while an unrelated GST fusion protein (GST-NPFL) failed to co-sediment (Fig. 3.6A). In control experiments, MAP-2 sedimented only in the presence of microtubules, whereas BSA did not sediment under any condition (Fig. 3.6B). Thus, the ENTH domain can interact directly with both microtubules and soluble tubulin *in vitro*.

Stimulation of E/ANTH domain binding to tubulin following tubulin phosphorylation by casein kinase II--In a number of instances, the interaction between tubulin and its binding proteins is influenced by phosphorylation. For example, AP180 was previously shown to co-immunoprecipitate with tubulin and this interaction is enhanced by casein

kinase II-mediated tubulin phosphorylation (Kohtz and Puszkin, 1989; Puszkin et al., 1989). This finding is of particular relevance as casein kinase II is accepted as a major kinase of clathrin-coated vesicles (Bar-Zvi and Branton, 1986) and it mediates the principal phosphorylation of tubulin in brain (Diaz-Nido et al., 1990). Since the ANTH domain of AP180 can bind tubulin (shown above), we investigated if AP180's increased binding to phosphorylated tubulin was a property extensible to other E/ANTH domain-containing proteins. Purified tubulin was incubated with casein kinase II and limiting amounts of [^{32}P-γ] ATP (such that a small fraction of tubulin was radiolabeled upon phosphorylation). This mixture was incubated with the MP90 ENTH domain fusion protein immobilized on glutathione-Sepharose beads. The bound material, along with an aliquot of the starting material, was analyzed by Western blotting with antibodies against α-tubulin and βIII-tubulin, as well as by autoradiography (Fig. 3.7). The binding of phosphorylated and non-phosphorylated tubulin to GST-MP90 ENTH was measured as a ratio of the signal density in the bead fractions versus the starting material. As seen in Figure 3.7, whereas the phosphorylated form of tubulin was enriched 46.5 ± 2.9 fold (mean ± s.e.m., n=3) in the beads over the starting material, the total pools of α-tubulin and βIII-tubulin, which are comprised predominantly of non-phosphorylated material, are enriched only 5.4 ± 2.5 fold and 4.3 ± 2.2 fold, respectively. ANOVA statistical analyses revealed that the enrichment of the phosphorylated form was significantly greater (p-value < 0.001) than that of the non-phosphorylated forms. Thus, casein kinase II-mediated phosphorylation of tubulin stimulates tubulin binding to E/ANTH domains.

In vivo interaction of epsin and tubulin--To determine whether tubulin interacts with ENTH-domain containing proteins *in vivo*, we performed co-immunoprecipitation assays for epsin from soluble rat brain extracts. Immunoprecipitation with epsin antibodies 2345 or 2346 revealed immunoreactive species at 94 and 74 kDa (Fig. 3.8) as determined by Western blotting with the 2345 antisera, in agreement with the reported molecular mobility of rat epsin 1 and 2, respectively (Chen et al., 1998; Rosenthal et al., 1999). In addition, the epsin anti-sera immunoprecipitates contained the clathrin adaptor AP-2, a previously described binding partner of epsin (Chen et al.,

1998; Owen et al., 1999; Traub et al., 1999) and tubulin (Fig. 3.8). As control for specificity, we found that another component of the endocytic machinery, synaptojanin, was not recovered in the same immune complex (Fig. 3.8). Thus, based on co-immunoprecipitation data epsins 1 and 2 may exist in protein complexes with tubulin and other endocytic proteins in rat brain extracts.

To further address the interaction between epsin and tubulin *in vivo*, we assessed the localization of endogenous epsins 1 and 2, relative to the microtubule cytoskeleton, in dissociated cortical neurons. Consistent with the previously reported cellular distribution of rat epsin 1 and 2 to clathrin-coated pits (Chen et al., 1998; Drake et al., 2000; Rosenthal et al., 1999), epsins displayed a punctate staining pattern (Fig. 3.9) that was often co-localized with clathrin (data not shown). Epsin-positive punctae were distributed throughout the cell body, neuronal processes (Fig. 3.9, top panels) and within growth cones (Fig. 3.9, bottom panels). While not exclusively associated with microtubule filaments, punctate staining of endogenous epsin 1 and 2 in neuronal processes was coincident with microtubules as revealed with α- and βIII-tubulin antibodies, and co-localization was particularly evident within growth cones (Fig. 3.9), where microtubules are splayed and distinct filaments are easily discernable (Dent and Kalil, 2001; Schaefer et al., 2002). Staining of the actin cytoskeleton in the same cell preparations revealed the full extent of the growth cone (Fig. 3.9). These data demonstrate that epsin can interact with tubulin *in vivo* as illustrated by immunoprecipitation analyses, and that a significant component of epsin punctae are associated with the microtubule cytoskeleton in dissociated cortical neurons, indicative of a physiological interaction between the ENTH domain and microtubules.

E/ANTH domains stimulate neurite outgrowth--To investigate the potential functional relevance of the interaction between tubulin and E/ANTH domains in neuronal processes and growth cones, we examined for alterations in neurite outgrowth in PC12 cells overexpressing ENTH domains. These cells require tubulin polymerization and re-organization of the microtubule cytoskeleton in order for neurite outgrowth to occur (Drubin et al., 1985; Lin and Forscher, 1993; Tanaka et al., 1995; Teichman-Weinberg et al., 1988; Williamson et al., 1996). Over-expression of GFP tagged fusion proteins

encoding the individual E/ANTH domains of epsin or AP180 specifically stimulated outgrowth of processes, defined as extensions greater than one cell body (Fig. 3.10). In contrast, over-expression of a GFP-expressing vector or full-length GFP-tagged epsin did not enhance neurite outgrowth (Fig. 3.10). As the observed processes were generated in the absence of exogenous nerve growth factor (NGF) it appears that over-expression of the E/ANTH domain is self-sufficient to stimulate outgrowth within PC12 cells.

DISCUSSION

The E/ANTH domain is an evolutionarily conserved protein module, composed of a super-helix of α-helices, located within the N-terminus of a variety of proteins. Recently it was demonstrated that this module functions as a mediator of lipid interactions (Ford et al., 2001; Itoh et al., 2001). Previous studies have indicated that the domain also binds to proteins (Hyman et al., 2000). In this study, we have characterized a novel interaction between tubulin and the E/ANTH domain isolated from several different species. Moreover, we have provided evidence that E/ANTH domain tubulin-binding elements are distinct from those implicated in lipid interactions. In this regard, the E/ANTH domain is reminiscent of other modular domains such as PH domains that bind to both inositol phospholipids and proteins (Lemmon et al., 2002). For example, the PH domain of the β-adrenergic receptor kinase binds to both PtdIns(4,5)P_2 and the G-protein β/γ subunit and in fact, simultaneous interaction with both ligands is necessary for the recruitment of the kinase to membranes (Pitcher et al., 1995). Moreover, PH domains from several proteins including Bruton's tyrosine kinase bind to both phosphoinositides and actin (Yao et al., 1999). C2 domains also interact with multiple ligands including Ca^{2+}, phospholipids, inositol polyphosphates, and proteins (Nalefski and Falke, 1996). Thus, like other modules, the E/ANTH domain appears to be multi-functional in terms of its ligand partners. Interestingly, this idea is consistent with recent studies demonstrating that as yet unidentified protein binding partners are essential for E/ANTH domain function in yeast (Aguilar et al., 2002).

Based on our findings, we propose that the interaction between E/ANTH domains and tubulin/microtubules represents a general mechanism for linking functionally disparate E/ANTH domain-containing proteins tò the cytoskeletal architecture. Of the numerous proteins characterized and shown to harbour an E/ANTH domain, there is propensity of these to function in clathrin-mediated membrane budding. It has long been demonstrated that tubulin is a component of clathrin-coated vesicles (Kelly et al., 1983; Zisapel et al., 1980). Thus, an interesting possibility is that tubulin interactions could help recruit E/ANTH proteins to endocytic vesicles. A functional link between endocytosis and the cytoskeleton has been examined in numerous studies. For example,

in yeast, mutations in several genes, including the epsin genes, ENT1 and ENT2 (Wendland et al., 1999), disrupt both endocytosis and the actin cytoskeleton. However, a study examining the dynamics of clathrin-coated pits in living cells indicates that while the actin cytoskeleton is involved in restricting pit mobility, actin is not entirely capable of determining where pits will form, nor in guiding vesicle movements (Gaidarov et al., 1999). Thus, the authors suggested that dynamic interactions between pits and other cytoskeletal structures, such as the microtubule network, is likely involved in spatial regulation of coated pit dynamics. Evidence supporting a direct role for microtubules in endocytosis consists of the observations that endocytic vesicles can be captured by microtubules below the cell cortex and actively transported inward along microtubules (Goltz et al., 1992; Oda et al., 1995). Moreover, this movement is sensitive to various microtubule destabilizing or stabilizing drugs (Novikoff et al., 1996). Ostensibly, endocytic E/ANTH domain-containing proteins such as epsin and/or HIP1 and HIP12 could bridge endocytic vesicles comprising these proteins directly to microtubules for microtubule-based transport through the cell cortex or subsequent trafficking through the endosomal pathway. Consistent with this notion, the endocytic protein Eps15, an *in vivo* interacting partner for epsin (Chen et al., 1998), has been demonstrated to move from the plasma membrane to various intracellular compartments of the endocytic pathway in a microtubule-dependent manner (Torrisi et al., 1999). Our observations could also provide a link between the actin and tubulin based cytoskeletal networks. For instance, the ANTH domain-containing protein HIP12 is a clear example of a molecule that physically links the actin cytoskeleton with components of clathrin-mediated endocytosis (Engqvist-Goldstein et al., 2001). With our demonstration that ANTH domains bind tubulin, HIP12 could additionally bridge these components to the microtubule cytoskeleton for coordinated regulatiòn of endocytosis with actin networks.

The interaction between E/ANTH domains and tubulin is regulated by phosphorylation via casein kinase II, an enzyme widely accepted as a regulator of both the microtubule cytoskeleton and vesicular trafficking (Diaz-Nido et al., 1990). In addition to being a major kinase of clathrin-coated vesicles (Bar-Zvi and Branton, 1986) this enzyme is the principal mediator of tubulin phosphorylation in brain (Diaz-Nido et al., 1990). It is likely that this enzyme is important in membrane trafficking, as

blocking casein-kinase II activity can disrupt receptor-mediated endocytosis (Cotlin et al., 1999; Shi et al., 2001). Our data demonstrating that casein kinase II mediated tubulin phosphorylation increases binding to the E/ANTH domains supports a notion that casein kinase II activity on the cytoskeleton and in endocytic events are in fact mechanistically interconnected.

Casein kinase II mediated phosphorylation of tubulin (Diaz-Nido et al., 1990) has also been implicated in promoting neuritogenesis *in vivo* (Aletta, 1996; Ulloa et al., 1993). Thus, the enhanced tubulin binding of E/ANTH domains following casein kinase II mediated phosphorylation may modulate E/ANTH's ability to stimulate neurite outgrowth by targeting these proteins to phosphorylated microtubules. While the precise mechanisms by which E/ANTH domains stimulate neurite outgrowth remain to be elucidated, it is possible the process is similar to that suggested for other tubulin binding proteins. For instance, it was recently suggested that the tubulin binding protein Collapsin Response Mediator Protein-2 (CRMP-2) stimulates neurite outgrowth, in conjunction with low doses of NGF, by drawing together tubulin heterodimers beyond a critical concentration required for polymerization (Fukata et al., 2002; Gu and Ihara, 2000). Our finding that the isolated ENTH domain stimulates neurite outgrowth, while full-length epsin does not, is quite analogous to the demonstration that over-expression of full-length epsin has no effect on receptor trafficking, while expression of its carboxy terminus inhibits endocytosis (Chen et al., 1998). These findings indicate that while the ENTH and carboxy termini of epsin can have clear and independent functions, the intact epsin molecule is dependent on the co-operative function of both elements. Ultimately, whether E/ANTH domains interactions with tubulin are principally involved in microtubule-based vesicular trafficking or in linking actin and microtubule networks to function in neuritogenesis await further investigation.

ACKNOWLEDGEMENTS

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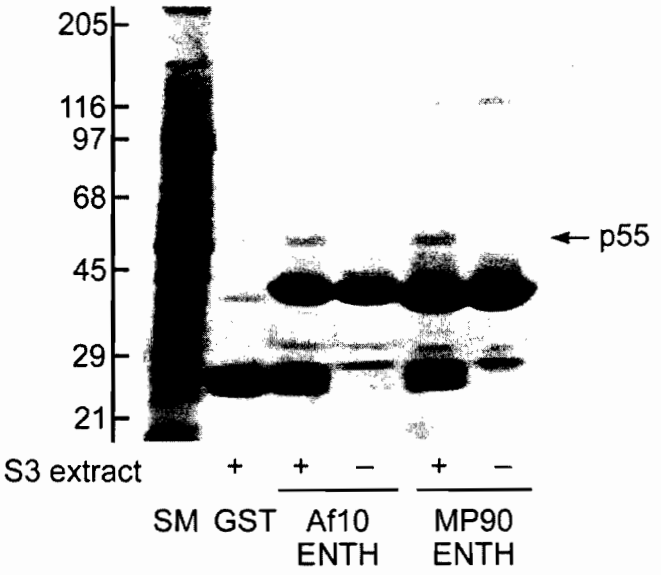

Figure 3.1 Identification of a 55 kDa protein from rat brain extracts that binds ENTH domains of Af10 and MP90. Approximately 30 μg of GST alone or GST fused to the ENTH domains of Af10 or MP90 were pre-coupled to glutathione-Sepharose and then incubated with 2 mg of a soluble rat brain (S3) extract (+ extract) or with buffer alone (- extract). The samples were washed extensively and the proteins bound to the beads were resolved by SDS-PAGE, along with an aliquot of the soluble starting material (SM), and detected by Coomassie Brilliant Blue staining. The arrow indicates the position of a 55 kDa affinity selected protein (p55) that does not bind to GST alone and is not detected in GST fusion protein preparations (in the absence of brain extract).

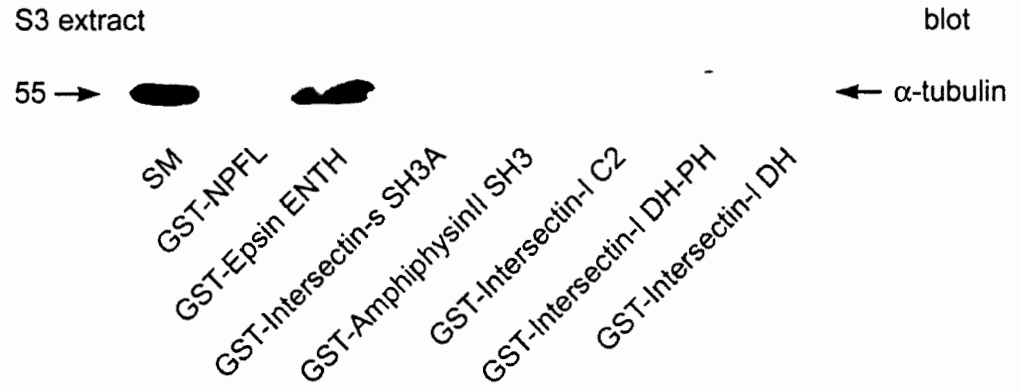

Figure 3.2 ENTH domains bind tubulin from rat brain extracts. Approximately 30 μg of GST alone, GST fused to the pentapeptide motif TNPFL (GST-NPFL), GST-Epsin ENTH, GST-Intersectin SH3A, GST-amphiphysin II SH3, GST-Intersectin-l C2, GST-Intersectin-l DH-PH and GST-Intersectin-l DH were pre-coupled to glutathione-Sepharose and used in binding assays with soluble rat brain (S3) extracts. Bound proteins were resolved by SDS-PAGE and processed for Western blot analysis with a monoclonal antibody against α-tubulin.

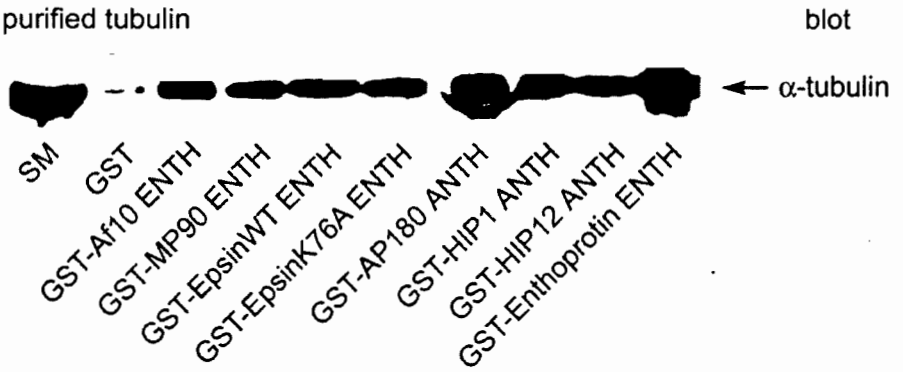

Figure 3.3 E/ANTH domains display conserved binding to purified tubulin. GST-ENTH domains from Af10, MP90, wild-type rat epsin 1 (EpsinWT ENTH), rat epsin 1 encoding the point mutation K76A (epsinK76A ENTH), and enthoprotin, and GST-ANTH domains from AP180, HIP1, and HIP12, as well as GST alone, were pre-coupled to glutathione-Sepharose and incubated with 200 nM purified tubulin. The samples were washed extensively and the proteins bound to the beads were resolved by SDS-PAGE, along with an aliquot of the soluble starting material (SM). The proteins were transferred to nitrocellulose and blotted with an antibody against α-tubulin.

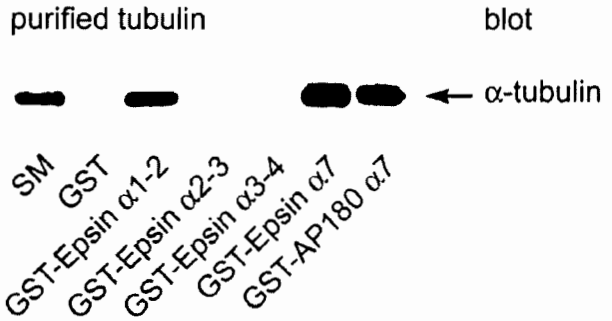

Figure 3.4 Specific E/ANTH domain helical segments mediate tubulin binding. Approximately 30 μg of GST alone, or GST fused to peptides encoding the helical regions α1-2, α2-3, α3-4, and α7 of rat epsin 1 and α7 of AP180, were pre-coupled to glutathione-Sepharose and used in binding assays with purified tubulin. Bound proteins were resolved by SDS-PAGE and processed for Western blot with an antibody against α-tubulin.

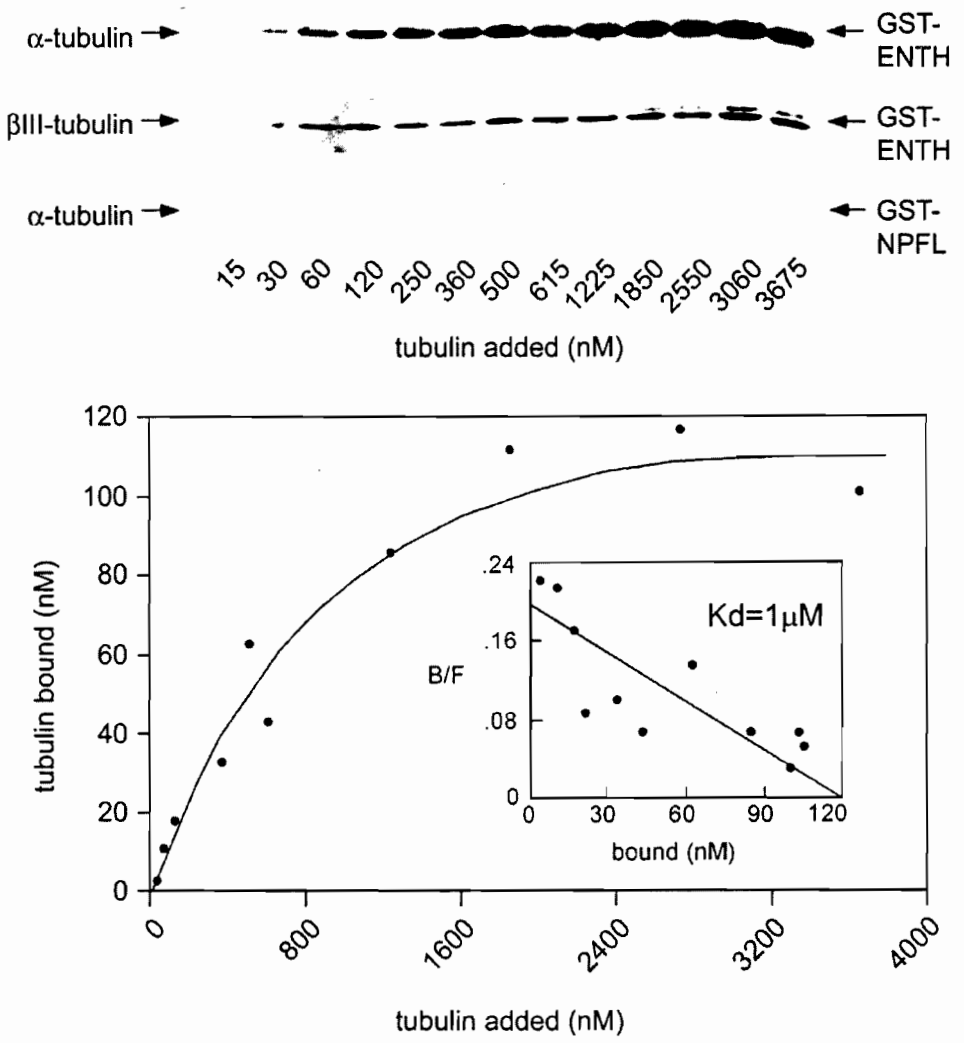

Figure 3.5 Saturation binding analysis of ENTH domain/tubulin interactions. Increasing concentrations of purified tubulin was added to GST-MP90 ENTH or GST-NPFL fusion proteins, pre-coupled to glutathione-Sepharose, and the tubulin specifically bound to the beads was detected with antibodies against α-tubulin or βIII-tubulin. The amount of α-tubulin bound to the beads was quantified as described in "Experimental Procedures" and saturation binding curves and Scatchard plots were derived from the data.

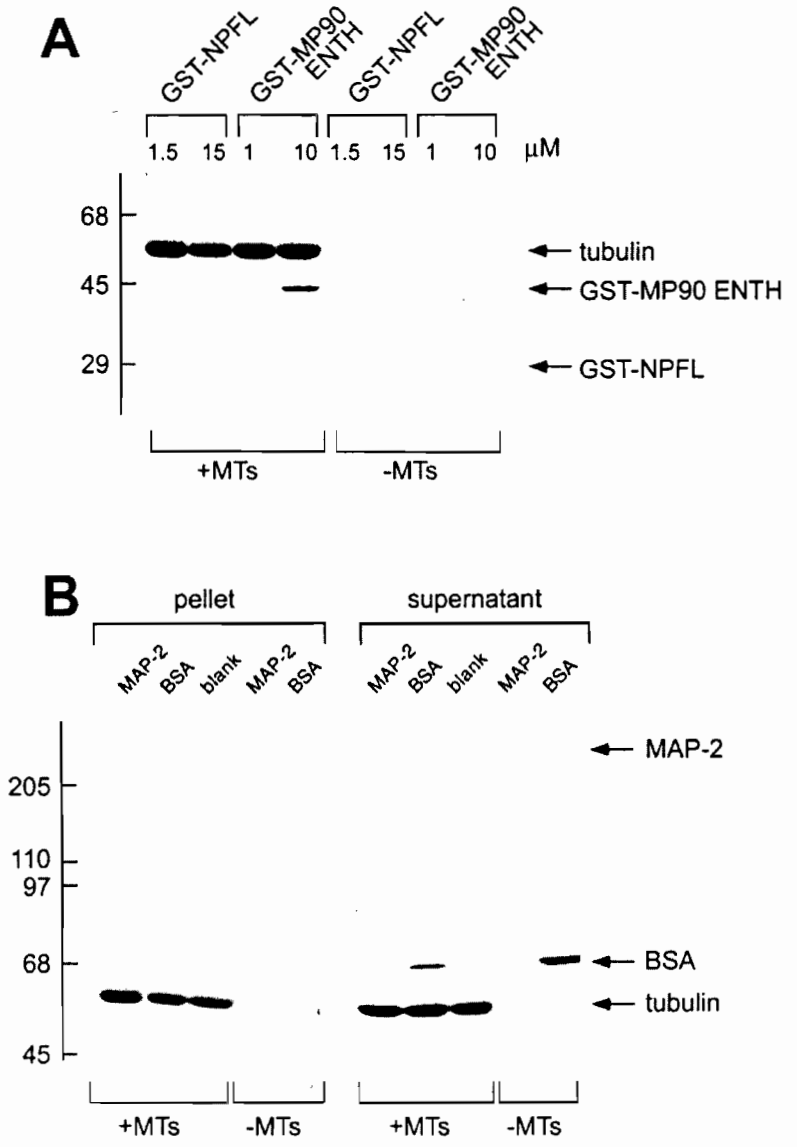

Figure 3.6 ENTH domain binds assembled microtubules. (A) GST-NPFL (1.5 or 15 μM) or GST-MP90 ENTH (1 or 10 μM) were incubated with assembled, taxol-stabilized microtubules (+MTs), or with buffer alone (-MTs). After incubation at 30oC, proteins were centrifuged and the pellet fraction was resolved by SDS-PAGE and stained with Coomassie Brilliant Blue; arrows on the right indicate the mobilities of the tubulin subunits and the two GST fusion proteins. (B) A sample of assembled microtubules stabilized with taxol (+MTs), or of buffer lacking microtubules (-MTs) was incubated with MAP-2, BSA, or buffer alone (blank) as indicated. Following incubation at 30°C the samples were centrifuged and the proteins in the pellets (left panel) and supernatants (right panel) were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue; arrows on the right indicate the mobilities of the tubulin subunits as well as MAP-2 and BSA.

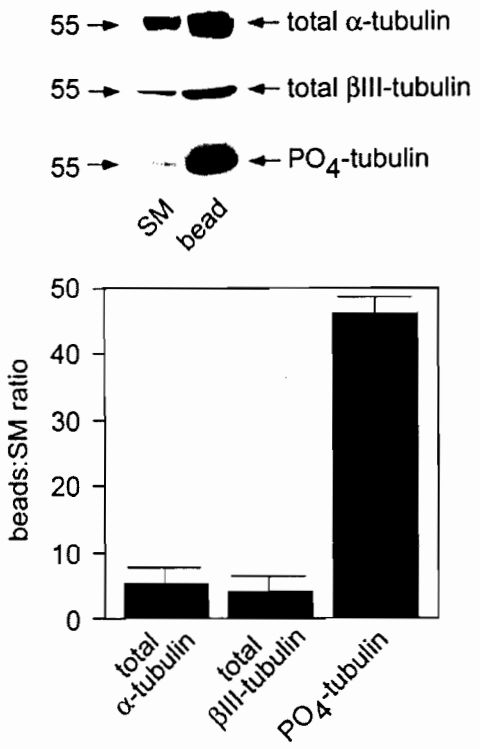

Figure 3.7 Effect of tubulin phosphorylation on ENTH domain binding. Purified tubulin was phosphorylated with casein kinase II and limiting amounts of [32P-γ] ATP, such that a small fraction of tubulin was radiolabeled upon phosphorylation. This mixture was then incubated with GST-MP90 ENTH immobilized on glutathione-Sepharose beads, and the tubulin specifically bound to the beads (bead), along with an aliquot of the starting material (SM), equal to 1/50th of the sample added to the beads, was analyzed by Western blot with antibodies against α-tubulin and βIII-tubulin, as well as by autoradiography (PO_4-tubulin). The binding of each form of tubulin was plotted as a ratio of the signal density in the bead fraction *versus* the starting material. Bars represent mean ± s.e.m., n=3.

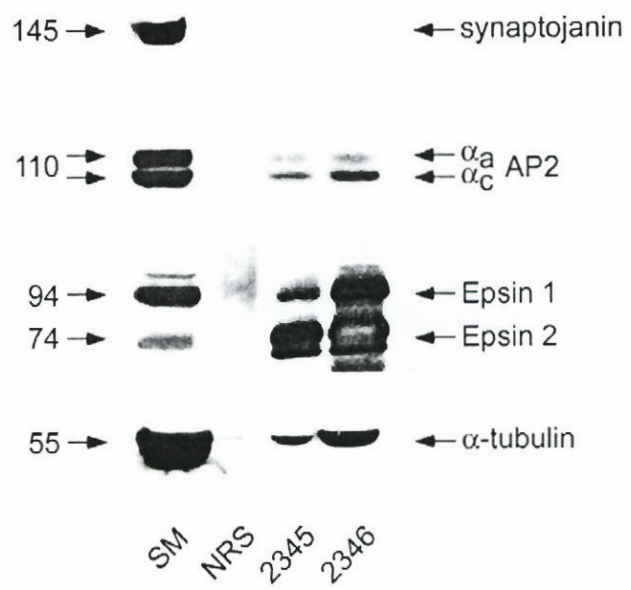

Figure 3.8 Co-immunoprecipitation of tubulin with epsin 1 and 2. Epsin antisera 2345 and 2346 (epsin antibody), along with pre-immune sera 2345 (NRS) were pre-coupled to protein A Sepharose and incubated with 1 mg of a soluble rat brain extract overnight at 4°C. The beads were extensively washed and the material specifically bound to the beads was recovered and processed for Western blot analyses with antibodies against the αa and αc-subunits of AP-2, against synaptojanin, and affinity purified epsin antibody 2345 as indicated. SM, starting material.

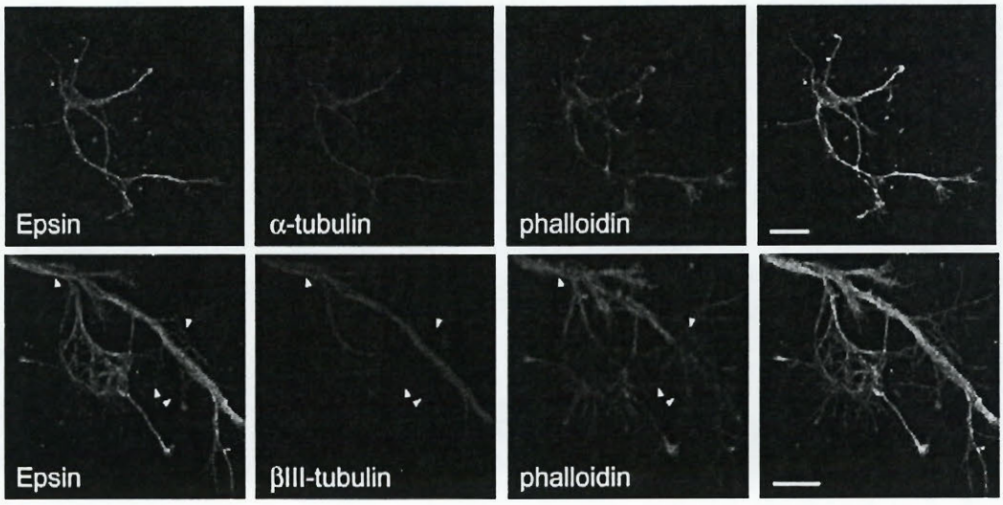

Figure 3.9 Endogenous epsin partially co-localizes with microtubules in cortical neurons. Dissociated cortical neurons isolated from embryonic day 15-16 mice were (E15-16) cultured for three days (3DIV) and subsequently processed for immunofluorescence using affinity purified antibodies specific for epsin 1and 2 (2345) (green), α-tubulin or βIII-tubulin monoclonal antibody (blue) and phalloidin staining (red). These neurons were analyzed by laser scanning confocal microscopy to determine the extent of co-localization between microtubules in images capturing an entire neuron (top panels) or focused on the growth cone (bottom panels). Punctate epsin staining co-localized with microtubules, (arrows highlight some of these regions) is seen upon superimposition of these micrographs (top-right and bottom-right panels). Scale bars represent 20 μm.

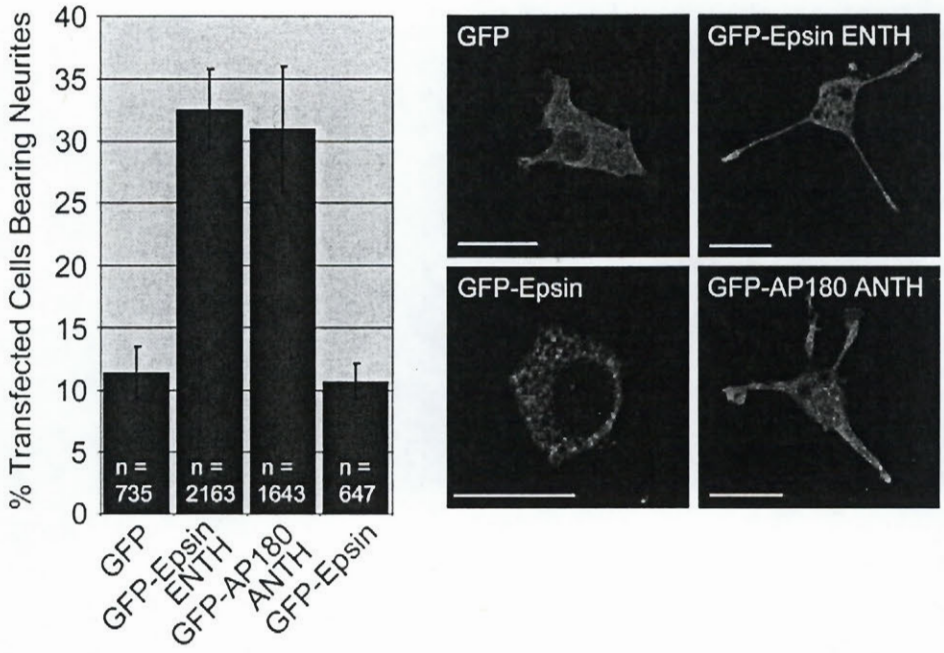

Figure 3.10 E/ANTH domains of epsin and AP180 stimulate neurite outgrowth in PC12 cells. PC12 cells transiently expressing GFP vector alone, or GFP fusion proteins encoding epsin, epsin ENTH or AP180 ANTH domains were cultured for five days, processed for immunofluorescence and analyzed by laser scanning confocal microscopy. All transfected cells were quantified and analysed for neurite outgrowth, defined as processes greater than one cell body. Left, graphical analyses of neurite outgrowth (four experiments were conducted, where n = the total number of transfected cells counted as indicated in the bar graph for each construct). Right, representative cells for GFP, GFP-Epsin, GFP-Epsin ENTH, and GFP-AP180 ANTH. Scale bars represent 20 μm.

PREFACE TO CHAPTER 4: Characterizing partners of intersectin-s carboxy-terminal SH3 domains

In addition to its amino-terminal EH domains, intersectin-s contains five Src homology 3 (SH3) domains (identified as SH3A-SH3E) (Guipponi et al., 1998; Yamabhai et al., 1998). SH3 domains are found in a diverse array of proteins, including those involved in signaling transduction, cytoskeletal organization, and synaptic vesicle recycling (Cohen et al., 1995; McPherson, 1999; Pawson, 1995). Given the diversity of function attributed to SH3 domains, we were intrigued by the prospective role(s) of these modules within intersectin-s.

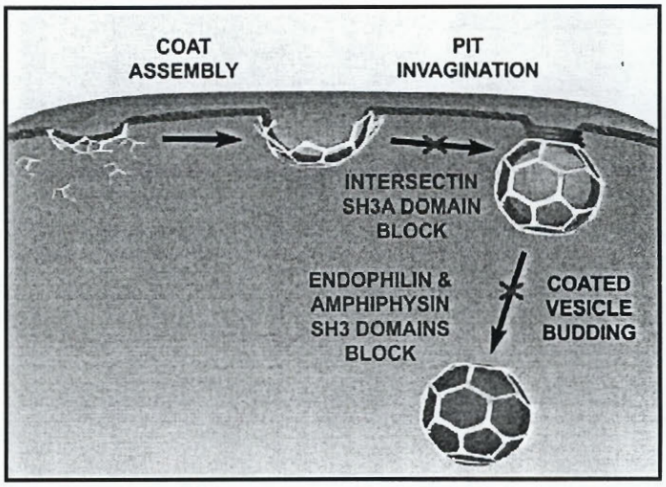

Figure 4.0 SH3 domains function at distinct steps in clathrin-mediated endocytosis.

Consistent with a function in synaptic vesicle recycling, we and others have demonstrated that intersectin interacts with dynamin and synaptojanin *in vitro* in an SH3 domain dependent manner (Okamoto et al., 1999; Roos and Kelly, 1998; Sengar et al., 1999; Yamabhai et al., 1998). Furthermore, in collaboration with Fiona Simpson and Sandra Schmid, we determined that the SH3 domains of endophilin I (de Heuvel et al., 1997; Ringstad et al., 1997), amphiphysin II (Ramjaun et al., 1997), syndapin I (Qualmann et al., 1999), as well as SH3C and SH3E of intersectin specifically inhibit the late stages of transferrin receptor endocytosis which involve membrane fission (Simpson et al., 1999). Intriguingly, SH3A of intersectin was uniquely capable of inhibiting early stages of endocytosis leading to the formation of constricted coated pits

(Simpson et al., 1999). In effort to elucidate intersectin-s functions further, we initiated a screen to identify cellular ligands unique to the SH3A domain of intersectin.

CHAPTER 4. THE ENDOCYTIC PROTEIN INTERSECTIN IS A MAJOR BINDING PARTNER FOR THE RAS EXCHANGE FACTOR mSOS1 IN RAT BRAIN

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Key words: clathrin/EH domain/endocytosis/Ras/SH3 domain

Running title: Intersectin/mSos1 interactions

ABSTRACT

We recently identified intersectin, a protein containing two EH and five SH3 domains, as a component of the endocytic machinery. The N-terminal SH3 domain (SH3A), unlike other SH3 domains from intersectin or various endocytic proteins, specifically inhibits intermediate events leading to the formation of clathrin-coated pits. We have now identified a brain-enriched, 170 kDa protein (p170) that interacts specifically with SH3A. Screening of combinatorial peptides reveals the optimal ligand for SH3A as Pp(V/I)PPR, and the 170 kDa mammalian son-of-sevenless (mSos1) protein, a guanine-nucleotide exchange factor for Ras, contains two copies of the matching sequence, PPVPPR. Immunodepletion studies confirm that p170 is mSos1. Intersectin and mSos1 are co-enriched in nerve terminals and the two proteins are co-immunoprecipitated from brain extracts. SH3A competes with the SH3 domains of Grb2 in binding to mSos1, and the intersectin/mSos1 complex can be separated from Grb2 by sucrose gradient centrifugation. Interestingly, over-expression of the SH3 domains of intersectin functions in a dominant-negative manner to block EGF-mediated Ras activation. These results suggest that intersectin functions in cell signaling in addition to its role in endocytosis and may in fact link these two important cellular processes.

INTRODUCTION

Src homology 3 (SH3) domains, 50-70 amino acids modules, mediate protein-protein interactions by binding specific proline-rich peptide sequences within cellular ligands. A well studied example of an SH3 domain-mediated interaction is the recruitment of the Ras guanine-nucleotide exchange factor, mammalian son-of-sevenless (mSos), to the plasma membrane by the adaptor Grb2 (Baltensperger et al., 1993; Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993). mSos contains a proline-rich C-terminus that mediates its stable association with the SH3 domains of Grb2, a protein composed of two SH3 domains flanking a central SH2 domain (Lowenstein et al., 1992). Through its SH2 domain, Grb2 interacts with activated growth factor receptors either directly or through the adaptor protein SHC (Ceresa and Pessin, 1998), thereby recruiting mSos to the membrane where it in turn activate Ras (Aronheim et al., 1994; Quilliam et al., 1994). However, recent evidence suggests that mSos can be recruited to the membrane by additional mechanisms. Specifically, activation of Ras by mSos can occur in the absence of Grb/mSos interactions (Karlovich et al., 1995; McCollam et al., 1995; Wang et al., 1995b) suggesting that other domains of mSos, such as its Dbl Homology (DH) or Pleckstrin Homology (PH) domains, play important roles in mSos membrane targeting and activity (Chen et al., 1997; Qian et al., 1998), or that targeting of mSos to the membrane can be mediated through the actions of other adaptor proteins.

SH3 domain mediated protein-protein interactions also function in vesicular trafficking, particularly in endocytosis (McPherson, 1999). For example, intersectin, a novel protein involved in clathrin-mediated endocytosis, contains two N-terminal Eps15 homology (EH) domains, a central helical region, and five C-terminal SH3 domains (termed SH3A-SH3E) (Guipponi et al., 1998; Yamabhai et al., 1998). Intersectin is homologous to Dap160 in Drosophila (Roos and Kelly, 1998), as well as to Ese1 in mouse (Sengar et al., 1999) and EHSH1 in rat (Okamoto et al., 1999). An alternatively spliced variant of intersectin, referred to as intersectin-long (intersectin-l), contains a C-terminal extension with DH, PH, and C2 domains (Guipponi et al., 1998; Hussain et al., 1999). Via its EH domains, intersectin interacts with epsin, a protein that binds to

clathrin and the clathrin adaptor complex, AP-2 (Chen et al., 1998), and through its SH3 domains (Okamoto et al., 1999; Sengar et al., 1999; Yamabhai et al., 1998), intersectin interacts with synaptojanin and dynamin, two proteins with critical enzymatic activities involved in the formation of clathrin-coated vesicles (Cremona et al., 1999; Sever et al., 1999). In addition, intersectin heterodimerizes through the formation of coiled-coils with Eps15 (Sengar et al., 1999), another accessory protein in endocytosis (Salcini et al., 1999). Thus, intersectin is believed to function as a scaffolding protein that assembles certain proteins at sites of clathrin-coated pit formation.

To further study the role of the SH3 domains of intersectin in endocytosis, we tested their ability to block transferrin receptor uptake using a permeabilized cell assay (Simpson et al., 1999). Intriguingly, while the SH3C and SH3E domains of intersectin, as well as the SH3 domains from the endocytic proteins endophilin I (de Heuvel et al., 1997; Ringstad et al., 1997), amphiphysin II (Ramjaun et al., 1997), and syndapin I (Qualmann et al., 1999) selectively inhibit late events of clathrin-coated vesicle formation involving membrane fission, intersectin SH3A was unique in its ability to block earlier stages (Simpson et al., 1999).

To follow up on this observation, we performed overlay assays with a GST-SH3A fusion protein and identified an SH3A domain-specific binding partner of 170 kDa (p170). Analysis of the optimal ligand preference of the SH3A domain suggested that p170 was mSos1, a result confirmed by a variety of methods. Like intersectin, mSos1 is enriched in nerve terminals, and the two proteins strongly co-immunoprecipitate from embryonic brain extracts. Intersectin and Grb2 compete for binding to mSos1, and sucrose density gradient analysis indicates that in brain, intersectin and mSos1 form a stable complex that primarily excludes Grb2. Interestingly, over-expression of the SH3 domains of intersectin strongly attenuates EGF-dependent GTP-loading of Ras. Thus, it appears that intersectin may function as a scaffold molecule for protein components of both the endocytic machinery and signal transduction pathways.

RESULTS

Identification of an intersectin SH3A domain-specific binding partner--In previous collaborative studies, we determined that the SH3A domain of intersectin is unique amongst a wide variety of SH3 domains from endocytic proteins, including the other SH3 domains of intersectin, in its ability to block intermediate steps leading to the formation of a sequestered clathrin-coated pit (Simpson et al., 1999). We thus performed overlay assays using a GST-SH3A fusion protein in order to identify specific SH3A-binding partners. The intersectin SH3A domain, as well as the SH3 domains from amphiphysin I and II and endophilin I, II, and III, each interact with bands at 100 and 145 kDa (Figure 4.1). The 100 and 145 kDa bands are most likely dynamin and synaptojanin, respectively, which have been observed to interact with the SH3 domains used here in several studies (Micheva et al., 1997a; Ramjaun et al., 1997; Yamabhai et al., 1998). Interestingly, SH3A also interacted with an additional band at 170 kDa (Figure 4.1). The 170 kDa band (p170) was readily detectable at concentrations of fusion protein as low as 100 ng/ml, but was not detected in overlay assays with the other four SH3 domains of intersectin nor with the SH3 domains from the amphiphysins or the endophilins, even at concentrations as high as 10 μg/ml (data not shown).

GST-SH3A overlays of tissue extracts revealed that p170 was most enriched in brain, although significant levels were detected in all tissues tested, and in several tissues, p170 was the only protein species identified (Figure 4.2A). Within the brain, subcellular fractionation revealed that p170 was present in both soluble and particulate fractions. Its overall distribution was similar to that of dynamin and synaptojanin (Figure 4.2B), proteins which are enriched in pre-synaptic nerve terminals (McPherson et al., 1994b), although a small pool of the protein was found in the P_1 fraction, possibly in association with nuclei.

Identification of p170 as mSos1--SH3 domains interact with proline-rich peptides with the core sequence PXXP (where X is any amino acid). To define the ligand specificity of the intersectin SH3A domain, peptides were affinity selected from a phage-displayed

X6PXXPX6 peptide library (Sparks et al., 1996b). The peptides that were selected from the library (Figure 4.3A) share a six amino acid motif with the consensus Pp(V/I)PPR, where p is typically proline. This motif represents a class II ligand for SH3 domains (Feng et al., 1994; Lim et al., 1994) and overlaps with the specificity of the Src SH3 domain (Sparks et al., 1996b). A computer search of GenBank revealed two matching sequences (PPVPPR) in mSos1 (Figure 4.3B), a protein of 170 kDa (Chardin et al., 1993; Li et al., 1993). To determine if these two sites may serve as peptide ligands for the SH3A domain of intersectin, the two sequences, along with a third highly related sequence from mSos1 (Figure 4.3B), were tested for binding to the five SH3 domains of intersectin expressed individually as GST fusion proteins. As seen in Figure 4.3C, only the proline-rich sequences within mSos1 that matched the defined consensus bound to the SH3A domain, whereas none of the peptides bound to the other four SH3 domains.

To determine if p170 is indeed mSos1, we performed GST-SH3A overlays on proteins immunoprecipitated from embryonic day 18 (E18) rat brain extracts with a polyclonal mSos1 antibody (C23). C23 efficiently immunoprecipitates mSos1 (Figure 4.4, top panel) and that the immunoprecipitated protein is strongly reactive in a GST-SH3A domain overlay (Figure 4.4, middle panel). Further, immunodepletion of mSos1 from rat brain extracts depletes p170 (Figure 4.4, bottom panel), confirming that this interacting protein is mSos1. Moreover, the tissue distribution of mSos1 as determined by Western blot (data not shown) closely parallels that of p170 (demonstrated in Figure 4.2A), suggesting that mSos1 is equivalent to p170 in all tissues tested.

Intersectin and mSos1 interact in neurons--To explore the potential interaction between mSos1 and intersectin in situ, we first performed immunoperoxidase staining to examine the regional distributions of the two proteins. Intersectin and mSos1 were expressed in discrete and highly overlapping neuronal populations in rat brain cortex, caudate, and ventral pallidum (data not shown). Previously, it was demonstrated that Dap160, the Drosophila homologue of intersectin, was present in nerve terminals from third instar larva (Roos and Kelly, 1998). Staining of 1 day old neuron cultures from the CA3 region of hippocampus revealed that intersectin is broadly distributed in neurons including an enriched pool in growth cones (Hussain et al., 1999/ and data not shown).

Interestingly, like intersectin, mSos1 is present throughout the hippocampal neurons and is enriched at the tip of their growth cones (Figure 4.5A). This enrichment is due to an increase in the density of mSos1 positive punctae in the growth cone area (Figure 4.5B) as well as to an increase in the content of mSos1 per punctae (Figure 4.5C).

We next performed co-immunoprecipitation experiments using a cytosolic fraction of E18 rat brain extracts. Two different antibodies against intersectin (2173, 2174) immunoprecipitate both the long (intersectin-l) and the short (intersectin-s) forms of intersectin and lead to a strong co-immunoprecipitation of mSos1 (Figure 4.5D; note the enrichment of mSos1 in the intersectin immunoprecipitated samples relative to the starting material). mSos1 also strongly co-immunoprecipitates with intersectin from a Triton X-100 solubilized particulate fraction suggesting that the proteins also interact at the membrane (Figure 4.5E).

Intersectin competes with Grb2 for binding to mSos1--To explore the relationship between mSos1, intersectin and Grb2, we immunoprecipitated mSos1 from soluble E18 rat brain extracts and performed Western blots with antibodies against the various proteins. Intersectin-l, intersectin-s, and Grb2 all co-immunoprecipitate with mSos1 (Figure 4.6). However, whereas intersectin-l was enriched in the immunoprecipitate relative to the starting material, Grb2 was not (Figure 4.6), even though in overlay assays, GST-Grb2 and GST-SH3A fusion proteins interact equally well with blotted mSos1 (data not shown). One plausible explanation is that intersectin and Grb2 compete for binding to mSos1, and that the greater co-immunoprecipitation of intersectin versus Grb2 is due to intersectin being more abundant than Grb2 in the brain.

mSos1 contains many SH3 domain-binding consensus sequences (i.e., PXXP). To determine if Grb2 and SH3A compete for binding to mSos1, GST-SH3A was used in overlay assays on E18 rat brain extracts in the presence of increasing concentrations of (His)6-tagged Grb2. Addition of Grb2 significantly reduced SH3A binding to mSos1 in a concentration dependent manner, with binding completely eliminated at a molar ratio of 5:1 (Figure 4.7). Thus, Grb2 and SH3A may compete for binding to the same or overlapping site(s) in mSos1 although steric hindrance cannot be excluded. Previous mapping experiments have demonstrated that Grb2 binds to human Sos1 at three sites

(PXXP cores at amino acids 1151-1154, 1212-1215, and 1290-1293) (Li et al., 1993; Rozakis-Adcock et al., 1993). The first and third of these sites are the same predicted by phage-display experiments to be ligand sites for the SH3A domain (Figure 4.3), further suggesting that intersectin and Grb2 bind mSos1 in a equivalent manner.

To further explore this issue, we infected undifferentiated PC12 cells with an adenovirus construct encoding the five tandem SH3 domains of intersectin fused at their N-terminus to GFP (GFP-SH3A-E). Following expression of the construct, soluble cell extracts were prepared and processed for anti-GFP immunoprecipitation followed by blotting for co-immunoprecipitating mSos1. The anti-GFP antibody immunoprecipitates the GFP-SH3A-E fusion protein (which migrates with the expected molecular mass of 85 kDa) (Figure 4.8, middle panel) and leads to a strong co-immunoprecipitation of mSos1 (Figure 4.8, top panel). However, Grb2 is not detected in the immunoprecipitated samples (Figure 4.8, bottom panel), consistent with the observation that intersectin and Grb2 bind competitively to mSos1.

We next investigated the association of intersectin with mSos1 by subjecting a soluble rat brain protein extract to sucrose density gradient centrifugation and analyzing the distribution of the proteins by Western blotting (Figure 4.9, top panels; the gradient was fractionated from the bottom such that lower fraction numbers correspond to a greater density of sucrose). Intersectin-l was most abundant in fraction 9, but was detectable even in the densest gradient fractions. Interestingly, mSos1 migrated on the gradients with a molecular mass that was similar, but not identical, to that of intersectin-l. Both intersectin-l and mSos1 exhibited higher native molecular masses than either clathrin triskelia (approximately 650 kDa) or the AP-2 heterotetramer (approximately 300 kDa), the migratory positions of which were revealed by Western blots of the clathrin heavy chain or the α-subunit of AP-2, respectively. Intriguingly, Grb2 remained at the top of the gradient and was effectively separated from mSos1.

To confirm that the extremely large native molecular mass for mSos1 was due, at least in part, to its interactions with intersectin, we performed co-immunoprecipitation experiments from the gradient fractions (Figure 4.9, bottom panels). mSos1 could be co-immunoprecipitated with intersectin from each of the gradient fractions containing the two proteins. Overall, the distribution of mSos1 seemed to more closely parallel that

of intersectin-l than intersectin-s. Whether this reflects a relatively higher affinity of intersectin-l versus intersectin-s for mSos1 or is due to the apparent greater abundance of intersectin-l in brain is not known. Regardless, these data suggest that in brain, a pool of the mSos1 protein is in a stable, large molecular weight complex with intersectin that predominantly excludes Grb2.

Over-expression of the SH3 domains of intersectin blocks Ras activation--To determine if the SH3 domain-mediated interactions of intersectin with mSos1 might affect Ras activation, we measured the levels of Ras-GTP in cells with a GST fusion protein encoding the Ras-binding domain of Raf1. This fusion protein binds to GTP-Ras but has significantly weaker affinity for Ras in its GDP bound form (de Rooij and Bos, 1997; Marais et al., 1998). In HEK-293 cells treated with EGF for 2 minutes, a substantial pool of the total cellular Ras binds to GST-Raf1 beads as detected by Western blots with a Ras monoclonal antibody, whereas little binding is detected in the absence of EGF stimulation (Figure 4.10, top panel). Over-expression of GFP has little effect on the ability of EGF to stimulate GTP-loading on Ras (Figure 4.10, bottom panel; note that the ratio of Ras bound to the beads relative to that in the starting material is similar to that seen in the EGF challenged non-infected cells in the top panel). In contrast, over-expression of the GFP-SH3A-E construct sharply attenuates EGF-activated GTP loading on Ras (Figure 4.10, bottom panel). There is no effect of the over-expression of either construct on the basal activity of Ras measured in the absence of EGF (data not shown).

DISCUSSION

The identification and characterization of accessory proteins in endocytosis has received a great deal of attention in recent years. One of these proteins has been identified in Drosophila, Xenopus, and mammals and is variously referred to as Dap160, intersectin, Ese, and EHSH (Guipponi et al., 1998; Hussain et al., 1999; Okamoto et al., 1999; Roos and Kelly, 1998; Sengar et al., 1999; Yamabhai et al., 1998). This family of proteins contains two EH domains, followed by a putative helical region and four to five tandem SH3 domains. Intersectin is localized in part to clathrin-coated pits and interacts *in vivo* through its EH and SH3 domains with several proteins involved in clathrin-mediated endocytosis. In addition, intersectin interacts with another accessory component of the endocytic machinery, Eps15, through its coiled-coil region (Sengar et al., 1999). Thus, intersectin may function as a scaffolding protein in the assembly of clathrin-coated pits.

We have now identified mSos1 as a protein that interacts with the SH3A domain of intersectin. Previously, Leprince *et al.* (Leprince et al., 1997) had isolated amphiphysin II in a two-hybrid screen using the proline-rich C-terminus of human Sos1. In experiments reported here, we have found in a side-by-side comparison using overlay assays, that the SH3A domain of intersectin can bind mSos1 better than the SH3 domains from amphiphysin I and II and endophilin I, II, and III. These data suggest that intersectin can bind mSos1 with higher affinity than other SH3 domain-containing endocytic proteins but do not rule out a role for amphiphysin II in regulating mSos1 function. Intersectin and mSos1 are both expressed at high levels in growth cones of developing neurons, and immunoprecipitation analysis from embryonic brain extracts confirms that the two proteins are associated in situ. In fact, sucrose-density gradients, coupled with co-immunoprecipitation analysis, suggest that in brain, intersectin and mSos1 are components of a large molecular mass protein complex that primarily excludes Grb2. Thus, the interaction of the SH3A domain with mSos1 appears specific and likely biologically relevant.

Given that intersectin is involved in the formation of clathrin-coated pits, and that the intersectin SH3A domain interacts specifically with cellular targets that function

early in the formation of a clathrin-coated bud (Simpson et al., 1999), it is interesting to speculate that mSos1 may also play a role in clathrin-coated pit formation, possibly through activation of Ras. Many vesicular budding events that are mediated by coat proteins are initiated by the activation of small GTP-binding proteins through the actions of guanine-nucleotide exchange factors (Schekman and Orci, 1996). Very recently, Nakashima et al. (1999) have demonstrated that over-expression of mutant forms of Ras, as well as the small GTP-binding protein Ral, which plays a major role in mediating downstream Ras function (Feig et al., 1996), blocks the internalization of the epidermal growth factor (EGF) receptor. Further, mSos1 can activate Rac (Nimnual et al., 1998; Scita et al., 1999), which has been implicated in transferrin receptor endocytosis (Lamaze et al., 1996). Finally, it should be noted that the long form of intersectin, which is generated by alternative splicing in neuronal tissues, contains DH, PH, and C2 domains (Guipponi et al., 1998; Hussain et al., 1999; Okamoto et al., 1999; Sengar et al., 1999). Comparison of the primary structure of the DH and PH domains with other proteins suggests that the long form of intersectin may be a guanine-nucleotide exchange factor for Rho. Further work is necessary to clarify the involvement of GTP-binding proteins in clathrin-mediated endocytosis.

Another possible function of the intersectin/mSos1 complex is to couple the molecular machineries for endocytosis and signal transduction. For example, it has been demonstrated that dynamin-dependent endocytosis of the EGF receptor is necessary for EGF-dependent activation of the MAP-kinase pathway (Vieira et al., 1996). The ability of insulin-like growth factor-1 (IGF-1) to activate the SHC/MAP-kinase pathway, but not the insulin receptor substrate 1 pathway, is also dependent on clathrin-mediated endocytosis of the IGF receptor (Chow et al., 1998). Further, endocytosis of the β2-adrenergic receptor is necessary for coupling to MAP-kinase activation (Daaka et al., 1998; Luttrell et al., 1999). Specifically, over-expression of a mutant form of β-arrestin, which prevents the β2-adrenergic receptor from targeting to clathrin-coated pits, blocks agonist activation of MAP-kinase. Thus, it is possible that the clathrin-coated pit can function as a membrane microdomain, directing the assembly of signaling complexes, much as has been proposed for caveoli (Anderson, 1998). In fact, activation of the EGF

receptor can lead to the formation of signaling complexes that include mSos1 and which are localized largely in endosomes (Di Guglielmo et al., 1994).

Given the evidence for a link between endocytosis and signaling, it is interesting to speculate that intersectin could play an important role in bringing together endocytic proteins such as dynamin, with signaling molecules such as mSos1. In fact, our data demonstrating that over-expression of the SH3 domains of intersectin functions in a dominant-negative manner to block EGF-dependent Ras activation strongly supports a role for intersectin in cell signaling. Moreover, human intersectin has been found to interact by yeast two-hybrid screening with the proto-oncogene product, c-Cbl (Robertson et al., 1997), a tyrosine kinase substrate with ubiquitin ligase activity (Joazeiro et al., 1999), and transfection experiments have revealed that full-length intersectin functions in cell signaling pathways leading to activation of the Elk-1 transcription factor (John O'Bryan, National Institute of Environmental Health Science, personal communication). Finally, in a genetic screen in *Drosophila*, Dap160, the *Drosophila* homologue of intersectin (Roos and Kelly, 1998), was selected as a negatively regulating component of the Sevenless receptor tyrosine kinase/MAP-kinase pathway (Felix Rintelen and Ernst Hafen, University of Zurich, personal communication). Thus, intersectin appears to have a dual function in both endocytosis as well as signal transduction pathways, and it may play a role as an interface between these two important cellular processes.

EXPERIMENTAL PROCEDURES

Antibodies--Polyclonal antibodies against intersectin were previously described (Hussain et al., 1999). Antibodies against clathrin (Simpson et al., 1996) and α-adaptin (Robinson et al., 1987) were a generous gift of Dr. Margaret Robinson (Cambridge University). Polyclonal antibodies against mSos1 (C23) and GFP were purchased from Santa Cruz Biotechnology and Molecular Probes, respectively. Monoclonal antibodies against Grb2 and Ha-Ras were purchased from Transduction Laboratories.

Generation of fusion protein constructs--GST-fusion protein constructs encoding the SH3 domains of amphiphysin I (David et al., 1994), amphiphysin II (Ramjaun et al., 1997), endophilin I (Micheva et al., 1997a), the individual SH3 domains of intersectin (Yamabhai et al., 1998), and full length Grb2 (McPherson et al., 1994a) were previously described. The SH3 domains of endophilin II and III were generated by PCR using full length cDNAs (Sparks et al., 1996a) as templates in PCR reactions with Vent DNA Polymerase (New England Biolabs) and the following primer pairs: endophilin II, forward primer 5'-GCGGGATCCGACCAGCCAAGCTGCAAG (nucleotides 919-936) and reverse primer, 5'-GCGGAATTCTCACTGAGGCAGAGGCACCAG (nucleotides 1104-1087); endophilin III, forward primer 5'-GCGGGATCCGACCAGCCCTGCTGTCG (nucleotides 856-872) and reverse primer, 5'-GCGCCCGGGTCACGGAGGTAAAGGCACAATG (nucleotides 1041-1023). The resulting PCR products were subcloned into the corresponding *Bam*HI-*Eco*RI and *Bam*HI-*Sma*I sites of pGEX-2T (Pharmacia Biotech Inc.), respectively. For (His)6-tagged Grb2, the GST-Grb2 construct in pGEX2T was digested with *Bam*HI plus *Eco*RI enzymes and the liberated insert was subcloned into pTrcHisA (Invitrogen) using the same enzymes.

Generation of recombinant adenovirus--A recombinant adenovirus encoding the five tandem SH3 domains of intersectin with an N-terminal GFP tag was produced using the system of He *et al.* (He et al., 1998). First, the appropriate region of intersectin was

amplified by PCR using a full length cDNA (Yamabhai et al., 1998) with Vent DNA Polymerase (New England Biolabs) and with the forward primer 5'- GCGCTCGAGGGAGAAAGCCCCTCTAACG (nucleotides 2352-2370) and the reverse primer, 5'-GCGGAATTCCTACGGTATTCCACCTGCTGG (nucleotides 3840-3823). The resulting PCR product was digested with *Xho*I and *Eco*RI and subcloned, in-frame, into the corresponding sites of pEGFP-C2 (Clontech) adding the N-terminal GFP tag. The resulting fusion protein construct was liberated with *Nhe*I and *Sma*I and subcloned into the compatible *Kpn*I and *Eco*RV sites of pShuttle-CMV (He et al., 1998). The resulting plasmid was recombined with pAdEasy-1 in BJ5183 cells and recombinant plasmids were selected on kanamycin and identified by *Eco*RI and *Bst*X1 digests (He et al., 1998). Recombinant adenovirus was produced and amplified in HEK-293 cells and purified on $CsCl_2$ gradients as described (He et al., 1998).

Tissue and subcellular distribution experiments--Various rat tissues were homogenized in buffer A (10 mM HEPES-OH, pH 7.4, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonylfluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin). Post-nuclear supernatants were obtained by centrifugation for 5 min at 800 x g_{max} and were then separated into cytsolic and membrane fractions by ultracentrifugation at 205,000 x g_{max} for 1hr at 4°C. Differential centrifugation of rat brain extracts leading to the defined subcellular fractions in Figure 4.2B was as described (McPherson et al., 1994b). Dissociated cell cultures were prepared from the CA3 and dentate regions of hippocampi from P1 rat pups as described (Hussain et al., 1999).

Overlay assays--Overlay assays with GST fusion proteins were performed as described (McPherson et al., 1994a). For competition experiments, GST-SH3A was mixed with increasing concentrations of (His)6-tagged Grb2 immediately prior to addition to the nitrocellulose transfers.

Immunoprecipitation analysis--Cytosolic extracts, prepared as described above, were made to 1% in Triton X-100 and precleared by incubation with protein A-Sepharose. The precleared samples were then incubated with various antibodies precoupled to

protein A-Sepharose, and following an overnight incubation at 4°C, were washed three times in buffer A containing 1% Triton X-100 before the proteins specifically bound to the beads were eluted and processed for SDS-PAGE. In some cases, membrane fractions, generated as described above, were incubated for 30 min at 4°C in buffer A containing 1% Triton X-100, the samples were centrifuged at 205,000 x g_{max} for 1hr at 4°C, and the soluble supernatants were used for immunoprecipitation. For immunoprecipitations from infected cells, PC12 cells were incubated with recombinant adenovirus encoding GFP-SH3A-E in six well plates at a multiplicity of infection of 100. The media was changed the next day, and the cells were left for an additional 48 hr. The cells were then washed, scrapped off the plate in buffer B (buffer A with 5 mM EGTA, 5 mM EDTA, 50 mM sodium flouride, 20 mM sodium pyrophosphate, and 1 mM sodium vanadate), lysed, Triton X-100 was added to 1%, and the extracts were centrifuged in a Beckman TLA 100.2 rotor at 75,000 rpm for 15 min. The solubilized extracts were precleared with protein A-Sepharose before the addition of anti-GFP antibody precoupled to protein A-Sepharose. Following 2 hr incubation, the samples were washed three times in buffer B containing 1% Triton X-100, and the proteins specifically bound to the beads were eluted and processed for SDS-PAGE.

Sucrose density gradient centrifugation--Proteins from a rat brain cytosolic fraction were layered onto linear gradients of 5-20% sucrose prepared in buffer A. The gradients were centrifuged in a STEPSAVER 50 V39 vertical rotor (Sorvall) at 195,000 x g for 2.5 hr and were then collected into 19 fractions of 2 ml each from the bottom. Aliquots of each fraction were analyzed by SDS-PAGE and Western blotting. For immunoprecipitations, 750 µl of each fraction was made to 1% in Triton X-100 before the addition of protein-A Sepharose beads that had been precoupled to intersectin antibody. Following an overnight incubation at 4°C, the samples were washed three times in buffer A containing 1% Triton X-100, and the proteins specifically bound to the beads were eluted and processed for SDS-PAGE.

Determination of SH3A binding specificity by phage display--GST-SH3A was used to affinity select phage from a library of bacteriophage M13 displaying combinatorial peptides at the N-terminus of mature protein III. The library displayed peptides of the form X_6PXXPX_6, where X represents any of the 20 naturally occurring amino acids and P represents invariant proline residues (Sparks et al., 1996b). Three different 14-mer peptides from mSos1 were fused to the N-terminus of secreted bacterial alkaline phosphatase (Yamabhai and Kay, 1997) and their binding to various GST-SH3 domains, which had been immobilized on the surface of microtiter dishes, was monitored with the substrate para-nitrophenyl phosphate. The optical absorbance (OD) of duplicate wells was determined with a plate spectrophotometer at 405 nm wavelength.

Ras assays--Recombinant adenoviruses encoding GFP-SH3A-E or GFP alone were added to HEK-293 cells platted on poly-L-lysine substrate in 10 cm^2 dishes at a multiplicity of infection of 100. The media was changed after 2 hr and the cells were left for an additional 48 hr. The cells were then transferred into serum free media (along with non-infected controls), and following an overnight incubation, were treated with 100 ng/ml EGF for 2 min at 37°C or were left untreated. The cells were then washed and immediately processed for Ras assays as described (de Rooij and Bos, 1997; Marais et al., 1998).

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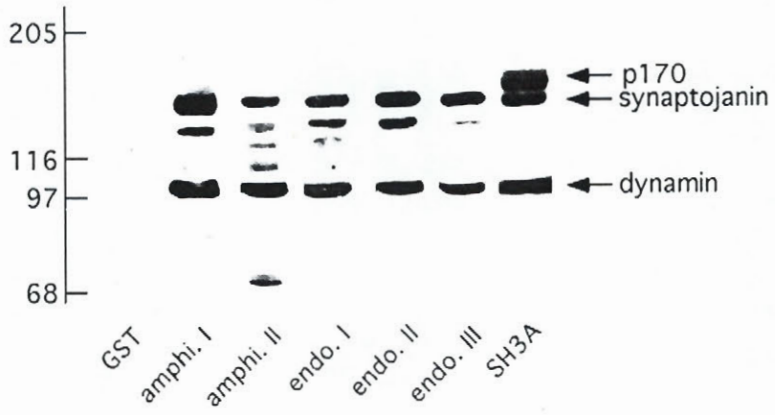

Figure 4.1 Identification of a 170 kDa protein (p170) that binds specifically to the SH3A domain of intersectin. Strips of rat brain cytosolic fraction (100 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with GST alone or GST fused to various SH3 domains of the proteins indicated on the blots (amphiphysin, amphi.; endophilin, endo.; SH3A domain of intersectin, SH3A). The positions of dynamin, synaptojanin, and p170, which are recognized by various SH3 domains, are indicated by arrows on the right. The identities of the additional bands in the overlays are unknown.

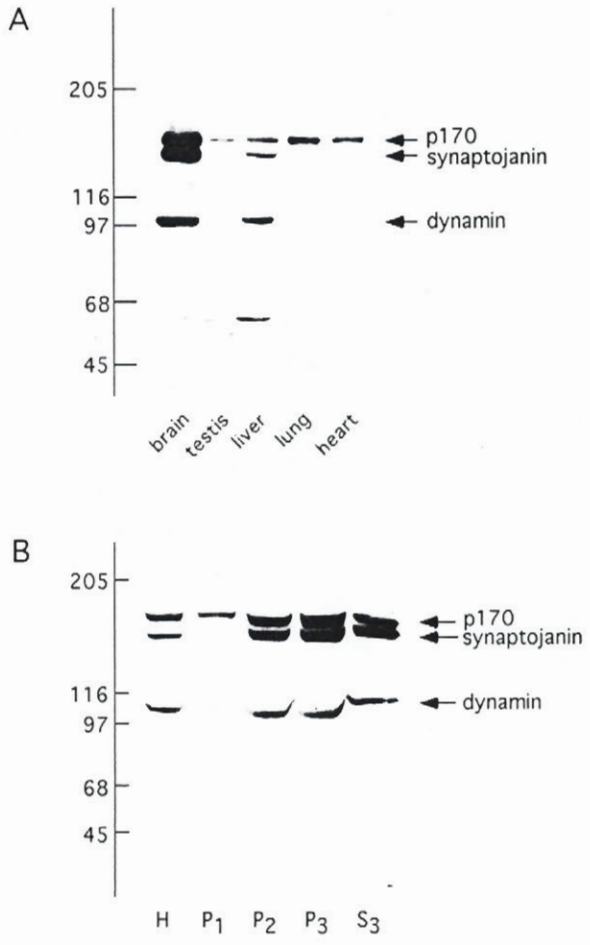

Figure 4.2 Tissue and subcellular distribution of p170. (A) Proteins of cytosolic fractions from various adult rat tissues (100 μg/tissue) were separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with a GST fusion protein encoding the SH3A domain of intersectin. (B) Proteins of brain subcellular fractions (100 μg/fraction) were separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with a GST fusion protein encoding the SH3A domain of intersectin. Subcellular fractions were prepared as described (McPherson et al., 1994b). (homogenate, H; pellet, P; supernatant, S). For both figures, the migratory positions of dynamin, synaptojanin, and p170 are indicated by arrows on the right.

A.

```
HVAGAS PPIPPR MAEL
 FASGA PPVPPR VGKLL
LYIPKR PEVPPR RHEA
YAHVAL PPIPPR SAFD
ALERPL PKVPLR SAEI
FKWGAA PAVPLR AHEL
YGWDKR PPVPPR IYEK
LPGLTP PAVPPR SYEA
 FWEEL PPIPPR AGASV
 MHRDP PPIPPR SAGEV
LVTESP PQVPLR QHER
   RGL PPVPPR MGDAC

      PpVPPR  consensus
        I
```

B.

```
...PVP PPVPPR RRPES... aa 1148-1161
...IAG PPVPPR QSTSQ... aa 1287-1300

..PESP PLLPPR EPVR...  aa 1208-1221
```

C.

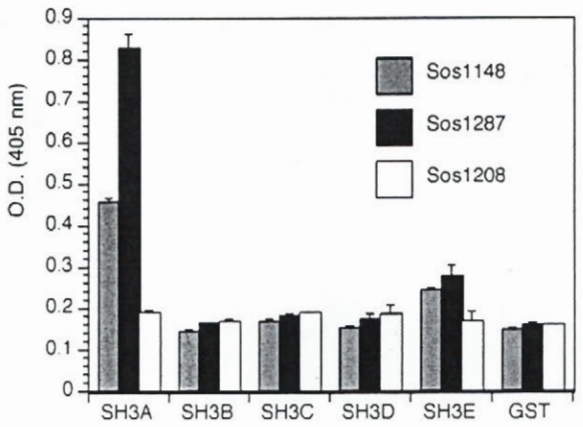

Figure 4.3 Identification of consensus-binding sites for SH3A in mSos1. (A) The sequence of twelve peptides, affinity selected from a phage-displayed X_6PXXPX_6 peptide library (where X is any amino acid) using the SH3A domain from intersectin are listed. The peptides define the SH3A-binding consensus sequence Pp(V/I)PPR, where p is typically proline. (B) Two putative ligand sites for the SH3A domain, PPVPPR, occur within human Sos1 at sequences between amino acids 1148 and 1161 and 1287 and 1300, and a third related site is found between amino acids 1208-1221, as indicated. (C) The segments of human Sos1 shown in B were fused to the N-terminus of secreted alkaline phosphatase and tested for binding to GST fusion protein encoding the individual SH3 domains of intersectin or to GST alone.

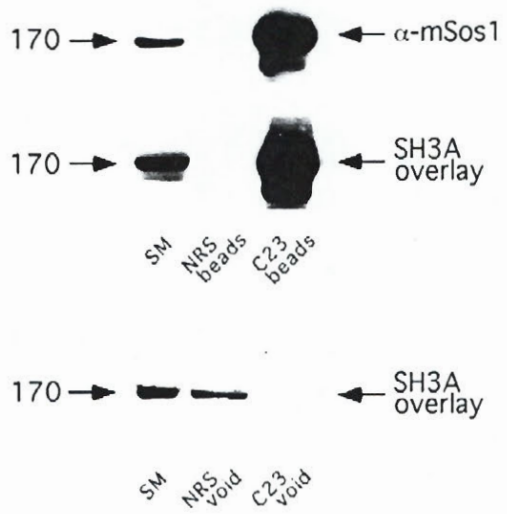

Figure 4.4 Confirmation of p170 as mSos1. Aliquots of antisera C23 against mSos1, as well as normal rabbit sera (NRS), were pre-coupled to protein A-Sepharose beads. Pre-coupled beads were washed, incubated overnight at 4°C with an E18 rat brain cytosolic fraction, and extensively washed the next day. The material specifically bound to the beads (top two blots labeled beads) was eluted and processed, along with an aliquot of the cytosolic extract (starting material, SM), for Western blot analysis with the anti-mSos1 antibody (top blot), or for SH3A domain overlay (middle blot). The proteins that did not bind to the beads (void) were also subjected to an SH3A domain overlay assay (bottom blot).

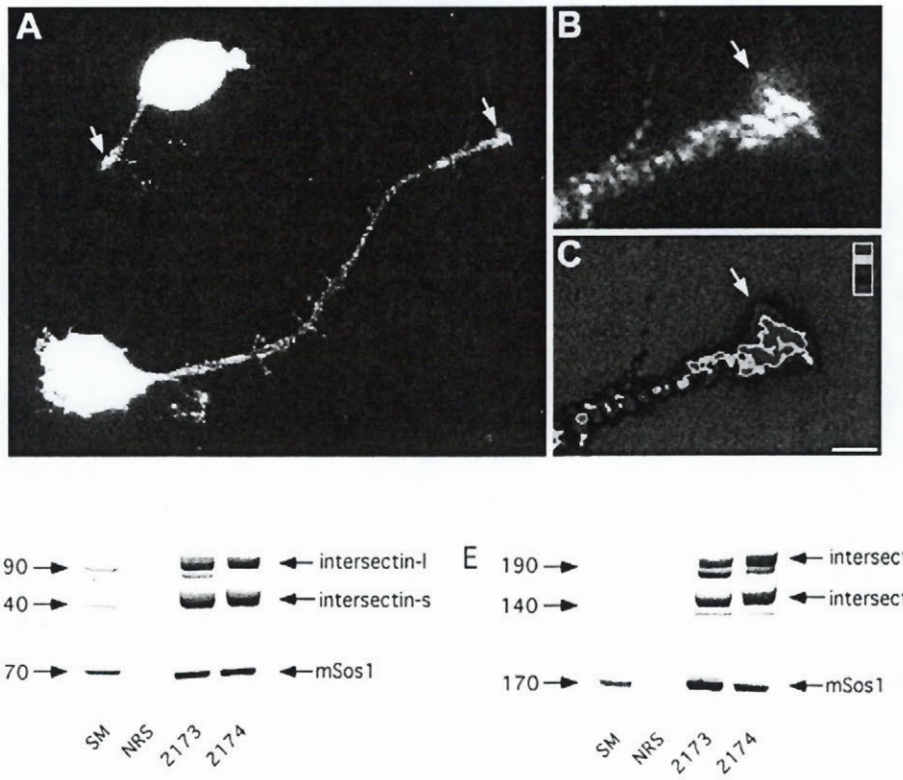

Figure 4.5 Interaction of intersectin and mSos1 in situ. (A) mSos1 is found in punctae that are expressed throughout the cell body and neurites of hippocampal neurons maintained in culture for one day. The punctae are relatively homogeneous along the neurite but are enriched at growth cones (arrows). (B) A higher magnification image of the growth cone in A reveals that the density of mSos1 positive punctae is higher at the tip of the growth cone than along the neurite. (C) Color coding of fluorescent intensities of the area in B indicates that the intensity of individual mSos1 positive punctae is higher in the growth cone (red color) than in other regions of the dendrite. Scale bar: (A)=10 μM, (B,C) = 1.5 μM. (D) Aliquots of antisera 2173 and 2174 against intersectin, as well as pre-immune 2173 sera (NRS), precoupled to protein A-Sepharose beads, were incubated overnight at 4°C with an E18 rat brain cytosolic fraction. The beads were extensively washed and the material specifically bound to the beads was eluted and processed for Western blot analysis with polyclonal antibodies against mSos1 and intersectin. (E) As for D except that immunoprecipitations were performed from Triton X-100 solubilized membrane fractions. The antigens and their approximate molecular weights (kDa) are denoted with arrows on the right and left sides of the figure, respectively.

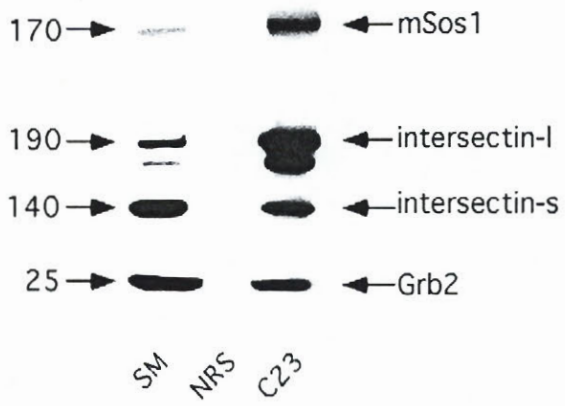

Figure 4.6 Co-immunoprecipitation of intersectin and Grb2 with mSos1. Aliquots of antisera C23 against mSos1, as well as normal rabbit sera (NRS), were pre-coupled to protein A-Sepharose beads. Pre-coupled beads were washed, incubated overnight at 4°C with an E18 rat brain cytosolic fraction, and extensively washed the next day. The material specifically bound to the beads was eluted and processed for Western blot analysis, along with an aliquot of the cytosolic extract (starting material, SM), with polyclonal antibodies against mSos1 and intersectin, and a monoclonal antibody against Grb2. The antigens and their approximate molecular weights (kDa) are denoted with arrows on the right and left sides of the figure, respectively.

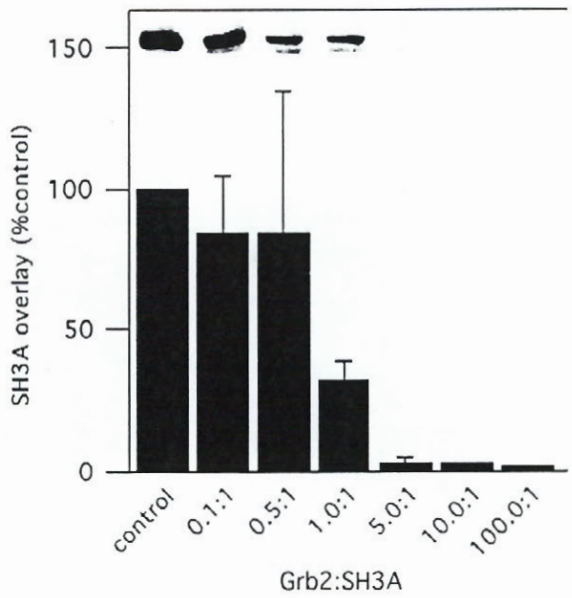

Figure 4.7 *In vitro* competition binding assays of intersectin SH3A domain and Grb2 to mSos1. E18 rat brain cytosolic fractions were separated on SDS-PAGE, transferred to nitrocellulose membranes, and strips of the membrane were processed for overlay assays with GST-SH3A at 200 ng/ml. The overlay assays also contained (His)6-tagged Grb2 at increasing molar ratios of Grb2 to SH3A ranging from 0:1 (control) to 100:1 as indicated at the bottom of the figure. An example of the overlay results are shown at the top of the graph. The intensity of the stained mSos1 band was determined by densitometry of the autoradiographs and was normalized to control. The bars represent the mean ± the s.e.m. from three separate experiments.

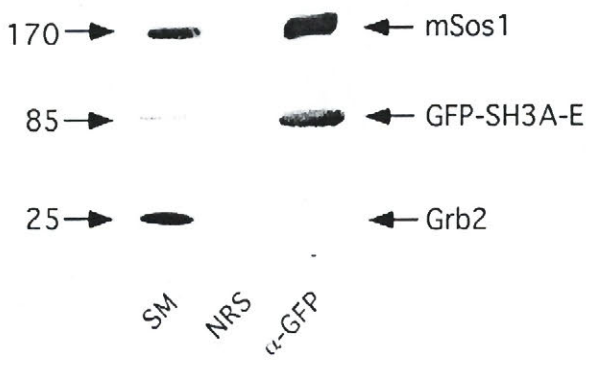

Figure 4.8 Co-immunoprecipitation of mSos1 with the SH3 domains of intersectin. Undifferentiated PC12 cells were infected with a recombinant adenovirus encoding the five tandem SH3 domains of intersectin fused to GFP (GFP-SH3A-E). Following protein expression, a soluble cell lysate was prepared and incubated overnight at 4°C with anti-sera against GFP or normal rabbit sera (NRS) pre-coupled to protein A-Sepharose beads. The beads samples were extensively washed the next day, and the material specifically bound to the beads was eluted and processed for Western blot analysis, along with an aliquot of the cell extract (starting material, SM), with polyclonal antibodies against mSos1 and GFP, and a monoclonal antibody against Grb2. The antigens and their approximate molecular weights (kDa) are denoted with arrows on the right and left.

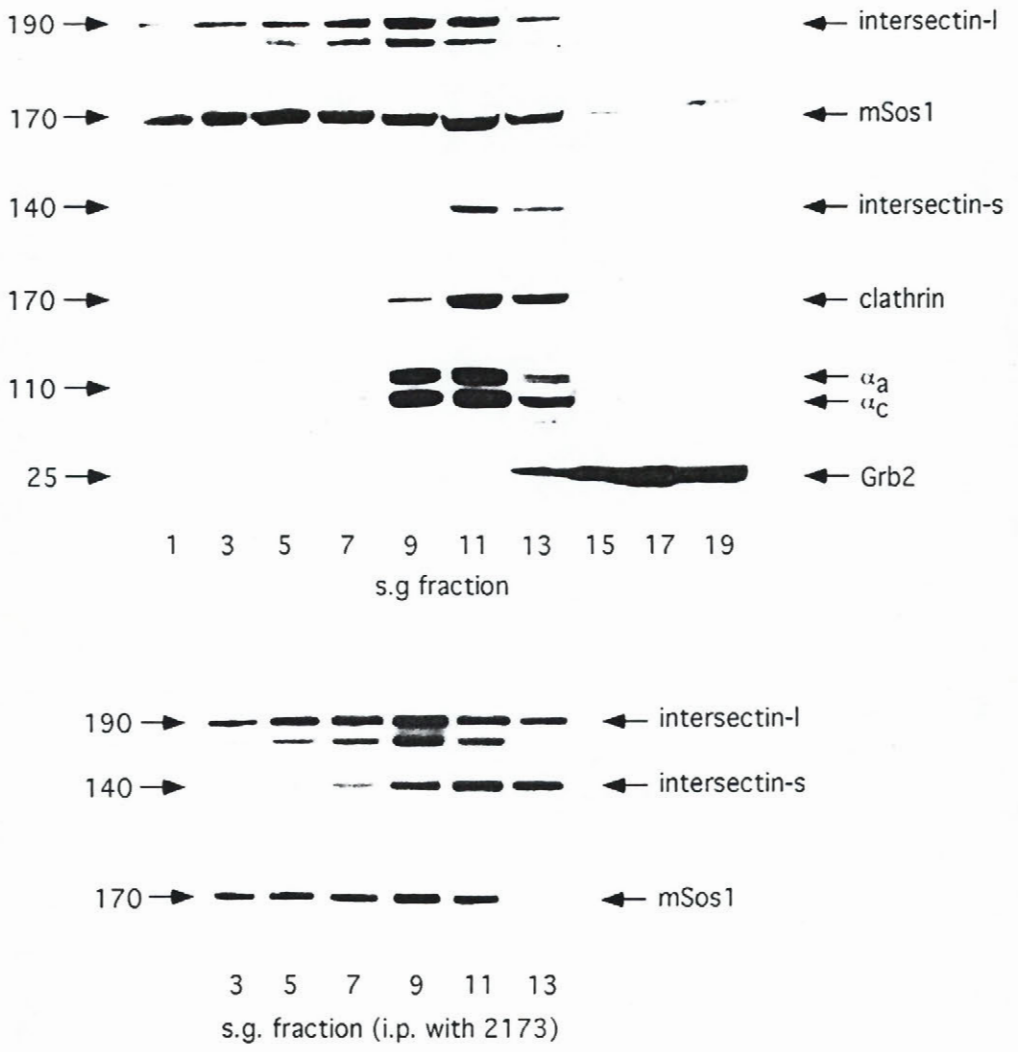

Figure 4.9 Sucrose-density gradient analysis of mSos1 interactions. Proteins from a cytosolic extract of rat brain were separated on 5-20% linear sucrose-density gradients by centrifugation at 195,000 x g for 2.5 hr and the gradients were then collected into 19 fractions of 2 ml each from the bottom. Top panels: Aliquots (100 µl) of odd gradient fractions (s.g. fraction) were analyzed by Western blot using a variety of antibodies (the antigens and their approximate molecular masses in kDa are denoted with arrows on the right and left sides of the figure, respectively). Bottom panels: Aliquots (750 µl) of the indicated gradient fractions were incubated with protein-A Sepharose beads pre-coupled to intersectin antibody (2173). Following an overnight incubation at 4°C, the samples were washed and the proteins specifically bound to the beads were eluted with gel sample buffer and processed for SDS-PAGE and Western blot analysis with intersectin and mSos1 antibodies. The antigens and their approximate molecular masses in kDa are denoted with arrows on the right and left sides of the figure, respectively.

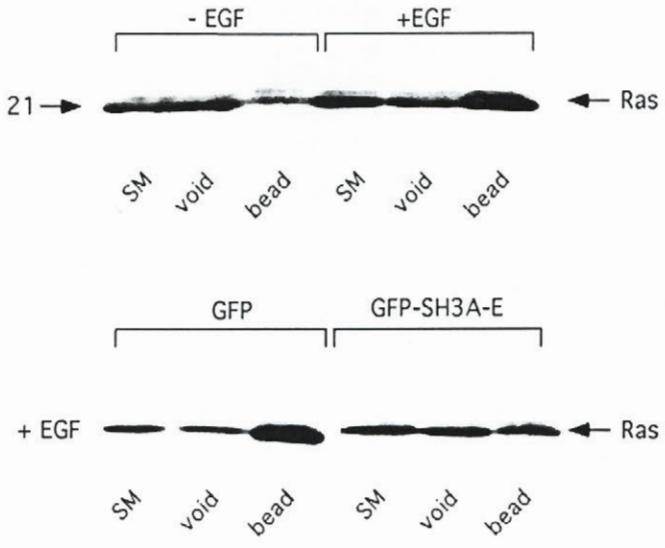

Figure 4.10 Inhibition of Ras activation by the SH3 domains of intersectin. (Top panel) HEK-293 cells were serum starved overnight and were then challenged for 2 min with serum free media (-EGF) or with serum free media containing 100 ng/ml EGF (+EGF). The cells were then washed, lysates prepared, and incubated with a GST fusion protein encoding the Ras-GTP binding domain of Raf1 coupled to glutathione-Sepharose beads. Following incubation, the beads were washed and material bound to the beads (bead) was processed for SDS-PAGE, along with an aliquot of the cell lysate (starting material, SM) (equal to one-tenth the amount added to the beads) and an equal aliquot of the unbound material (void). The samples were processed for Western blots with a monoclonal antibody against Ras as indicated on the figure. (Bottom panel) HEK-293 cells infected with recombinant adenovirus encoding GFP alone (GFP) or the five tandem SH3 domains of intersectin coupled to GFP (GFP-SH3A-E) were processed for Ras assays following a 2 min exposure to EGF.

PREFACE TO CHAPTER 5: Characterizing partners of intersectin-l carboxy-terminal DH-PH domain

In the study described in Chapter 4, we provided evidence that intersectin-s forms a complex in brain with mSOS, a guanine nucleotide exchange factor (GEF) for Ras and Rac (Nimnual et al., 1998). GEFs are molecular switch proteins that can specifically activate signaling cascades to regulate a variety of cellular processes including transcription, transformation, and cell growth (Etienne-Manneville and Hall, 2002; Manser, 2002). The typical function attributed to Ras is to regulate the MAPK pathway, while Rac is best characterized for its ability to regulate the actin cytoskeleton (Hall, 1998). Rac has been shown to interact with microtubules, and although much remains to be delineated regarding this interaction, it is possible that Rac can additionally regulate the microtubule cytoskeleton (Glaven et al., 1999; Hu et al., 2002; Ory et al., 2002). Thus, these data suggest an additional and possibly complementary role for the endocytic protein intersectin-s in signaling mechanisms and/or actin cytoskeleton dynamics.

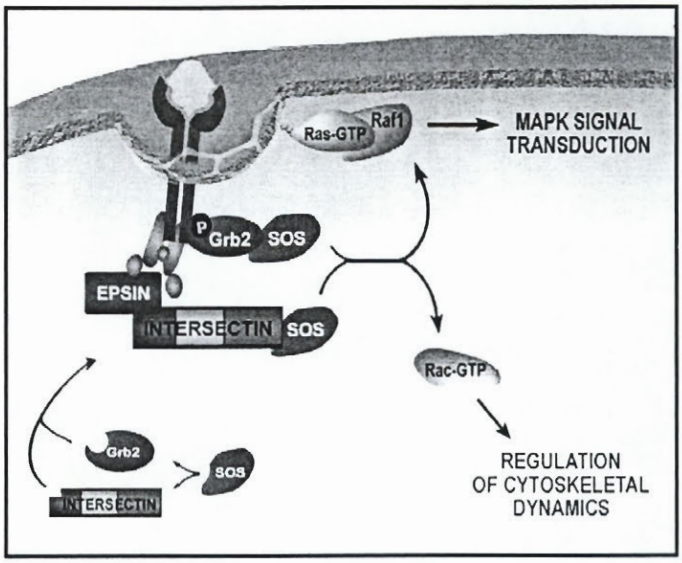

Figure 5.0 Schematic of the links between endocytic and signal transduction components provided by intersectin binding to Sos.

The neuronal enriched protein intersectin-l may be additionally linked to the cytoskeleton by virtue of the structure of its carboxy-terminus. Intersectin-l is contrasted from intersectin-s by the presence of a carboxy-terminal extension that includes a Dbl homology (DH) in tandem with a pleckstrin homology (PH) domain. This structure is a characteristic feature of all GEFs for the Rho GTPase family of proteins, which functionally regulate the architecture of the actin cytoskeleton (Bishop and Hall, 2000). Thus, the final component of my thesis focused on characterizing the possible role of the carboxy-terminal extension specific to intersectin-l.

CHAPTER 5. THE ENDOCYTIC PROTEIN INTERSECTIN-1 REGULATES ACTIN ASSEMBLY VIA CDC42 AND N-WASP

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ABSTRACT

Intersectin-s is a modular scaffolding protein regulating the formation of clathrin-coated vesicles (Sengar et al., 1999; Simpson et al., 1999). In addition to the Eps15 homology (EH) and Src homology 3 (SH3) domains of intersectin-s, the neuronal variant (intersectin-l) also has Dbl homology (DH), pleckstrin homology (PH) and C2 domains (Guipponi et al., 1998; Hussain et al., 1999; Okamoto et al., 1999; Roos and Kelly, 1999; Sengar et al., 1999; Yamabhai et al., 1998) . We now show that intersectin-l functions through its DH domain as a guanine nucleotide exchange factor (GEF) for Cdc42. In cultured cells, expression of DH-domain-containing constructs cause actin rearrangements specific for Cdc42 activation. Moreover, *in vivo* studies reveal that stimulation of Cdc42 by intersectin-l accelerates actin assembly via N-WASP and the Arp2/3 complex. N-WASP binds directly to intersectin-l and upregulates its GEF activity, thereby generating GTP-bound Cdc42, a critical activator of N-WASP. These studies reveal a role for intersectin-l in a novel mechanism of N-WASP activation and in regulation of the actin cytoskeleton.

RESULTS AND DISCUSSION

Intersectin-s functions as a scaffolding protein in the formation of endocytic vesicles. Whereas the EH domains of intersectin-s target the protein to clathrin-coated pits (Hussain et al., 1999), its SH3 domains recruit dynamin to these endocytic structures (Okamoto et al., 1999; Roos and Kelly, 1999; Sengar et al., 1999; Yamabhai et al., 1998). Intersectin-l, an alternatively spliced form of intersectin-s, includes a carboxy (C)-terminal extension with a DH domain in tandem with a PH domain (Fig. 5.1a), an organizational feature of GEFs that activate Rho family GTPases by catalysing the exchange of GDP for GTP (Whitehead et al., 1997). Rho GTPases control a variety of cellular functions including membrane trafficking and axon guidance (Van Aelst and D'Souza-Shorey, 1997), but are best characterized as regulators of the actin cytoskeleton (Hall, 1998). Specifically, RhoA induces the formation of actin stress fibres and Rac1 and Cdc42 cause actin assembly, leading to the formation of lamellipodia and filopodia, respectively (Hall, 1998).

To explore the potential GEF function and substrate specificity of intersectin-l, we examined its binding to various GTPases in their nucleotide-free state, a condition under which GEF–GTPase interactions are most stable (Hart et al., 1994). Green fluorescent protein (GFP) fusions encoding the DH–PH domains, the complete tail domain (DH–PH–C2) or the isolated C2 domain (Fig. 5.1a) were expressed in cultured cells and tested for binding to RhoA, Rac1 and Cdc42 glutathione-*S*-transferase (GST) fusion proteins. The DH–PH and tail constructs, but not the C2 domain, bound to Cdc42 but not to RhoA, Rac1 or GST (Fig. 5.1b). Conversely, Myc-tagged Cdc42 bound to GST–DH–PH–C2 and GST–DH–PH, but not to GST–C2 or GST alone (see *Supplementary Information Chapter 5*). Furthermore, anti-GFP antibodies efficiently immunoprecipitated Myc-tagged Cdc42 along with a variety of DH-domain-containing GFP-tagged constructs, whereas no immunoprecipitation was seen with the isolated PH or C2 domains or with GFP alone (Fig. 5.1c). Hence, the DH domain of intersectin-l binds specifically to Cdc42. This interaction is direct because purified Cdc42 cleaved from GST by thrombin binds to purified GST–DH–PH but not to GST alone (Fig. 5.1d and data not shown).

We next tested whether the nucleotide state of Cdc42 affected its interaction with intersectin-l. A GST construct encoding the isolated DH domain bound strongly to wild-type, nucleotide-free Cdc42, with moderate binding to the constitutively GDP-bound Cdc42N17 mutant and no binding to the GTPase-defective mutant Cdc42L61 (Fig. 5.1e). The lack of binding of Cdc42L61 indicates that intersectin-l does not interact with the GTP-bound form of Cdc42. This pattern is consistent with that seen for other GEFs in binding to their substrate GTPases (Whitehead et al., 1997). To assess intersectin-l GEF activity directly, we followed the time-dependent incorporation of GTP-γ [^{35} S] onto GDP-preloaded Cdc42 in the presence of GFP-tagged intersectin-l tail constructs immunoprecipitated from transfected cells. GFP–DH mediated significant GEF activity compared with that observed with GFP–PH, GFP alone or mock immunoprecipitates (Fig. 5.1f). The activity of the isolated DH domain was consistently lower than that of constructs that also included the PH domain (Fig. 5.1f). The amount of GFP construct added to the assays was assessed by anti-GFP western blots (Fig. 5.1f).

To test for intersectin-l GEF activity towards Cdc42 in intact cells, alterations in the actin cytoskeleton mediated by Cdc42 activation were assessed in Swiss 3T3 fibroblasts (Nobes and Hall, 1995). Cells microinjected with an effector mutant of Cdc42 that is constitutively GTP bound but does not cascade to Rac activation (Myc-Cdc42L61A37) (Lamarche et al., 1996) showed robust filopodium formation (Fig. 5.2), characteristic of Cdc42 activation (Nobes and Hall, 1995). By contrast, cells microinjected with Flag-tagged intersectin-s, which lacks the DH domain, had a similar morphology to non-injected cells. As predicted, cells expressing Flag–intersectin-l displayed extensive filopodia (Fig. 5.2). Intersectin-l expression was detected in filopodia, whereas a punctate staining pattern was observed for intersectin-s. To confirm that cytoskeletal reorganization induced by intersectin-l was mediated by Cdc42, we injected intersectin-l along with a GST fusion protein encoding the Cdc42/Rac interactive-binding motif (CRIB) of the Wiskott–Aldrich syndrome protein, WASP (Burbelo et al., 1995). This domain binds specifically to GTP-bound Cdc42 and inhibits Cdc42-induced signaling pathways (Symons et al., 1996). Microinjection of WASP CRIB blocked filopodium formation induced by intersectin-l, showing the Cdc42 specificity of the observed

effects (Fig. 5.2). No lamellipodia or stress fibres were observed after expression with the CRIB domain, as would be expected if intersectin-l also displayed GEF activity towards Rac1 or RhoA. The ability of intersectin-l to induce filopodia is localized to its DH domain, because cells microinjected with GFP–DH, GFP–DH–PH and GFP–DH–PH–C2 each produced extensive filopodia (see *Supplementary Information Chapter 5*). Although there was some degree of variability in cell shape after the injection of different DH-domain-containing constructs, similar variability was seen between cells injected with Myc-Cdc42L61A37 and, in all cases, filopodium formation was the salient morphological change (Fig. 5.2).

Filopodia were detected in 94±3.6% of the cells injected with Myc-Cdc42L61A37, whereas they were detected in only 11±3.9% of cells injected with intersectin-s. Intersectin-l expression caused 53±6.6% of the expressing cells to display filopodia, whereas this was reduced to 2.3±2.3% in cells also expressing the WASP CRIB domain. Each of the isolated tail constructs containing the DH domain displayed greater activity than did full-length intersectin (GFP–DH, 83±7%; GFP–DH–PH, 77±6.2%, GFP–DH–PH–C2, 77±5.6%).

To test for intersectin-l GEF activity towards Cdc42 in a neuronal model, we used N1E-115 neuroblastoma cells, which show Cdc42-dependent neurite extension in response to serum starvation (Kozma et al., 1997; Sarner et al., 2000). Five hours after serum withdrawal, a significantly higher proportion of cells expressing GFP–DH–PH–C2 elaborated neurites (38.3±1.9%) than did cells expressing GFP alone (24.3±0.9%) (see *Supplementary Information Chapter 5*). Not only is activated Cdc42 necessary for neurite outgrowth in N1E-115 cells but also its activation is sufficient to stimulate neurite outgrowth, even in the presence of serum (Sarner et al., 2000). GFP–DH–PH–C2 expression was sufficient to overcome serum-mediated suppression of neurite outgrowth because the proportion of cells elaborating neurites increased threefold in GFP–DH–PH–C2-expressing cells (12.5±1.1%) relative to GFP-expressing cells (4.4±0.2%) (see *Supplementary Information Chapter 5*).

An important protein linking Cdc42 activation to the regulation of the actin cytoskeleton is the neuronal-enriched homologue of WASP, N-WASP (Miki et al., 1996; Miki et al., 1998). N-WASP regulates actin polymerization by stimulating the

actin-nucleating activity of the Arp2/3 complex (Machesky and Insall, 1999). To examine the effector pathway by which the GEF activity of intersectin-l mediates actin assembly *in vivo*, we used a recently developed actin nucleation assay based on permeabilized, acutely isolated human neutrophils (Glogauer et al., 2000). When compared with a control (GST alone), the addition of DH-domain-containing constructs stimulated actin nucleation, as indicated by robust increases in the number of free actin barbed ends (GST–DH–PH, 308±30% of control; GST–DH, 179±27% of control) (Fig. 5.3). The addition of 3 μM CA, an N-WASP-derived peptide that inhibits N-WASP interactions with Arp2/3 and subsequent Arp2/3-induced actin nucleation (Glogauer et al., 2000), completely blocked (102±30% of control) actin nucleation induced by GST–DH–PH (Fig. 5.3). Addition of the CA peptide in the absence of the DH–PH fusion protein had no effect on basal actin nucleation levels (data not shown). Thus, intersectin-l regulates actin assembly by stimulating N-WASP-dependent activation of the Arp2/3 actin-nucleating complex.

N-WASP contains multiple SH3-domain-consensus binding sites (Miki et al., 1996), raising the possibility that intersectin-l, a GEF for Cdc42, interacts directly with N-WASP, a major Cdc42 effector protein (Miki et al., 1996; Miki et al., 1998). To test for this interaction, we used pull-down assays with GST fusion proteins encoding the SH3 domains of intersectin. GFP–N-WASP from transfected cells bound variably to the five SH3 domains with no binding to GST alone (Fig. 5.4a). Similar results were obtained when examining endogenous N-WASP in pull-down assays from brain extracts (data not shown). Furthermore, immunoprecipitated GFP-tagged N-WASP was readily detected by overlay with GST–SH3A of intersectin, whereas no bands were detected in immunoprecipitates from GFP-transfected cells or by overlay with GST alone (see *Supplementary Information Chapter 5*). GST–SH3B of intersectin bound very weakly to N-WASP in overlay and binding was only detected after extended exposure (see *Supplementary Information Chapter 5*). These results show that intersectin-l interacts directly with N-WASP through selected SH3 domains.

Following expression of GFP–N-WASP and Flag–intersectin-l in fibroblasts, immunofluorescence analysis revealed that a portion of the transfected cells had a highly punctate staining pattern for GFP–N-WASP that was partially localized with

Flag–intersectin-l (Fig. 5.4b). Co-staining with fluorescent phalloidin revealed that many of the structures displayed assembled actin adjacent to the N-WASP (Fig. 5.4b). N-WASP has been previously reported to assemble actin on endocytic vesicles as a mechanism of vesicle propulsion (Taunton et al., 2000).

Immunoprecipitation assays revealed that GFP–N-WASP was specifically purified with Flag–intersectin-l after immunoprecipitation from fibroblasts (see *Supplementary Information Chapter 5*). To examine the interaction of intersectin and N-WASP *in vivo*, we performed immunoprecipitation experiments from rat brain extracts. An antibody that recognizes both intersectin-s and intersectin-l (2173) immunoprecipitated both proteins from a Triton-X-100-solubilized membrane fraction and also led to the specific immunoprecipitation of N-WASP, whereas no N-WASP was purified in immunoprecipitations performed with normal rabbit serum (Fig. 5.4c). Intersectin did not purify with the abundant brain protein tubulin in the same experiment (Fig. 5.4c). We next examined the relative distributions of intersectin and N-WASP in endocytic vesicles. A pool of intersectin-s and intersectin-l was found to distribute with N-WASP and actin in highly purified clathrin-coated vesicles prepared from rat brain (Fig. 5.4d). By contrast, endophilin (an abundant brain protein that is primarily soluble (de Heuvel et al., 1997)) was not associated with the purified clathrin-coated vesicles. Together, these data show that intersectin interacts specifically and directly with N-WASP both *in vitro* and *in vivo*.

We next assessed the effect of N-WASP binding on intersectin-l function. In previous experiments, we showed that the isolated DH domain of intersectin-l interacts with the constitutively GDP-bound Cdc42N17 mutant (Fig. 5.1e). Surprisingly, no immunoprecipitation of Myc-Cdc42N17 with Flag-tagged full-length intersectin-l was detected after transfection of the two constructs in fibroblasts (Fig. 5.5a). However, Cdc42N17 did immunoprecipitate with intersectin-l when the cells were also transfected with GFP–N-WASP (Fig. 5.5a). As expected, Cdc42N17 did not immunoprecipitate directly with N-WASP (Fig. 5.5a). Thus, N-WASP interactions with intersectin-l seem to stabilize the interaction of full-length intersectin-l with GDP-bound Cdc42.

In *in vitro* exchange assays, GFP–DH–PH strongly stimulated guanine nucleotide exchange on Cdc42 compared with GFP alone, whereas GFP–intersectin-l (in the presence of GST) displayed a more moderate stimulatory effect (Fig. 5.5b). This result is consistent with the earlier experiments showing that the isolated tail constructs of intersectin-l were more effective than the full-length protein at stimulating the formation of filopodia in Swiss 3T3 fibroblasts. Our data suggest that N-WASP stabilizes intersectin-l–Cdc42 interactions, and so we examined whether the binding of N-WASP to intersectin-l affected its GEF activity. The addition of the isolated proline-rich domain of N-WASP (amino acids 265–391 of rat N-WASP) expressed and purified as a GST fusion protein (GST–PRD) caused a dose-dependent stimulation of guanine nucleotide exchange on Cdc42 catalysed by intersectin-l (Fig. 5.5b). Guanine nucleotide exchange on Cdc42 was not enhanced by the addition of GST–PRD in the absence of GFP–intersectin-l (Fig. 5.5b). The amount of intersectin-l added to each assay is estimated to be 1 μM based on Coomassie Blue staining (data not shown). Thus, the binding of N-WASP to the SH3 domains of intersectin-l is a potential mechanism for stimulating the GEF activity towards Cdc42 in cells.

Collectively, our results suggest a model (Fig. 5.5c) in which N-WASP binding to intersectin-l enhances the ability of the DH domain to interact with GDP-bound Cdc42 and to catalyse its conversion to GTP-bound Cdc42. Activated Cdc42 could subsequently interact with N-WASP to stimulate Arp2/3-dependent actin nucleation and assembly (Fig. 5.5c). Thus, by regulating the enzymatic activity of intersectin-l, N-WASP might participate in its own activation. This regulation of intersectin-l GEF activity is reminiscent of that recently described for p190RhoGEF (van Horck et al., 2001). Although the isolated DH–PH domains of p190RhoGEF activate exchange on RhoA, the full-length protein has no effect, suggesting that the protein requires as-yet-unknown binding partners to unmask its GDP and GTP exchange activity (van Horck et al., 2001). Similarly, the DH-domain-dependent GEF activity of both mSos and RasGRF1 are only seen upon their binding to regulatory signaling proteins (Kiyono et al., 1999; Scita et al., 1999).

Emerging data suggest a role for the actin cytoskeleton in synaptic vesicle endocytosis (Qualmann et al., 2000). Filamentous actin is most prominently detected in

nerve terminals after treatments that lead to increased synaptic vesicle endocytosis (De Camilli et al., 2000). Moreover, these actin networks are manifest in the area surrounding the active zone, a region specialized for endocytosis (De Camilli et al., 2000). Specialized sites of endocytosis dependent on the actin cytoskeleton have also been described in fibroblasts (Gaidarov et al., 1999) and in *Drosophila* neuromuscular junctions (Roos and Kelly, 1999). In non-neuronal systems, actin-rich networks referred to as 'actin comet tails' have been detected on endocytic vesicles *in vitro* (Merrifield et al., 1999; Taunton et al., 2000). The assembly of actin comet tails, which generate propulsive forces to drive endocytic vesicle transport, depends on the activation of N-WASP (Taunton et al., 2000). Here, we show that intersectin-l stimulates actin assembly in an N-WASP-dependent manner. Thus, intersectin-l might function with N-WASP and Arp2/3 at an interface between the formation of clathrin-coated vesicles and the regulation of the actin cytoskeleton. The yeast endocytic protein Pan1p has been recently shown to bind and activate the Arp2/3 complex directly (Duncan et al., 2001). Eps15, the mammalian homologue of Pan1p, forms heterodimers with intersectin (Sengar et al., 1999), further suggesting a link of intersectin to the actin regulatory machinery. Recent studies have identified several proteins that might function at an interface between endocytosis and actin regulation (Qualmann et al., 2000). However, intersectin-l is the only described component of the endocytic machinery with an enzymatic activity directed towards actin assembly. The recent identification of a ubiquitously distributed form of intersectin that also carries a DH domain (Pucharcos et al., 2000; Sengar et al., 1999) could allow intersectin to link endocytosis to actin regulation in multiple cell types.

EXPERIMENTAL PROCEDURES

Antibodies and cDNA constructs--Antibodies against intersectin (Hussain et al., 1999) and endophilin (Micheva et al., 1997a) were as previously described. An anti-N-WASP antibody (Miki et al., 1996) was a gift from H. Miki. Antibodies against tubulin (Sigma), actin (ICN Biomedicals), GFP (Molecular Probes), Myc (PharMingen International), Cdc42 (Santa Cruz Biotechnology) and Flag (M1, Sigma) were obtained commercially. The production of constructs encoding various regions of human intersectin-l tail regions fused to GST and GFP, full-length human intersectin-s and intersectin-l fused to GFP or the FLAG epitope, and GFP-tagged N-WASP are described in the *Supplementary Information Chapter 5*. A GST fusion protein construct in baculovirus encoding the proline-rich domain of rat N-WASP amino acids 265–391 was a gift from H. Miki. GST fusion proteins with RhoA, Rac1, Cdc42 (Nobes and Hall, 1995), the WASP CRIB domain (Miki et al., 1996) and the individual SH3 domains of *Xenopus* intersectin (Yamabhai et al., 1998) were as described previously. Myc-tagged Cdc42, Cdc42N17 and Cdc42L61 were generous gifts of M. Olson. Cdc42L61A37 was as described previously (Lamarche et al., 1996).

Affinity-selection assays--HEK-293 cells were washed into serum-free media 24 h after transfection with various constructs. After an additional 24 h, the cells were lysed (0.5 ml per 10 cm^2 dish) in nucleotide-free (NF) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 0.5 μg ml^{-1} aprotinin and 0.5 μg ml^{-1} leupeptin). Lysates were sonicated and centrifuged at 245,000 g_{max} for 15 min and the supernatants (800 μg protein) were incubated with ~50 μg of GST fusion protein constructs precoupled to glutathione–Sepharose beads. When GST–GTPases were coupled to glutathione–Sepharose (see *Supplementary Information Chapter 5*), the beads were washed in 10 mM EDTA before incubation with soluble extracts. GST–Cdc42 purified after bacterial expression was cleaved with thrombin to remove the GST tag and was then incubated with GST–DH–PH coupled to glutathione–Sepharose in NF buffer (Fig. 5.1d). After incubation at 4 °C, the beads were washed in NF buffer and specifically bound proteins were eluted with SDS gel sample buffer and processed for

western blot analysis. Some assays were performed as described above except that cells were left in serum-containing medium and lysates were prepared in G buffer (NF buffer with 10 mM $MgCl_2$ substituted for 10 mM EDTA; Fig. 5.1e) or lysates were made in L buffer (10 mM HEPES, pH 7.4, containing 1% Triton X-100, 0.5 μg ml^{-1} aprotinin, 0.5 μg ml^{-1} leupeptin; Fig. 5.4b). Overlay assays with GST fusion proteins were performed as described (McPherson et al., 1994a).

Immunoprecipitation assays--Soluble extracts were prepared from transfected cells in NF buffer (Fig. 5.1c), L buffer (see *Supplementary Information Chapter 5*) or G buffer (Fig. 5.5a) as described above. The extracts were precleared by incubation with protein-A–Sepharose or protein-G–Sepharose beads before the addition of anti-GFP or anti-Flag antibodies, respectively, precoupled to the corresponding Sepharose beads. After a 2 h incubation, the samples were washed in the corresponding buffer and proteins specifically bound to the beads were eluted and processed for SDS-PAGE and western blot or overlay analyses. Immunoprecipitation assays from brain extracts were performed as described previously (Tong et al., 2000).

Immunofluorescence of transfected cells--HEK-293 cells were plated on poly-L-lysine-coated coverslips as previously described (Hussain et al., 1999). 48 h after transfection, cells were fixed in 3% paraformaldehyde and processed for indirect immunofluorescence with various antibodies and for direct immunofluorescence with TRITC-conjugated phalloidin as described (Lamarche-Vane and Hall, 1998). Images were captured with a scanning laser confocal microscope.

In vitro exchange assays--48 h after transfection, HEK-293 cells were processed in 0.5 ml per 10 cm^2 dish of lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5 μg ml^{-1} aprotinin, 0.5 μg ml^{-1} leupeptin). The samples were sonicated and spun at 245,000 g_{max} for 15 min and the supernatants were incubated with anti-GFP antibodies precoupled to protein-A–Sepharose. After an overnight incubation at 4 °C, the beads were washed with assay buffer (lysis buffer excluding Triton X-100). For assays performed in the presence of GST fusion proteins, immunoprecipitated beads

were preincubated with purified fusion proteins for 2 h at 4 °C before performing exchange assays. Guanine-nucleotide exchange assays were performed essentially as described (Horii et al., 1994). Briefly, 0.4 μM purified, GDP-loaded Cdc42 was incubated with 5 μM GTPγ[^{35}S] (0.25 mCi mmol^{-1}) and the immunoprecipitated samples suspended in a final volume of 30 μl in assay buffer containing 0.5 mM DTT. At the indicated times, 5 μl of the reaction was removed and passed through nitrocellulose filters, which were washed and counted using a liquid scintillation counter.

Microinjection experiments--Sub-confluent, serum-starved Swiss 3T3 cells plated on fibronectin were prepared as previously described (Lamarche et al., 1996). The cells were injected with 0.15 mg ml^{-1} of DNA. In some cases, DNA was injected with 0.6 mg ml^{-1} purified GST–WASP-CRIB domain. Cells were processed for indirect immunofluorescence with various antibodies and for direct immunofluorescence with TRITC-conjugated phalloidin as described (Lamarche-Vane and Hall, 1998).

Neurite outgrowth--N1E-115 neuroblastoma cells were transfected with Optimem, 10 μl of lipofectamine and 2 μg of DNA. After 5 h, the Optimem was removed and replaced with DMEM culture medium containing 10% foetal calf serum (FCS). The cells were allowed to recover overnight and were then scraped into media containing 10% FCS, triturated to obtain a suspension of single cells and plated on 35 mm plates coated with laminin at a density of 2 x 10^4 cells per plate (in DMEM with 10% FCS). 2 h later, the medium was switched to DMEM with 0.5 % bovine serum albumin or cells were left in serum-containing medium. After a 5 h incubation, the cells were fixed and transfected cells were identified by immunohistochemical staining with anti-GFP antibody. For each dish, 200 transfected cells were identified, and the number of neurites and the length of each neurite were recorded for each cell. The 200 cells were selected using a preset scanning pattern, such that the selected cells were evenly distributed over the dish. Data were recorded from each transfected cell that was encountered within this scanning pattern. A series of four dishes was used for each transfection.

Actin nucleation assays--Actin nucleation was measured in permeabilized neutrophils as previously described (Glogauer et al., 2000). The permeabilized cells were exposed to the various GST peptides (9 μg in 300 μl assay volume) for 3 min. Pyrene-labelled rabbit skeletal muscle G-actin (Hartwig, 1992) at 1 μM was then added to start the assays and its assembly was monitored as a fluorescence increase at excitation and emission wavelengths of 366 nm and 386 nm, respectively. To determine the contribution of actin filament barbed ends to the rate of fluorescence increase, we added 2 μM cytochalasin B to the assays and converted the data on the cytochalasin B-sensitive actin assembly rate into the number of nucleation sites as described previously (Hartwig, 1992).

Purification of clathrin-coated vesicles--Subcellular fractionation of rat brain extracts to generate highly purified clathrin-coated vesicles (CCVs) was performed as described (Maycox et al., 1992).

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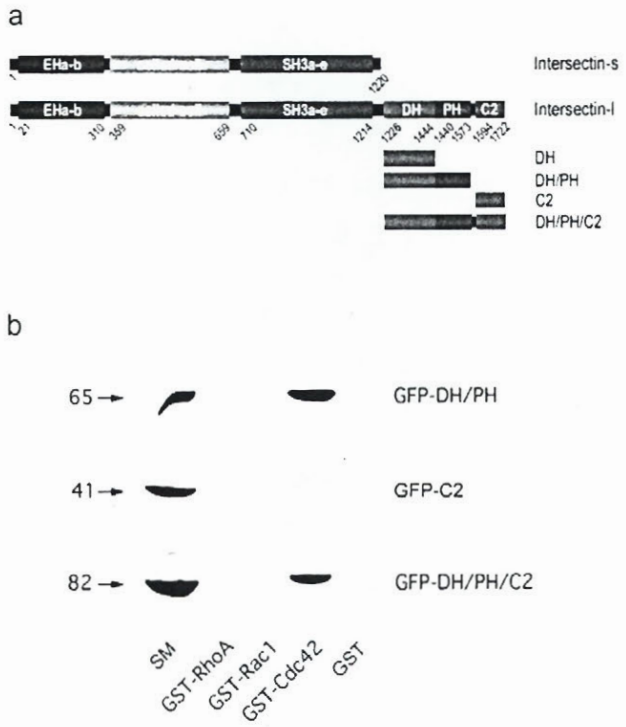

Figure 5.1 Intersectin-l has GEF activity towards Cdc42. a, Structure map of intersectin-s and -l. The amino acid numbers of domain boundaries are indicated below intersectin-l. b, Lysates from HEK-293 cells expressing GFP fusion protein constructs encoding various regions of intersectin-l were prepared in nucleotide free (NF)-buffer and incubated with GST fusion proteins of RhoA, Rac1, and Cdc42 or GST alone pre-coupled to glutathione-Sepharose. Specifically bound material was analyzed by α–GFP Western blot along with an aliquot of the lysate (starting material, SM). c, GFP-tagged constructs of intersectin-l or GFP alone were co-expressed with Myc-tagged Cdc42 in HEK-293 cells. Immunoprecipitations were performed in NF-buffer with α–GFP antibodies and immunoprecipitated proteins (IP) were detected by Western blot with α–GFP or α–Myc antibodies as indicated. d, GST-DH/PH was coupled to glutathione Sepharose and incubated in NF-buffer with bacterially expressed and purified Cdc42, cleaved from GST by thrombin. Specifically bound material was analyzed by α-Cdc42 Western blot along with an aliquot of starting material (SM) and unbound material (void). e, GST alone or GST-DH was coupled to glutathione-Sepharose and incubated with cell lysates expressing various forms of Myc-tagged Cdc42. Lysates were prepared in NF-buffer (Cdc42wt) or G-buffer (Cdc42L61, cdc42N17). Specifically bound material, as well an aliquot of the starting material (SM) was analyzed by α–Myc Western blot. f, GFP-tagged domains of intersectin-l were immunoprecipitated from transfected HEK-293 cells using α–GFP antibody and were tested for GEF activity on bacterially expressed and purified Cdc42, cleaved from GST by thrombin, and pre loaded with GDP. GEF activity was measured as the relative incorporation of GTP-γ[^{35}S] onto Cdc42 as a function of time. A fraction of the immunoprecipitated proteins were used for Western blots with α–GFP antibody (IP).

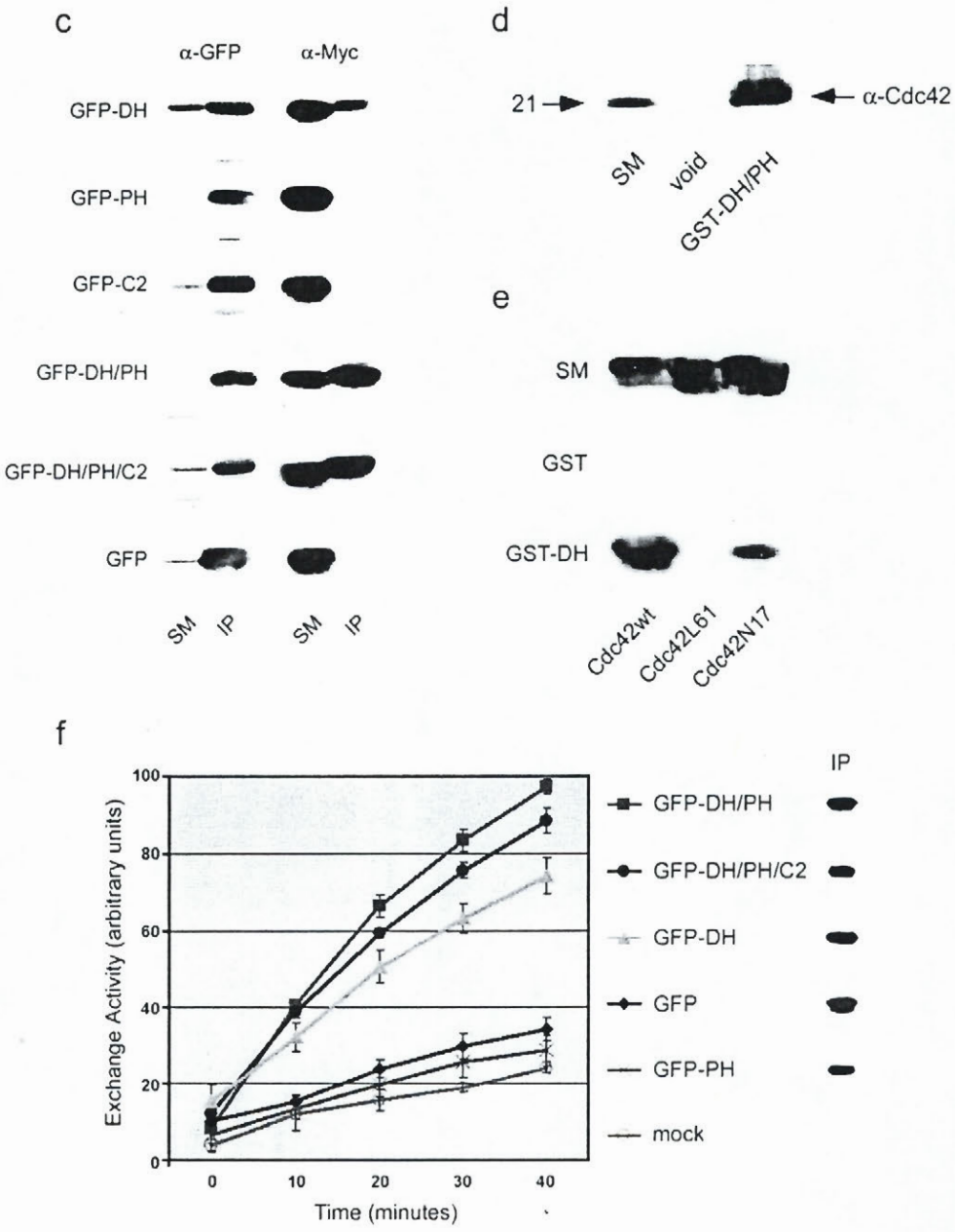

Figure 5.1 continued.

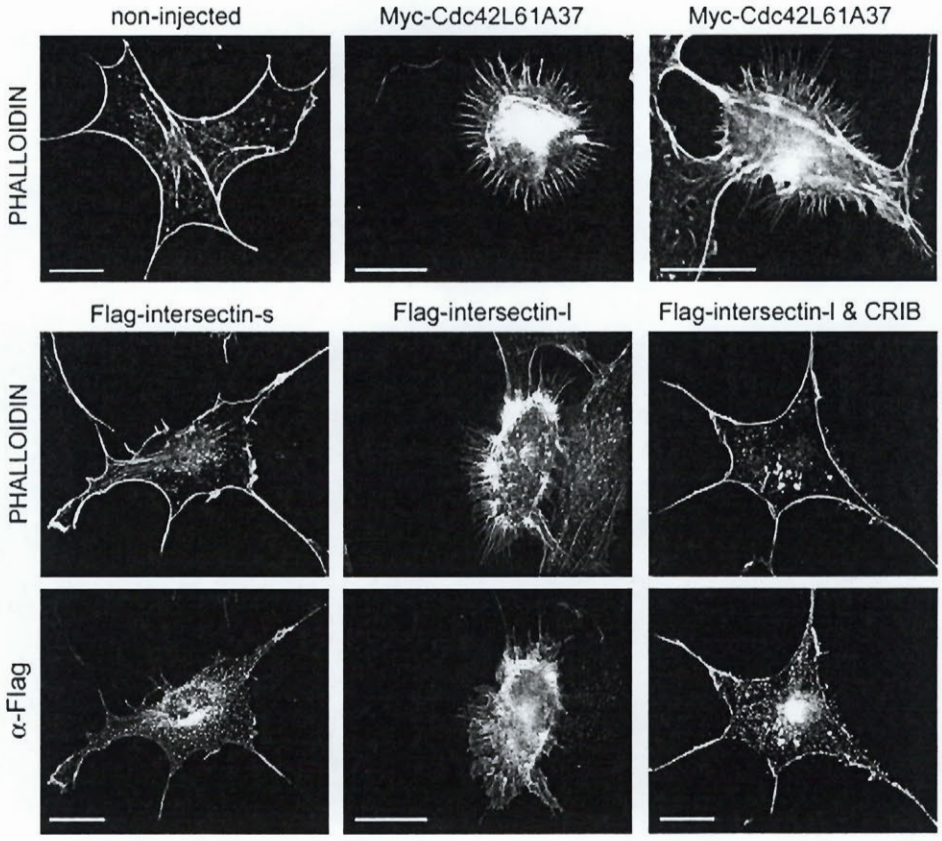

Figure 5.2 Intersectin-l causes a Cdc42 activation phenotype in Swiss 3T3 fibroblasts. Serum-starved sub-confluent Swiss 3T3 fibroblast were microinjected with mammalian expression constructs encoding a Myc-tagged mutant of Cdc42 (Myc-Cdc42L61A37), Flag-tagged intersectin-s, and Flag-tagged intersectin-l alone or co-injected with a GST fusion protein of the CRIB domain of WASP (amino acids 201-321). Microinjected cells expressing the proteins were revealed by indirect immunofluorescence with α–Myc (data not shown) or α–Flag antibodies and filamentous actin was detected by staining with TRITC-labeled phalloidin. Approximately 50 cells were injected per experiment for each construct (n≥3 experiments for each construct). Scale bars equal 20 μm.

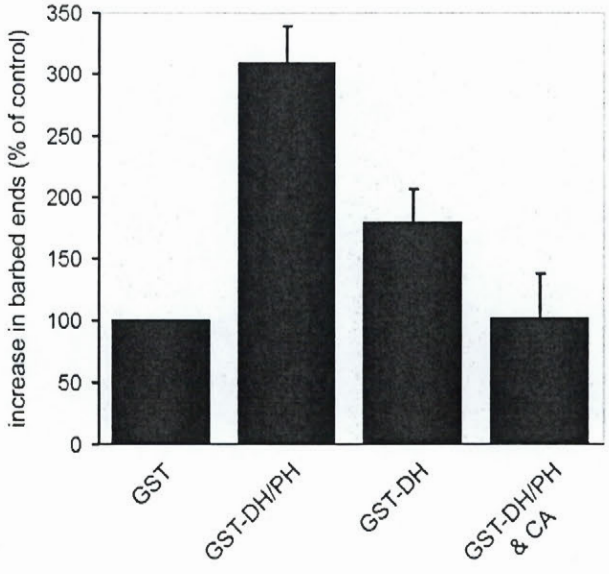

Figure 5.3 Intersectin-l stimulates actin nucleation through N-WASP-dependent activation of Arp2/3. Permeabilized, acutely isolate human neutrophils were exposed to GST, GST-PH, GST-DH/PH, or GST-DH/PH in the presence of 3 μM CA peptide for 3 minutes. The number of free barbed ends per cell was determined as described (Hartwig, 1992).

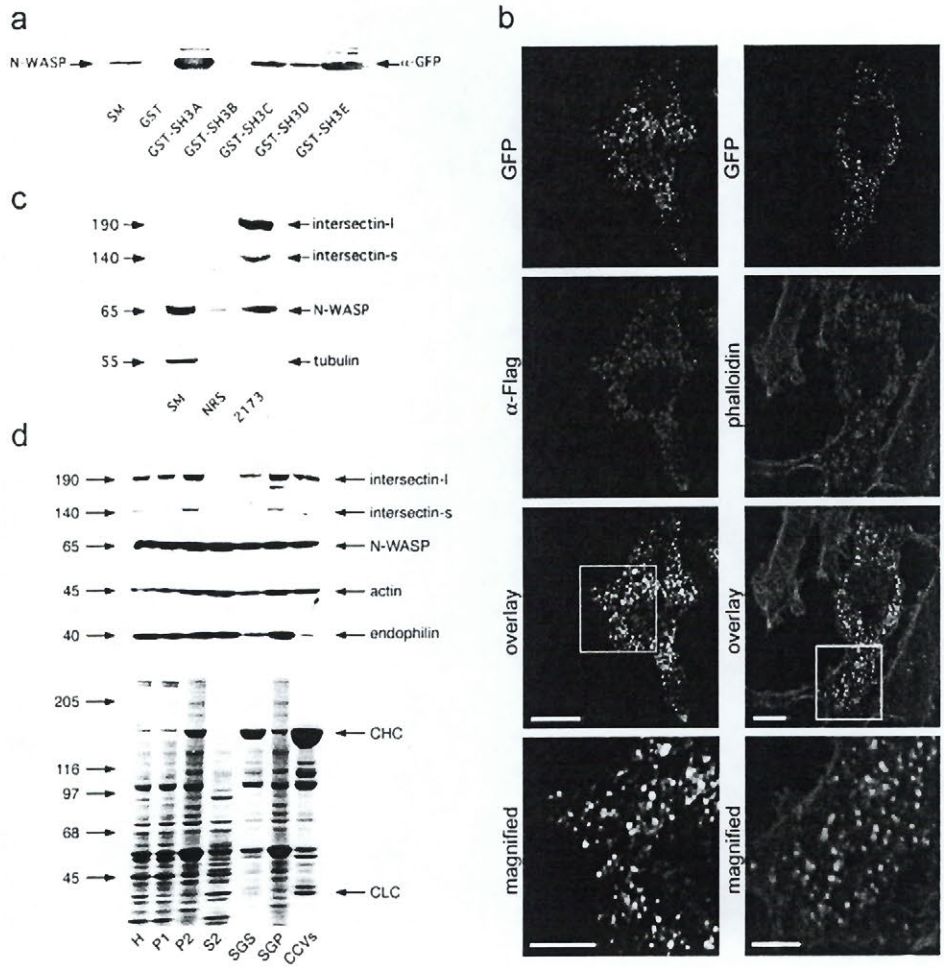

Figure 5.4 N-WASP interactions with intersectin-l. a, Lysates from HEK-293 cells expressing GFP-tagged N-WASP were incubated with GST fusion proteins encoding each of the five SH3 domains (GST-SH3A through GST-SH3E) of intersectin or GST alone pre-coupled to glutathione-Sepharose. Specifically bound material was analyzed by α–GFP Western blot along with an aliquot of the crude cell lysate (starting material, SM). b, HEK-293 cells transiently co-expressing GFP-N-WASP and Flag-intersectin-l were processed for immunofluorescence staining with α-Flag antibody or by staining with TRITC-labeled phalloidin , and examined by confocal microscopy. Super-imposition of the images (overlay) reveals the degree of co-localization (yellow) between GFP-N-WASP (green) and intersectin-l (red) or GFP-N-WASP (green) and phalloidin labeled actin (red). Scale bars equal 10 μm. The bottom panels are enlarged versions of the boxed regions of the superimposed images (scale bars equal 5 μm). c, Co-immunoprecipitation experiments from Triton X-100 solubilized membrane fractions of rat brain were performed using normal rabbit serum (NRS) or an antibody

against intersectin (2173) which immunoprecipitates both intersectin-s and –l. Precipitated proteins were detected by Western blot with antibodies against intersectin, N-WASP, or tubulin. d, Equal protein aliquots (40 μg) from various steps of a subcellular fractionation procedure leading to purified clathrin-coated vesicles were separated on SDS-PAGE, and stained with Coomassie blue (bottom panel), or transferred to nitrocellulose, and Western blotted (top panels) with antibodies against intersectin, N-WASP, actin, and endophilin as indicated (H, homogenate; P, pellet; S supernatant; SGS, sucrose-gradient supernatant; SGP, sucrose-gradient pellet; CCVs, clathrin-coated vesicles).

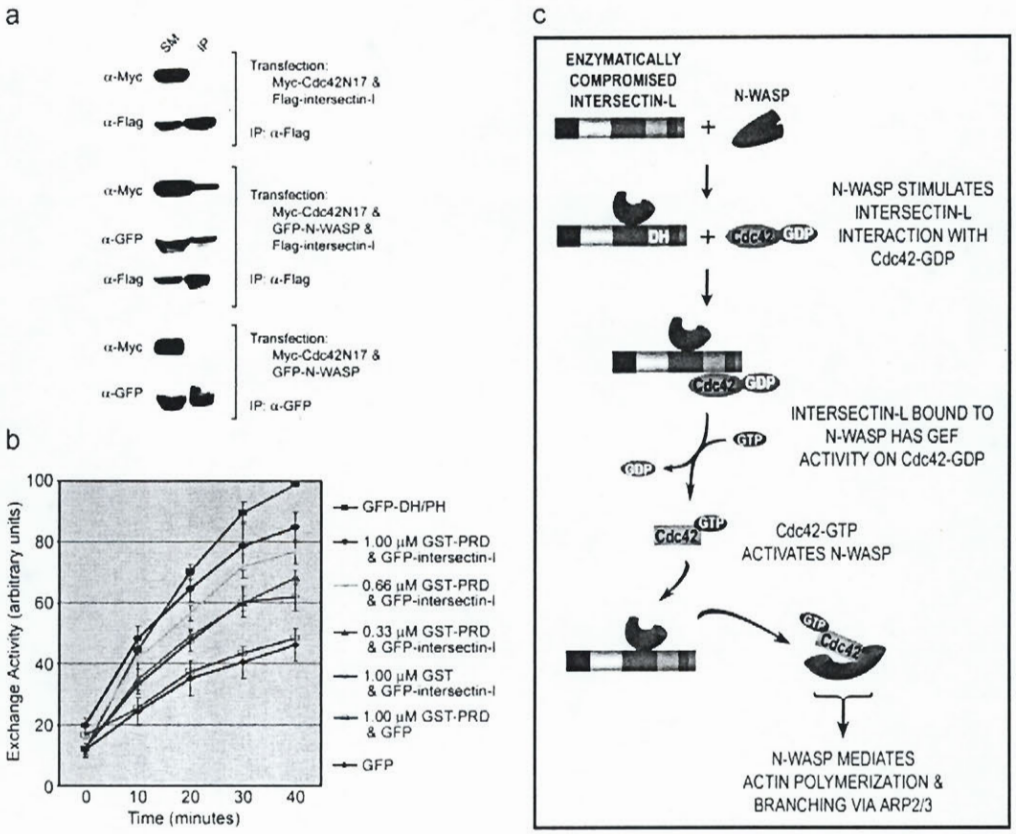

Figure 5.5 Intersectin-l guanine-nucleotide exchange factor (GEF) activity is upregulated by N-WASP binding. a, HEK-293 cells were transfected with DNA constructs encoding Myc–Cdc42N17, green fluorescent protein (GFP)-tagged NWASP or Flag-tagged intersectin-l, as indicated. Lysates were prepared in G buffer and immunoprecipitated with anti-Flag or anti-GFP antibodies, as indicated. The immunoprecipitated proteins (IP) and an aliquot of the cell lysate equivalent to onetenth of the cell extract added to the binding assays (starting material; SM) were processed for western blots with the antibodies indicated on the left. b, GFP alone, GFP–DH–PH or GFP–intersectin-l were immunoprecipitated from transfected HEK-293 cells using anti-GFP antibody and were used in GEF assays as described in Fig. 1. GFP-intersectin-l GEF activity was measured in the presence of purified glutathione- *S*-transferase (GST) or increasing concentrations of GST fused to the proline-rich domain of N-WASP (GST–PRD). The amount of intersectin-l added to each assay is estimated to be 1 μM based on Coomassie Blue staining (data not shown). c, Model of the functional role for intersectin-l in actin nucleation and assembly.

SUPPLEMENTAL INFORMATION CHAPTER 5

Method S1 Production of cDNA constructs. Human intersectin-l tail constructs fused to GST and GFP were prepared by PCR using a cDNA template with Vent DNA polymerase and the following primers (F and R denote forward and reverse, respectively): DHF2 (5'-CGCGTCGACTCTTGGATATGTTGACCCC-3');
DHR2 (5'-GCGGGGCCCGCGGCCGCTCAGCCTTCACACTGCACGTG-3');
PHF2 (5'-CGCGTCGACAGTGTGAAGGCCTGTC-3');
PHR (5'-GCGGGGCCCGCGGCCGCTCAGTAAAGTTCAGAAGCAGC-3');
C2F (5'-CGCGTCGACTTGGAAGGTTGATGGTGAAC);
C2R2 (5'-GCGGGGCCCGCGGCCGCCTACGCTCATCAAACAAC-3'). The PCR products were digested with *Sal* I/*Not* I or *Sal* I/*Apa* I and sub-cloned into the corresponding sites of pGEX-4T1 (Amersham Pharmacia Biotech) or pEGFP-C2 (Clontech), respectively. Constructs of human intersectin-s and -l with N-terminal Flag or GFP tags were prepared by PCR using cDNA templates of each in pBluescript-SK and the following primers:
FlagF (5'CGCGCGGCCGCCACCATGGACTACAAAGACGATGACGACGCTCA GTTCCAACACCTTTTTG-3') and FlagR (5'-GGGAACAGAAGATACTAGGG-3') for Flag-intersectin-s; FlagF and Bgl-INT-R (5'-CCACATCAAATGACTGTGCC-3') for Flag-intersectin-l;
5hINT-F (5'-CGCGCGGCCGCCTCGAGGGCTCAGTTTCCAACACC-3') and Bgl-INT-R for GFP-intersectin-s and -l. The PCR products were digested with *Not* I/*Bam*HI for Flag-intersectin-s or *Not* I/*Bgl* II for both Flag and GFP constructs of intersectin-s and -l and were sub-cloned to replace the N-termini of the cDNA templates of intersectin-s and -l in pBluescript-SK digested with the corresponding enzymes. The resulting Flag-tagged constructs were excised with *Not* I/*Sal* I, made blunt at their 5' end using T4 DNA polymerase, and were subcloned into the *Sma* I/*Sal* I sites of the mammalian expression vector pRK5. The resulting constructs intended for GFP tags were excised from pBluescript-SK by digest with *Xho* I (intersectin-s) or *Xho*I/*Sal*I (intersectin-l) and were sub-cloned into the corresponding sites of pEGFP-C2. GFP-N-

WASP was prepared by PCR using a cDNA template of N-WASP (gift of Dr. Hiroaki Miki) with Vent DNA polymerase and the following primers:
NWF (5'-GCGAATTCATGGACTACAAAGACGATGACGACAAGATCTCGAGGA GCTCGGGCCAG-3') and NWR (5'-CGCAAGCTTTCAGTCTTCCCACTCATC-3'). The resulting PCR product was digested with *Xho* I/*Hind* III and subcloned into the corresponding sites of pEGFP-C2.

Figure S1. Myc-tagged Cdc42 binds to the DH domain of intersectin-l. Lysates from HEK-293 cells expressing Myc-tagged Cdc42 were prepared in NF-buffer and incubated with GST fusion proteins of various regions of intersectin-l or GST alone pre-coupled to glutathione-Sepharose. Specifically bound material was analyzed by α–Myc Western blot along with an aliquot of starting material (SM).

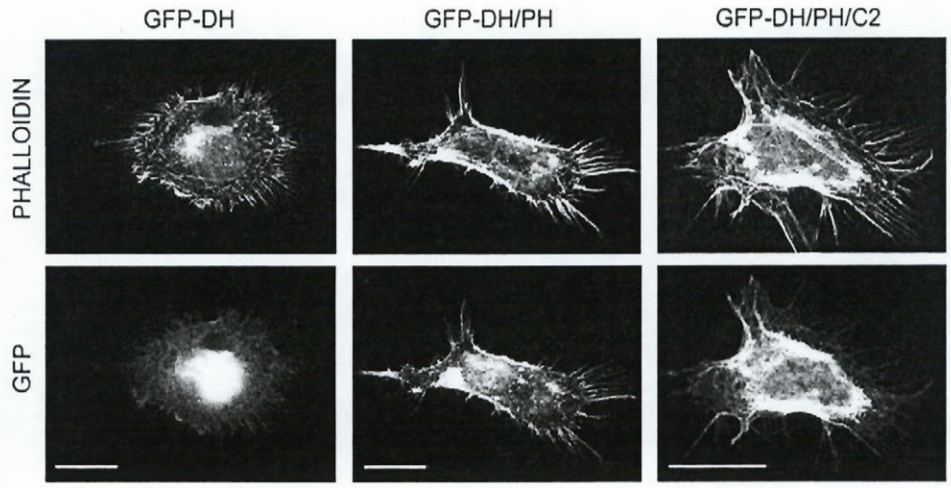

Figure S2. Intersectin-l tail constructs cause a Cdc42 activation phenotype in Swiss 3T3 fibroblasts. Serum-starved sub-confluent Swiss 3T3 fibroblast were microinjected with mammalian expression constructs encoding GFP-tagged regions of intersectin-l. Microinjected cells expressing the proteins were revealed by GFP fluorescence (GFP) and filamentous actin was detected by staining with TRITC-labeled phalloidin. Approximately 50 cells were injected per experiment for each construct (n≥3 experiments for each construct).

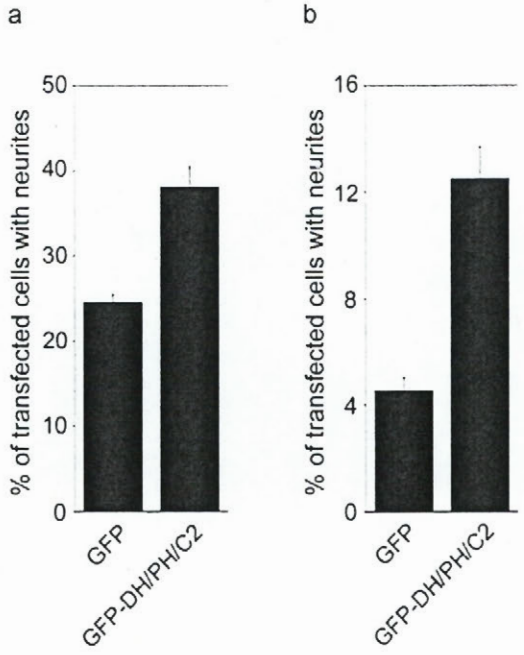

Figure S3. Intersectin-l tail constructs stimulate neurite outgrowth in N1E-115 cells. a, and b, N1E-115 neuroblastoma cells were maintained in culture for 16 h following transfection with plasmids encoding GFP or GFP-DH/PH/C2. The cells were then re-plated into a, serum-free media or b, serum-containing media for 5 h. Graphs represent the percentage of GFP-positive cells with neurites in which only neurites greater than 25 μm in length were recorded. The bars represent mean ± s.e.m.

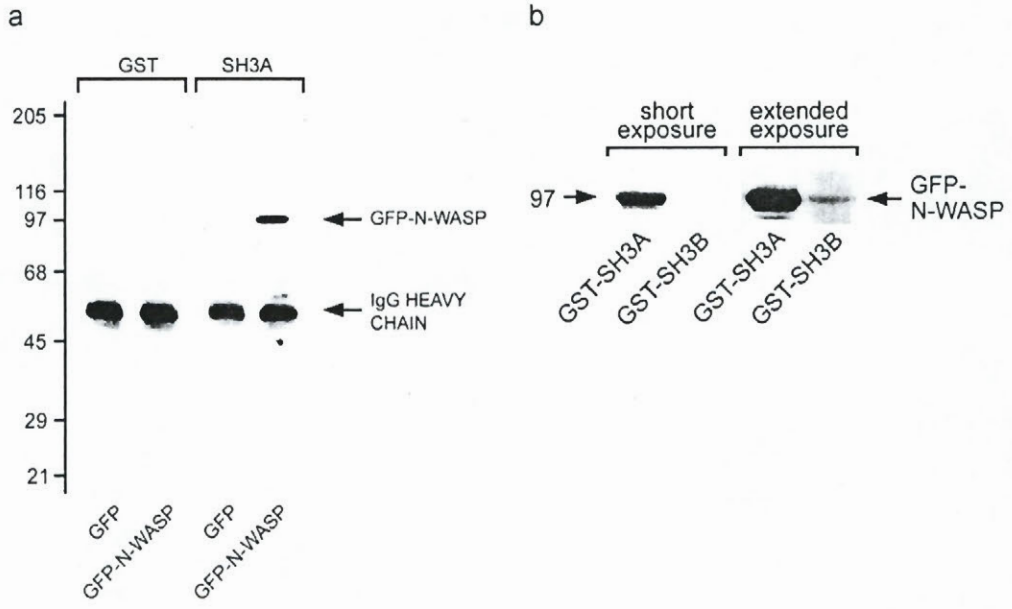

Figure S4. Overlay of N-WASP with intersectin SH3 domains. a, Lysates from HEK-293 cells transiently expressing GFP or GFP-N-WASP were used for immunoprecipitation with α–GFP antibody. Immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and were overlaid using 10 μg ml^{-1} of purified GST-SH3A domain of intersectin or GST alone. b, Immunoprecipitated GFP-N-WASP was overlaid with 10 μg ml^{-1} GST-SH3A or GST-SH3B of intersectin. Two different exposures of the same experiment are shown.

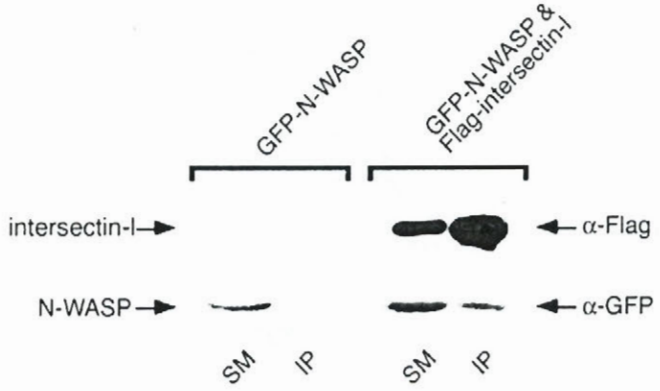

Figure S5. Co-immunoprecipitation of intersectin-l and N-WASP from co-transfected cells. Cell lysates from HEK-293 cells transiently expressing GFP-N-WASP alone or N-WASP co-expressed with Flag-intersectin-l were used for immunoprecipitation assays with α–Flag antibody. Immunoprecipitated proteins were detected with α–Flag and α–GFP Western blots.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

Comprehensive studies of vesicular recycling processes have revealed that a myriad of proteins are associated with clathrin-mediated-endocytosis (Figure 1.4). This molecular complexity may reflect a high degree of physiological control over this process. The orchestration of each step in formation of endocytic vesicles is critically dependent upon the precise localization of proteins that regulate vesicular recycling (for reviews see Brodin et al., 2000; Higgins and McMahon, 2002). Our investigations of the intersectin proteins suggest that they function as scaffolding proteins to control the localization of proteins associated with clathrin-mediated endocytosis. In addition, they link endocytic processes to cell signaling and the cytoskeleton.

B. Major Findings and Scientific Contributions

Both intersectin-s and intersectin–l have the ability to organize several components of the endocytic machinery at sites of clathrin-mediated endocytosis (**Chapter 2** (Hussain et al., 1999)). These data were among the first to raise the possibility that these molecules play an important regulatory function on recycling in nerve terminals (Hussain et al., 1999; Okamoto et al., 1999; Roos and Kelly, 1998; Roos and Kelly, 1999; Sengar et al., 1999).

Through characterizing intersectin mediated interactions, we have identified endocytic links to both microtubule (**Chapter 3** (Hussain et al. *to be submitted*)) and actin cytoskeletal networks (**Chapter 5** (Hussain et al., 2001)). Many of the specific functions of microtubules, including their roles in cellular division, intracellular membrane trafficking, and the maintenance of cyto-architecture, are dependent upon their dynamic properties (Mandelkow and Mandelkow, 1992). Microtubules are dynamic filaments created by the perpetual cycling of tubulin between a soluble αβ

expression of intersectin 2-l in Jurkat cells potentiated T-cell receptor internalization (McGavin et al., 2001). These data further support an association between the regulation of actin cytoskeletal dynamics and endocytosis.

Analyses of intersectin 1 and 2 expression patterns in brain reveal that transcripts of each are found in nearly every region of the brain. While both were often found in overlapping cell populations, their relative levels of expression were variable (Pucharcos et al., 2001). Pucharcos *et al.* (2001) identified specific expression pattern differences in developing mice (post-natal day ten) within the cerebellum, suggesting that intersectin 1 is expressed in both proliferating (i.e. granule) and differentiating (i.e. Purkinje) neurons, while intersectin 2 expression is restricted to Purkinje neurons. A recent comparative study of intersectin 1-s versus intersectin 1-l suggest that proliferating neurons primarily express the short form, whereas differentiating and mature neurons primarily express long intersectin 1 in newborn rat brain (Ma et al., 2003). A similar analysis of the short and long forms of intersectin 2 has yet to be completed. Further investigations will be required to clarify the distribution of these proteins and importantly, their potential differences and/or specializations in function within the nervous system and non-neuronal cells.

i. The Long and Short of Intersectin 1 and 2 Evolution

Numerous signal transduction pathways, including the Ras and Rho GTPase pathways, display substantial molecular fidelity throughout evolution (Etienne-Manneville and Hall, 2002; Rebay, 2002). Thus, investigations using organisms such as yeast, and *Drosophila* have greatly aided the characterization of mammalian signal transduction pathways. Biochemical characterization of synaptic vesicles and the study of membrane budding using yeast genetics has led researchers to suggest that many mechanisms involved in vesicle recycling are also evolutionarily conserved (Bennett and Scheller, 1993; Richmond and Broadie, 2002). Given our evidence for intersectin function in processes of signal transduction (**Chapters 4 and 5**) and clathrin-mediated endocytosis (**Chapter 2**), it is notable that the intersectin genes are also conserved throughout evolution. Although no intersectin genes exist in yeast, intersectin 1-short sequences have been identified in *Caenorphabditis elegans* (GenBank accession

number CAB55138), *Drosophila melanogaster* (Roos and Kelly, 1998) and *Xenopus laevis* (Yamabhai et al., 1998). *Xenopus* may additionally include the intersectin 1-long and 2-long proteins since analogous sequences have been deposited in expressed sequence tag databases. Homologues of both the short and long isoforms of intersectin 1 and 2 have been characterized in rodents and humans (Guipponi et al., 1998; Okamoto et al., 1999; Sengar et al., 1999).

Intersectin genes 1 and 2 have highly conserved exon boundaries, and share similar mechanisms for generating analogous splice variants (Guipponi et al., 1998; Pucharcos et al., 2000). Pucharcos *et al.* (2000) argue that these features indicate that intersectin genes 1 and 2 were the result of a gene duplication event. In addition, the fusion of two distinct genes to form a single intersectin gene (prior to duplication of the genome) provides an explanation for the evolution of the short and long isoforms of intersectin, both consistently identified within vertebrates (Guipponi et al., 1998; Pucharcos et al., 2000).

Several additional splice variants of intersectins 1 and 2 have been noted in rodents and humans, which may represent a mechanism that regulates intersectin functions (Guipponi et al., 1998; Pucharcos et al., 2001; Sengar et al., 1999). For instance, three mRNA splice variants affecting the coiled-coil domain of intersectin have been isolated. (Guipponi et al., 1998; Pucharcos et al., 2001). Hypothetically, if these coiled-coil variants are expressed as proteins, potential differences in their cellular distribution and relative expression levels, and in their abilities to bind Eps15R, Eps15 and/or SNAP-25 could influence the role of intersectins in receptor signaling, endocytosis and exocytosis.

An mRNA splice variant encoding the isolated EH domains (amino acids 1-586) of mouse intersectin 2 (Pucharcos et al., 2001) is of particular interest given our demonstration that the EH domains of intersectin 1 mediates its interaction with epsin, and indirect binding to clathrin and AP2 (**Chapter 2** and Yamabhai et al., 1998). Immunostaining of endogenous epsin or intersectin in cells highlight small punctae distributed throughout the cell that are coincident with clathrin (**Chapter 2** and Chen et al., 1998; Sengar et al., 1999). Additionally, intersectin is reported to stain larger, peri-nuclear punctae of as yet undefined organelle identity (our unpublished observations,

and Sengar et al., 1999). Our data support the notion that intersectin EH domains regulate the localization of epsin in cells (rather than epsin driving intersectin localization). We found that co-expression of either full-length intersectin, or its isolated EH domains, with *Xenopus* epsin (MP90) was sufficient to significantly re-direct the localization of MP90 from the small clathrin positive punctae, to the larger punctate peri-nuclear structures outlined above (*N.K.H. unpublished data*). Therefore, if full-length intersectin and the EH splice variant described above are co-expressed in cells, variability in their respective protein expression levels could have a profound effect on the localization and thus specific functions of epsin; our data support epsin function in tubulin binding/neurite outgrowth (**Chapter 3**) in addition to its ability to stimulate curvature of clathrin-coated membranes (Ford et al., 2002).

Although numerous intersectin mRNA splice variants have been identified, their particular protein expressions have not yet been formally investigated. Undoubtedly, future examination of intersectin function should consider the impact particular combinations of modular domains might have on intersectin-mediated arrangement of multi-protein complexes. It is conceivable that intersectin splice variant specific protein-complexes could establish different signaling cascades and differentially regulate clathrin-mediated endocytosis.

D. Intersectin and Signal Transduction

mRNA splice variants of intersectin specifically lacking either the SH3C or the SH3D domains have also been described (Pucharcos et al., 2001). As suggested above, these variants could influence the ability of intersectin to regulate specific stages of clathrin-mediated endocytosis. Consistent with other investigators analyses of intersectin SH3 domains, we found that over-expression of the five tandem SH3 domains in COS-7 cells was sufficient to disrupt constitutive and regulated endocytosis, presumably in a dominant negative fashion (**Chapter 2**, N.K.H. unpublished data, and Sengar et al., 1999). Further investigation of the isolated SH3 domains using an assay for clathrin-mediated endocytosis of the transferrin receptor revealed that SH3C and E specifically disrupt late events, while SH3A uniquely disrupts early events in clathrin-

mediated receptor uptake, whereas SH3B and D had no effect in the assay (Simpson et al., 1999).

As an extension of these studies, we identified cellular ligands unique to the SH3A domain of intersectin. We determined that the RAS exchange factor, mammalian mSOS1 binds specifically to the SH3A domain (**Chapter 4**). One interpretation of these findings is that stable intersectin/mSOS complexes in brain mechanistically couple endocytic and signal transduction machineries. Alternatively, mSOS may perform a more direct role in clathrin-coated pit formation, potentially via its stimulation of Ras and/or Rac signaling cascades.

Indirect evidence supporting a potential role for mSOS in endocytosis comes with the finding that over-expression of mutant Ras or the downstream regulator, GTP-binding protein Ral, disrupts endocytosis of the epidermal growth factor (EGF) receptor (Nakashima et al., 1999). Considering the fact that Rac is activated by mSOS1 (Nimnual et al., 1998), findings by Lamaze *et al.* demonstrating that activated Rac can regulate receptor mediated endocytosis lends further support for a role of mSOS1 function in endocytosis (Lamaze et al., 1996). Further linking Rac to endocytosis is the fact that Rac can bind to and recruit the endocytic protein synaptojanin 2 to membranes (Malecz et al., 2000).

Synaptojanin 1 and 2 are phosphatases that have the ability to hydrolyse PtdIns(4,5)P_2 on the plasma membrane, and assist in the uncoating of clathrin-coated vesicles liberated from the membrane (Cremona and De Camilli, 2001). Microdomains enriched in various lipids, in particular with PtdIns(4,5)P_2, on the plasma membrane are predicted to define nucleation sites, or 'hot spots' for clathrin-mediated endocytosis (see section 1D.i. *Nucleation of coat components*) (Martin, 2001). Thus, the ability of synaptojanin to manipulate lipid content within hot spots represents a critical mechanism in the regulation of clathrin-mediated endocytosis. Coupling the ability of activated Rac to disrupt endocytosis and to translocate synaptojanin 2 to the plasma membrane, this interaction is predicted to influence endocytic rates by regulating phospholipid content at the plasma membrane (Qualmann and Mellor, 2003). Although intersectin binds synaptojanin 1 *in vitro*, a similar interaction with synaptojanin 2 has not yet been directly tested.

While the interaction between intersectin SH3A and mSOS1 fosters exciting speculation as to their possible activities in regulating Ras/Rac activation at endocytic sites *in vivo*, the precise functional role of this complex in signaling and/or endocytosis remains to be explored.

Both intersectin-s and –l are capable of binding mSOS and may be implicated in regulation of the Ras/Rac pathways, however the neuronal protein intersectin 1-l additionally regulates Cdc42 signal transduction through its ability to bind and activate Cdc42 (**Chapter 5**). Similar to Rac, Cdc42 is a member of the Rho family of small GTPases, which are signal transduction molecules modulating a variety of cellular functions. Characterizing the signal transduction pathways mediated by specific Rho GTPases is complicated by the highly integrated nature of the circuitry between family members (for reviews see Etienne-Manneville and Hall, 2002; Van Aelst and D'Souza-Shorey, 1997). For instance, activated Cdc42 and Rac function co-operatively in regulating cell polarity (Etienne-Manneville and Hall, 2001), and stability of the microtubule network (Daub et al., 2001). Furthermore, the regulation of neurite outgrowth appears to be not only influenced by Cdc42 and Rac, but also by Ras-mediated signal transduction pathways (Kozma et al., 1997; Sarner et al., 2000). Our data supporting the potential function of intersectin 1-l in both Ras (**Chapter 4**) and Rho (**Chapter 5**) GTPase signal transduction leaves this molecule well placed to assist in their integration.

An additional level of intersectin-mediated control over signal transduction is achieved through its SH3 domain-dependent binding to CdGAP (Cdc42/Rac-specific GTPase-activating protein) (Jenna et al., 2002). When bound to intersectin SH3 domains, the GAP activity of CdGAP is inhibited, providing a mechanism whereby intersectin-s or -l could indirectly increase the levels of activated Cdc42 and Rac *in vivo* (Jenna et al., 2002). Unique to the long form of intersectin is its enzymatic activity directed toward Cdc42 activation, which in turn stimulates actin polymerization (**Chapter 5**, and Irie and Yamaguchi, 2002; Karnoub et al., 2001; Zamanian and Kelly, 2003). The ability of intersectin 1-l to activate Cdc42 and stimulate actin polymerization has been functionally linked to regulation of dendritic spine morphology in hippocampal neurons (Irie and Yamaguchi, 2002).

The principal pathway by which Cdc42 regulates actin dynamics is through activation of N-WASP, a protein we have demonstrated interacts directly with intersectin proteins. Although, N-WASP is localized to endocytic vesicles, it has not been determined whether this localization is mediated by its interaction with intersectin, or with other endocytic proteins such as endophilin A (Otsuki et al., 2003), or the pacsins/syndapins (Modregger et al., 2000; Qualmann and Kelly, 2000). N-WASP function in receptor-mediated endocytosis is supported by the fact that T-cell receptor internalization, in addition to the actin cytoskeleton, is disrupted in mice lacking the lymphocyte specific WAS protein (Zhang et al., 1999). However, the precise role of N-WASP in endocytosis remains unclear. One hypothesis is that N-WASP propels endocytic vesicles through a cell by stimulating a concentrated burst of actin polymerization at the neck of nascent endocytic clathrin-coated pits (Taunton et al., 2000). *In vivo* imaging of clathrin-mediated endocytosis has demonstrated that a burst of actin polymerization does precede the inward propulsion of endocytic vesicles (Merrifield et al., 2002). However, rather than being a propulsive force, an alternative interpretation of these results is that actin acts as scaffold to organize endocytic machinery in the pre-synapse (Sankaranarayanan et al., 2003). Contrary to both suggestions is the notion that actin polymerization is localized to the neck region of emergent vesicles to cause their constriction and help sever vesicles from the plasma membrane. Thus, while several studies indicate that actin filaments may be involved at multiple stages of clathrin dependent and independent endocytosis in yeast and mammalian cells, the precise function(s) of actin in endocytosis remains unclear (for reviews see McPherson, 2002; Qualmann and Mellor, 2003).

E. Actin 'Intersectin' with Microtubule Tracks

In the following sections, I will discuss potential reasons for integration between endocytic and cytoskeletal systems, and in particular possible functional roles of intersectin therein.

i. The Cytoskeleton Stabilizes Endocytic Loci on the Membrane

Several studies indicate that actin restricts the mobility and formation of vesicles on the plasma membrane (Gaidarov et al., 1999; Roos and Kelly, 1999; Wu et al., 2001). For instance, the disruption of the actin cytoskeleton in synapses using latrunculin A causes an increase in the release probability of synaptic vesicles docked within nerve terminal active zones, with no detectable alteration in their rate of refilling (Morales et al., 2000). In addition, image analyses of clathrin-coated pit dynamics in living cells demonstrate that actin destabilizing drugs disrupt the formation of clathrin-coated pits at defined 'hot spot' sites on the plasma membrane (Gaidarov et al., 1999; Roos and Kelly, 1999). However, investigations by Gaidarov *et al.* (1999) showed that while the cortical actin cytoskeleton does contribute to spatial regulation of coated pit formation, actin is not entirely capable of determining where pits will form, nor in completely guiding vesicle movements (Gaidarov et al., 1999). Thus, these authors suggested that dynamic interactions between actin and other cytoskeletal structures are likely involved in spatial regulation of coated pit genesis and dynamics (Gaidarov et al., 1999).

Another cytoskeletal system potentially acting in conjunction with actin to control pit dynamics is the microtubule network. *In vivo* studies suggest that intact microtubules are important for movement of endocytic vesicles (Freed and Lebowitz, 1970; Herman and Albertini, 1984). Scaffolding proteins present on vesicles and at specific loci on the plasma membrane, limited by actin and microtubule networks, are predicted to help establish the discrete membrane association described for clathrin-coated pits (Gaidarov et al., 1999). A number of factors support the notion that intersectin may satisfy this role as scaffolding protein, namely that it functions at an interface between components of clathrin-mediated endocytosis (**Chapter 2**), actin polymerization (**Chapter 5**), and the microtubule cytoskeleton (**Chapter 3**). Furthermore, preliminary studies conducted by Keen and colleagues demonstrate that intersectin is co-localized with clathrin on coated vesicles *in vivo*, and that together these proteins display discontinuous, non-Brownian movements (known as 'saltatory' movements indicative of travel along microtubules), which they interpret to represent vesicle motion along filamentous tracks (*J. Keen, personal communication*).

ii. The Vesicle Freeway: Actin and Microtubule Networks

A large body of evidence suggests that the microtubule cytoskeleton is involved in vesicular trafficking between early and late endosomes (Aniento et al., 1993; Bomsel et al., 1990; Gruenberg et al., 1989; Kelly, 1990; Matteoni and Kreis, 1987). Considerable cell biological and biochemical evidence indicates that in mammalian neurons microtubule-associated motor proteins (kinesins) function in the transport of vesicles between these organelles and to peri-nuclear regions of the cell (Brady, 1995 1077). However, within pre-synaptic nerve terminals and at the tips of neuronal growth cones, kinesins are actively degraded, making it improbable that microtubules are the principal transport networks in these areas, or are involved in the early stages of vesicular transport (Okada et al., 1995). In these regions of the cell, actin filaments dominate the cytoskeletal framework, and invasion by microtubules appears to occur only transiently (Dent and Kalil, 2001; Forscher and Smith, 1988; LeBeux and Willemot, 1975; Schaefer et al., 2002).

Actin filaments have recently been shown to mediate very early endocytic events in mammalian cells (Lamaze et al., 1997; Merrifield et al., 2002; Merrifield et al., 1999; Sankaranarayanan et al., 2003). In addition, the minus end directed (i.e. from cell periphery toward cell centre) actin filament motor protein myosin VI has been shown to directly bind clathrin, and could thereby direct travel of emergent endocytic vesicles along actin filaments (Buss et al., 2001). Based on these findings, it is tempting to speculate that within regions of the cell where microtubule networks are rare, the cortical actin cytoskeleton may function as a vesicular 'freeway', able to transport or push endocytic vesicles short distances along their length until they encounter microtubule rich cellular domains. In these regions, interaction between the actin and microtubule network could allow the vesicle to be transferred onto the microtubule route for long distance delivery of the vesicles to their final destinations. In support of this model, some studies have suggested that membranous organelles can travel along both actin and microtubule filaments within the same cell (Kuznetsof et al., 1992; van Deurs et al., 1995). Unfortunately, compelling evidence for a mechanism by which a single vesicle may transition from one network to another, while remaining a mobile structure, has been lacking. However, Huang *et al.* (1999) have determined that actin

and microtubule based motors can form direct interactions in yeast and mammalian cells. These investigators proposed that a single vesicle could form an integrated complex with microtubule and actin motors, and through tight regulation of motor activity such that only one specified motor would be active at any given moment, vesicles would be fully capable of transition between actin and microtubule networks (Huang et al., 1999).

A model where dynamic interactions between actin and other cytoskeletal structures could create vesicle freeways would likely require inputs from extracellular cues in order to integrate the two networks. Endocytic proteins that additionally integrate cytoskeletal networks could well serve this position. The ANTH domain-containing protein HIP12 is a clear example of a molecule that physically links the actin cytoskeleton with components of clathrin-mediated endocytosis (Engqvist-Goldstein et al., 2001; Legendre-Guillemin et al., 2002). Coupled with our demonstration that ANTH domains bind tubulin, HIP12 could additionally bridge these components to the microtubule cytoskeleton for coordinated regulation of endocytosis with actin networks (**Chapter 3**). Similarly, the ENTH domain-containing protein epsin may be linked to the actin cytoskeleton, albeit indirectly, via its interaction with intersectin-l (**Chapter 3**, **5**, and Sengar et al., 1999).

In line with the model described above, several studies have revealed the integrated fashion by which actin and microtubule networks function to collectively regulate growth cone turning and neuronal architecture in response to the internalization/endocytosis of external guidance factors (Forscher and Smith, 1988; Kabir et al., 2001; Zhou et al., 2002). Moreover, recent studies demonstrate that cell-surface receptor activation can specifically stimulate intersectin-l to integrate the internalization of extracellular factors with actin polymerization (Irie and Yamaguchi, 2002). The consequence of activating this intersectin 1-l mediated signaling pathway is alteration of neuronal architecture, an effect that likely involves reorganization of the microtubule network in addition to actin polymerization (Dent and Kalil, 2001; Irie and Yamaguchi, 2002; Schaefer et al., 2002). In fact, epsin is not intersectin's only link to the microtubule network, intersectin also binds CRMP-2 (Quinn et al., 2001), dynamin (Yamabhai et al., 1998) and WASP (Hussain et al., 2001), each of which are associated

with the microtubule cytoskeleton (Fukata et al., 2002; Scaife and Margolis, 1990; Shpetner and Vallee, 1989; Tian et al., 2000). This propensity for endocytic/actin-associated components to interact with proteins implicated in tubulin binding further suggests a convergence of these systems. Endocytic proteins such as HIP12, and protein complexes such as intersectin/epsin, which may associate with the actin cytoskeleton, but also include a tubulin binding E/ANTH domain, thus provide an ideal mechanism for linking these two cytoskeletal networks with the endocytic machinery.

The notion of actin and microtubule networks functioning as vesicular highways is consistent with microtubule/intersectin protein-complexes being associated with the later steps in clathrin-mediated endocytosis. Following endocytosis, the involvement of microtubules in retrograde-transport of synaptic vesicles is well established (Goltz et al., 1992; Matteoni and Kreis, 1987; Oda et al., 1995; Tooze and Burke, 1987). While accessory proteins likely function at multiple stages in endocytosis, specific functions for epsin and intersectin have been attributed to regulation of early stages in endocytic pit formation (Ford et al., 2002; Ford et al., 2001; Roos and Kelly, 1999; Simpson et al., 1999). Despite suggestive evidence to the contrary, these early steps are generally not considered to involve microtubules, (Gaidarov et al., 1999; Herskovits et al., 1993; Honda et al., 2002; Novikoff et al., 1996; Orzech et al., 2001; Shpetner and Vallee, 1992).

Based on the data generated within this thesis and in several other studies, I will outline one hypothesis for how intersectin and epsin interactions could couple both actin and microtubules to early events in clathrin-mediated endocytosis. (1) Following stimulation of intersectin 1-l by receptor activation, intersectin 1-l could recruit epsin, dynamin and N-WASP to clathrin-coated vesicles, where epsin ENTH-PtdIns(4,5)P_2 interactions at the plasma membrane would stimulate the curvature of clathrin lattices on the membrane. (2) Intersectin 1-l enzymatic activity could stimulate the burst of actin polymerization that purportedly precedes separation of endocytic vesicles from the plasma membrane (Merrifield et al., 2002). The greatest degree of N-WASP mediated stimulation of actin polymerization occurs when N-WASP is in complex with PtdIns(4,5)P_2, activated Cdc42, and an SH3 domain domain-containing protein (Carlier et al., 2000; Miki et al., 1996; Rohatgi et al., 2001). Intersectin 1-l functionally activates

Cdc42, binds to N-WASP via its SH3 domains and, by virtue of its interactions with epsin, may recruit this complex to regions enriched PtdIns(4,5)P_2. Therefore, intersectin 1-l is perfectly situated for temporal and spatial regulation of actin polymerization via N-WASP prior to vesicle scission by dynamin. (3) Many studies demonstrate that the enzymatic activity of dynamin is required to release clathrin-coated pits from the membrane to generate vesicles, and moreover that dynamin GTPase activity is stimulated by assembly and binding of microtubules (Herskovits et al., 1993; Shpetner and Vallee, 1992). Epsin-tubulin interaction and localized polymerization approximate to emergent vesicles could stimulate the GTPase activity of dynamin. In this fashion, targeted interaction between E/ANTH domains and tubulin at sites of clathrin-coated pits could possibly assist in the scission of endocytic vesicles from the membrane. Thus, while it remains to be formally tested, it is conceivable that the ENTH domain mediated interactions of epsin provide duplicitous function in endocytosis, first in instigating membrane curvature via lipid binding, followed by microtubule assembly putatively leading to stimulation of dynamin activity and thus scission of vesicles from the plasma membrane. Ultimately, it can be concluded that intersectin-l/epsin interactions provide a mechanistic framework for coupling the clathrin-mediated endocytic pathway with the actin and microtubule cytoskeleton. However, whether functionally integrating actin and microtubule networks with an intersectin/epsin complex is principally involved in microtubule-based vesicular trafficking or in regulating the early stages of endocytosis is an issue that requires further investigation.

FUTURE DIRECTIONS

Our investigations regarding intersectin function demonstrate that true to its moniker, intersectin is the centre for a myriad of protein interactions, each having their own set of possible cellular functions. Although numerous studies have made it clear that intersectin is involved in endocytosis, the precise function of this molecule in the process of clathrin-mediated endocytosis remains incompletely described. Thus, understanding the temporal, spatial, and potential multiplicity in intersectin functions in

clathrin-mediated endocytosis should be an area of future investigations. Furthermore, specific splice variants and/or particular protein complexes formed by intersectin-mediated interactions have not been directly investigated for their functional roles. Coupling recent findings regarding the protein distribution of intersectin-s versus intersectin-l in brain, with similar examinations of thus far uncharacterized intersectin splice variants, should undoubtedly advance our understanding of how clathrin-mediated endocytosis is regulated.

Information garnered from the conclusion of the human and mouse genome projects is an immeasurable asset for those investigating vertebrate molecular biology. This information could also be applied to further studies on intersectin and vesicular recycling. Examination of these genomes allows one to conclude that two separate intersectin genes exist in vertebrates (Guipponi et al., 1998; Pucharcos et al., 2000; Sengar et al., 1999), and thus, experiments to specifically disrupt intersectin 1 and 2 expression, via RNAi or knock-out technologies, can be better designed and executed to clearly investigate relevance of intersectin expression *in vivo*. In addition to potentially clarifying the function of intersectin in vesicular recycling, these *in vivo* analyses should elucidate the role of intersectin in other cellular functions such as synapse formation, neurite outgrowth and spine morphology, and regulation of signal transduction pathways.

The determination that intersectin can form distinct complexes with mSOS and with Cdc42 provides evidence for a mechanistic link between endocytic and signal transduction machineries. Although we specifically investigated the effects of intersectin in downstream signaling cascades leading to Ras activation and actin polymerization, the mSOS and Cdc42 transduction pathways actually regulate a variety of cellular functions including, cell growth, death and survival (Etienne-Manneville and Hall, 2002; Manser, 2002). Thus, intersectin functions in signal transduction may be even more complex than are indicated by our investigations. Future studies should examine the role of intersectin in other cellular mechanism, as these studies should not only lead to a better appreciation for the scope of intersectin functions, but also for specific interplay between pathways that may regulate seemingly distinct cellular functions.

Collectively, studies on intersectin allow one to predict that this molecule functions as a coincidence detector triggering the assembly of specific protein complexes in response to specific inputs activated by extracellular cues. Thus, intersectin may provide a rapid integrative mechanism to affect multiple cellular responses. Although it is clear that several functional interconnections exist based on intersectin-mediated interactions, the overall logic of this integration has yet to emerge. The challenge of the future will be to clarify the functional nature of these intersectin-integrated cellular processes. Meeting this challenge will undoubtedly require scientific approaches that are as multifaceted as the intersectin molecule itself.

CONCLUDING REMARKS

Consequent to synaptic vesicle exocytosis, neurons require an efficient mechanism of endocytosis, such that recovered vesicle components may be reused for the local assembly and packaging of transmitters into vesicles at the nerve terminal. Alterations in neurotransmitter release are associated with many psychiatric and neurological diseases, and defects in neurotransmission are found in flies and mice in which endocytosis has been disrupted through genetic manipulations (for review see Narayanan and Ramaswami, 2001; Pralong et al., 2002). Endocytosis is exhibited by all eukaryotic cells, and although endocytosis may occur through a variety of morphologically and biochemically distinct mechanisms, clathrin-mediated endocytosis is believed to be the principal means for recovering synaptic vesicles (Johannes and Lamaze, 2002; Mellman, 1996). Significant insights into the molecular mechanisms that govern clathrin-mediated endocytosis have been gained by examining vesicular transport within the pre-synaptic nerve terminal.

While the studies included in this thesis focused on characterizing intersectin and epsin molecules involved in vesicular recycling, we have uncovered their potentially complementary roles in signal transduction and regulation of the cytoskeleton. Further investigations conducted along a similar vein should not only deepen our understanding of the molecular mechanisms governing these processes, but may ultimately provide

insights needed to counter diseases or injuries caused by their disturbance (DeTulleo and Kirchhausen, 1998; O'Bryan et al., 2001; Yao et al., 2000).

Our progress is narrow: it takes a vast world unchallenged and for granted. This is why we will have to accept the fact that no one of us really will ever know very much. This is why we shall have to find comfort in the fact that, taken together, we shall know more and more.

--J. R. Oppenheimer

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APPENDIX

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Louise Mansi
PA/Secretary to Mike Peacey
Deputy Director of Research Integration and Services
Tel: 020 7269 3197
Fax: 020 7269 3585
E.mail: louise.mansi@cancer.org.uk

-----Original Message-----
From: Natasha Hussain [mailto:natasha.hussain@mail.mcgill.ca]
Sent: 11 April 2003 19:29
To: b.marte@nature.com
Subject: Copyright request

To Whom It May Concern: April 11, 2003

As an author on the Nature Cell Biology article published in 2001 (Oct;3(10):927-32) entitled "Endocytic protein intersectin-l regulates actin assembly via Cdc42 and N-WASP", I am writing to request permission to include this document in my Doctoral Thesis. If copyright permission is granted, I would greatly appreciate if you could email or fax a letter to this effect to the address included below.

Thank you for your consideration.

Sincerely,
Natasha K. Hussain

Department of Neurobiology
Montreal Neurological Institute, rm. 776
McGill University
3801 University Street
Montreal, P.Q. H3A 2B4
Phone: (514) 398-6644 x 00209
Fax: (514) 398-8106
Email: natasha.hussain@mail.mcgill.ca

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L.C. Godbolt Date 15/4/03

LISA GODBOLT/KAREN MOORE
EDITORIAL ASSISTANTS
NATURE PUBLISHING GROUP
The Macmillan Building
4 Crinan street
London, N1 9XW, UK
Tel: +44 (0)20 7843 4924
Fax: +44 (0)20 7843 4794

Subject: Re: Copyright release request
From: Peter McPherson <peter.mcpherson@mcgill.ca>
Date: Thu, 01 May 2003 10:06:56 -0400
To: natasha.hussain@mail.mcgill.ca

Natasha Hussain wrote:

> Dear co-Authors, April 30, 2003
>
> As you are all authors on the recently submitted article "The ENTH domain binds tubulin and microtubules" to the Journal of Biological Chemistry, I am writing to request permission from each of you to include this document in my Doctoral Thesis (letters of permission from each of you is required by McGill for final submission). Thus, I would greatly appreciate if you could email (a reply email with "permission granted" within the body of the text would be sufficient) or fax a letter to this effect to the address included below. Martina, could you also send this to Dr. Hayden, or provide his co-approval in an email or fax.
>
> Thank you all for your time and consideration.

Dear Natasha:

Permission granted.

Sincerely,

Peter McPherson

Subject: RE: Copyright release request
From: Stephen Ferguson <ferguson@rri.ca>
Date: Wed, 30 Apr 2003 20:50:25 -0700
To: natasha.hussain@mail.mcgill.ca

Natasha,
It is with pleasure that I relinquish copyright permission to you for the manuscript entitled ""The ENTH domain binds tubulin and microtubules" submitted to the Journal of Biological Chemistry, for inclusion in your thesis. Best of Luck with your defense.
Steve

Stephen S. G. Ferguson
Canada Research Chair in Molecular Neuroscience
Scientist, Robarts Research Institute
Director, Cell Biology Research Group
Associate Professor,
Department of Physiology and Pharmacology, UWO
100 Perth Dr.
London, Ontario, CAN
N6A 5K8

Tel: (519) 663-3825
FAX: (519) 663-3789
Email: ferguson@rri.ca

-----Original Message-----
From: Natasha Hussain [mailto:natasha.hussain@mail.mcgill.ca]
Sent: Wednesday, April 30, 2003 2:58 PM
To: Martina Metzler; Montarop Yamabhai; Asha Bhakar; Stephen S. G. Ferguson; Peter McPherson; Brian Kay
Subject: Copyright release request

Dear co-Authors, April 30, 2003

As you are all authors on the recently submitted article "The ENTH domain binds tubulin and microtubules" to the Journal of Biological Chemistry, I am writing to request permission from each of you to include this document in my Doctoral Thesis (letters of permission from each of you is required by McGill for final submission). Thus, I would greatly appreciate if you could email (a reply email with "permission granted" within the body of the text would be sufficient) or fax a letter to this effect to the address included below. Martina, could you also send this to Dr. Hayden, or provide his co-approval in an email or fax.

Thank you all for your time and consideration.

Sincerely,
Natasha K. Hussain

Department of Neurobiology
Montreal Neurological Institute, rm. 776
McGill University
3801 University Street
Montreal, P.Q. H3A 2B4
Phone: (514) 398-6644 x 00209
Fax: (514) 398-8106
Email: natasha.hussain@mail.mcgill.ca

Subject: Re: Copyright release request
From: Asha Bhakar <abhakar@hotmail.com>
Date: Thu, 01 May 2003 16:01:57 +0000
To: natasha.hussain@mail.mcgill.ca

Permission granted, Asha Bhakar

>From: Natasha Hussain
>Reply-To: natasha.hussain@mail.mcgill.ca
>To: Martina Metzler , Montarop Yamabhai , Asha Bhakar , "Stephen S. G. Ferguson" , Peter McPherson , Brian Kay
>Subject: Copyright release request
>Date: Wed, 30 Apr 2003 17:57:34 -0400
>
>Dear co-Authors, April 30, 2003
>
>As you are all authors on the recently submitted article "The ENTH
>domain binds tubulin and microtubules" to the Journal of Biological
>Chemistry, I am writing to request permission from each of you to
>include this document in my Doctoral Thesis (letters of permission
>from each of you is required by McGill for final submission). Thus,
>I would greatly appreciate if you could email (a reply email with
>"permission granted"! within the body of the text would be
>sufficient) or fax a letter to this effect to the address included
>below. Martina, could you also send this to Dr. Hayden, or provide
>his co-approval in an email or fax.
>
>Thank you all for your time and consideration.
>
>Sincerely,
>Natasha K. Hussain
>
>Department of Neurobiology
>Montreal Neurological Institute, rm. 776
>McGill University
>3801 University Street
>Montreal, P.Q. H3A 2B4
>Phone: (514) 398-6644 x 00209
>Fax: (514) 398-8106
>Email: natasha.hussain@mail.mcgill.ca
>
>
>

Subject: Re: Copyright release request
From: bkay@anl.gov
Date: Thu, 01 May 2003 15:33:45 -0500
To: natasha.hussain@mail.mcgill.ca

On 4/30/03 4:57 PM, "Natasha Hussain" <natasha.hussain@mail.mcgill.ca>
wrote:

> Thus, I would greatly appreciate if you could email (a reply email with
> "permission granted" within the body of the text would be sufficient) or

Permission granted.

Congratulations by the way on your defense!

Brian

Brian K. Kay, Ph.D.
Senior Biochemist & Group Leader
Argonne National Laboratory
9700 South Cass Avenue
Building 202, Room B209
Argonne, IL 60439
USA

office: 01-630-252-3824
fax: 01-630-214-0648
http://www.bio.anl.gov/research/brian_kay.html

Subject: Re: Copyright release request
From: Montarop Yamabhai <montarop@ccs.sut.ac.th>
Date: Fri, 02 May 2003 21:39:23 +0700
To: Natasha Hussain <natasha.hussain@mail.mcgill.ca>

Dear Natasha,

I am writing this letter to inform you that permission is granted for the publication of our recently submitted article entitled "The ENTH domain binds tubulin and microtubules" into your Doctoral thesis.

Sincerely yours,

Montarop Yamabhai

Dear co-Authors, April 30, 2003

As you are all authors on the recently submitted article "The ENTH domain binds tubulin and microtubules" to the Journal of Biological Chemistry, I am writing to request permission from each of you to include this document in my Doctoral Thesis (letters of permission from each of you is required by McGill for final submission). Thus, I would greatly appreciate if you could email (a reply email with "permission granted" within the body of the text would be sufficient) or fax a letter to this effect to the address included below. Martina, could you also send this to Dr. Hayden, or provide his co-approval in an email or fax.

Thank you all for your time and consideration.

Sincerely,
Natasha K. Hussain

Department of Neurobiology
Montreal Neurological Institute, rm. 776
McGill University
3801 University Street
Montreal, P.Q. H3A 2B4
Phone: (514) 398-6644 x 00209
Fax: (514) 398-8106
Email: natasha.hussain@mail.mcgill.ca

permission granted:
Dr. Metzler
Martina Metzler
Michael Hayde

A Role for ENTH/ANTH Domains in Tubulin Binding

Natasha K. Hussain[1], Montarop Yamabhai[2], Asha L. Bhakar[1],
Martina Metzler[3], Stephen S. Ferguson[4], Michael R. Hayden[3], Peter S. McPherson[1], and Brian K. Kay[5]

[1]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC, H3A 2B4, Canada

[2]School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

[3]The Centre for Molecular Medicine and Therapeutics, Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia V5Z 4H4, Canada

[4]Department of Cell Biology Research Group, Robarts Research Institute, London, Ontario N6A 5K8, Canada

[5]Biosciences Division, Argonne National Laboratory, Argonne, IL 60439 USA

Running title: ENTH domains bind tubulin

Correspondence should be addressed to:
Dr. Peter S. McPherson
Department of Neurology and Neurosurgery
Montreal Neurological Institute
McGill University
3801 University Ave.
Montreal, QC
H3A 2B4
Phone: (514) 398-7355
Fax: (514) 398-8106
E-mail: peter.mcpherson@mcgill.ca

Dr. Brian K. Kay
Biosciences Division
Argonne National Laboratory
Building 202,
9700 South Cass Ave.
Argonne, IL
60439
Phone: (630) 252-3824
Fax: (630) 252-3387
E-mail: bkay@anl.gov

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 3667
Investigator #: 950
Approval End Date: MARCH 31, 2003
Facility Committee: MNI

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 3667

Title (must match the title of the funding source application): Role of epsins in endocytic vesicular trafficking.

1. Investigator Data:

Principal Investigator: Peter McPHERSON **Office #:** 398-7355
Department: Neurology & Neurosurgery **Fax#:** 398-8106
Address: Montreal Neurological Institute **Email:** peter.mcpherson@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Peter McPherson **Work #:** 398-7355 **Emergency #:** 639-1249
Name: Elaine De Heuvel **Work #:** 398-6644 Ext. 00209 **Emergency #:** 630-4167

3. Funding Source:

External ☒
Source (s): NSERC
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: Apr 1, 2001-Mar 31, 2005

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

ACTION	✓	DATE
PI	✓	Feb 19/02
FACC	✓	"
RGO	✓	"
VET	✓	"
DB		

Approved

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed. e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: [signature] **Date:** Feb 19, 2002

Approval Signatures:

Chair, Facility Animal Care Committee:	[signature]	**Date:** March 25, 2002
University Veterinarian:	[signature]	**Date:** 4/2/02
Chair, Ethics Subcommittee(as per UACC policy):		**Date:**
Approved Period for Animal Use	**Beginning:** April 1, 2002	**Ending:** MARCH 31, 2003

☐ This protocol has been approved with the modifications noted in Section 13.

MAR 28 2002

Montreal Neurological Institute and Hospital

A Teaching and Research Institute at McGill University

Internal Radioisotope User Permit

Issued by
The Radiation Safety Committee of the
Montreal Neurological Institute and Hospital

Authorized by the Canadian Nuclear Safety Commission
CNSC Radioisotope Licence Number: 01187-2-03.0

1	Radioisotope User Permit Number	MNI_012
	Classification	BASIC
	Date of Issue	May 1, 2001
	Expiry Date	April 30, 2003
2	Name of Principal Investigator	McPHERSON, Peter
3	Department	CBET
4	Locations approved by this permit	740
5	Radioisotopes approved by this permit	^{3}H [redacted] ^{35}S ^{32}P [redacted] ^{125}I [redacted]
6.	Personal Dosimeters Required	YES
7	Method of Disposal	**Quantities higher than these limits must be disposed of as radioactive waste**

	^{3}H	[redacted]	^{35}S	^{32}P	[redacted]	^{125}I	[redacted]
Liquid/sewer (µCi/l)	10		0.1	0.1		1	
Solid/ garbage (µCi/Kg)	1000		10	10		100	
Gas/Atmosphere (µCi/ CubicMeter)	1		0.01	0.01		0.1	

8 Special Conditions

Gloves and labs coats mandatory
Weekly wipe tests required in areas where radioisotopes are used
Ring badges required for staff using > 50 M Bq of ^{32}P.
Monitoring of all work surfaces where ^{32}P is used at the end of the work day
Radio-iodinations: Must be carried out in a working fume hood. Schedule thyroid monitoring. Use of proper survey equipment during radio iodine manipulation

The Radiation Safety Officer, MNI: _____ (E. Meyer, RSO)

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: **Peter McPherson**

ADDRESS: **Montreal Neurological Institute**
3801 University Street

TELEPHONE: **398-7355**
FAX NUMBER: **398-8106**
E-MAIL: **mcpm@musica.mcgill.ca**

DEPARTMENT:

PROJECT TITLE: **Role of epsins in endocytic vesicular trafficking**

---

2. FUNDING SOURCE: MRC ☐ NSERC ☒ NIH ☐ FCAR ☐ FRSQ ☐
INTERNAL ☐ OTHER ☐ (specify)

Grant No.: Beginning date **April 1, 2001** End date **March 31, 2005**

---

3. Indicate if this is

☒ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date: March 31, 2001

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency Approval End Date

☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: SIGNATURE date: 24/04/2001 (day month year)

Chairperson, Biohazards Committee: SIGNATURE date: 27/04/2001 (day month year)

DR. DALIUS J. BRIEDIS
Dept. of Microbiology & Immunology
McGill University
3775 University Street
Montreal, Quebec H3A 2B4
Canada

Approved period: beginning 27/04/2001 (day month year) ending 31-03-2005 (day month year)

* as defined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996