The Role of Cdc42 GTPase Effector N-WASP in Neurite Outgrowth

A Thesis By

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To Mum for all your hard work and support over the years and giving me the opportunity to fulfil my dreams. Dad, even though you are not here to see this day, I know you are and always will be looking down from heaven over me, I hope I have made you proud. To Tom, for all your encouragement, support and making me smile during my lowest moments.
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The Rho family GTPase proteins Cdc42, Rac and Rho play critical roles in signalling pathways leading to cytoskeletal rearrangements and involved in cell growth and differentiation. In neuronal cells, the combined action of Cdc42 and Rac results in the formation of neurites, whereas Rho acts to collapse neurites and growth cones.

Overexpression of the Cdc42/Rac effector proteins N-WASP, IRS-58 and Pak (the latter with CAAX motif to promote membrane localisation) all promoted neurite outgrowth in neuroblastoma N1E-115 cells, with differing morphologies. Combinations of these effector molecules did not act synergistically in the promotion of neurite outgrowth. Overexpression of the downstream effectors of Pak, PIX and GIT1, inhibited outgrowth by the effectors N-WASP and IRS-58, instead they promoted cell motility.

N-WASP is comprised of several domains and the C-terminal (VCA) region has been shown to interact with the Arp2/3 complex, F-actin and to be involved in the formation of the autoinhibited structure. The verprolin homology domain (VH), which is believed to be responsible for the binding/severing of actin, inhibits neurite outgrowth induced by the effectors IRS-58, N-WASP, PakCAAX and low serum conditions, but does not inhibit filopodia formation. Live imaging studies of N1E-115 cells overexpressing N-WASP suggest its importance in filopodia turnover. Microinjection of NWASP -/- cells with IRS-58 observed in real time indicates the importance of N-WASP the formation of filopodia by IRS-58.

These data together suggest the existence of a balance between the induction of cell motility or neurite outgrowth by different Rho GTPase effectors. The observations made in this project indicate the requirement of N-WASP in neurite outgrowth and of IRS-58’s formation of filopodia.
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Abbreviations

AB/AM  Antibiotic/antimycotic tissue culture additive
ABP   Actin binding protein
ACH   Acetylcholine
ADF   Actin-depolymerising factor
ADP   Adenosine diphosphate
AMP   Adenosine monophosphate
Amp   Ampicilin
APS   Ammonium persulphate
ARF   ADP ribosylation factor
ARP   Actin related proteins
ATP   Adenosine triphosphate
BAIAP Brain-specific Angiogenesis inhibitor associated protein
BSA   Bovine serum albumin
Ca²⁺  Calcium ions
cAMP  Cyclic adenosine monophosphate
Cdc42 Cell division cycle 42
cGMP  Cyclic guanosine monophosphate
CIID  C-terminal intramolecular interaction domain
CIP4  Cdc42 interacting protein 4
CNS   Central nervous system
Cof   Cofilin homology domain of N-WASP
CRIB  Cdc42/Rac interacting binding region
CRMP  Collapsin response mediator protein
CSPG  Chondroitin sulphate proteoglycan
DAG   Diacylglycerol
ddH₂O Deionised purified water
DH    Dbl homology domain
DNA   Deoxyribonucleic acid
DOPA  Dihydroxyphenylalanine
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<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
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<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
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<td>EGF(R)</td>
<td>Epidermal growth factor (receptor)</td>
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<td>Ena</td>
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<td>GTPase binding domain</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide inhibitor protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GIT</td>
<td>G-protein coupled receptor kinase interacting targets</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-thio--D-galactoside</td>
</tr>
<tr>
<td>IQ</td>
<td>Calcium/Calmodulin binding motif</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase-activated protein kinase</td>
</tr>
<tr>
<td>LIM</td>
<td>LIN-1, ISL-1, MEC-3</td>
</tr>
<tr>
<td>LPA</td>
<td>Lyosphosphatidic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MDia</td>
<td>Mammalian diaphanous</td>
</tr>
<tr>
<td>Mena</td>
<td>Mammalian enabled</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MRCK</td>
<td>Myotonic Dystrophy kinase-related Cdc42-binding kinase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotine adenine dinucleotide phosphate plus a proton</td>
</tr>
<tr>
<td>NAK</td>
<td>Nef-associated kinase</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofilament</td>
</tr>
<tr>
<td>NGF</td>
<td>Neuron growth factor</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neural-Wiskott Aldrich Syndrome Protein</td>
</tr>
<tr>
<td>p21'</td>
<td>Rho GTPase family members</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF(R)</td>
<td>Platelet derived growth factor (receptor)</td>
</tr>
<tr>
<td>PDZ</td>
<td>Post synaptic density-95, Dlg, ZO1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology domain</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3 phosphate lipid kinase</td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphatidylinositol phosphate</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol biphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol triphosphate</td>
</tr>
<tr>
<td>PIX</td>
<td>Pak interacting exchange factor</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKN</td>
<td>Protein kinase N</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethyl-sulfonyl fluoride</td>
</tr>
<tr>
<td>POPX</td>
<td>Partner of PIX</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Post synaptic density protein of 95 kDa</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding domain</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride transfer membrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>RasGRP</td>
<td>Ras guanine nucleotide releasing protein</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homologous member A</td>
</tr>
<tr>
<td>ROK</td>
<td>Rho associated kinase</td>
</tr>
<tr>
<td>ROK</td>
<td>RhoA binding kinase</td>
</tr>
<tr>
<td>RPTK</td>
<td>Receptor protein tyrosine kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress activated protein kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine residue</td>
</tr>
<tr>
<td>SH</td>
<td>Scar homology domain</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3 domain</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethylethlenediamine</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine residue</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyoxyethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine residue</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VCA</td>
<td>Verprolin homology-cofilin homology-acidic region of WASP/N-WASP</td>
</tr>
<tr>
<td>VH</td>
<td>Verprolin homology domain of WASP family members</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott Aldrich Syndrome Protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP family verprolin homologous protein</td>
</tr>
<tr>
<td>WH</td>
<td>WASP homology domain</td>
</tr>
<tr>
<td>WIP</td>
<td>WASP interacting protein</td>
</tr>
<tr>
<td>WIP</td>
<td>Wiskott Aldrich Syndrome interacting protein</td>
</tr>
<tr>
<td>WIRE</td>
<td>WIP related protein</td>
</tr>
<tr>
<td>WISH</td>
<td>WASP interacting SH3 protein</td>
</tr>
<tr>
<td>WW</td>
<td>tryptophan tryptophan domain</td>
</tr>
</tbody>
</table>
Chapter One

Introduction
Introduction:

1.1: The Cytoskeleton:

1.1.1: Components of the Cytoskeleton:

The cytoskeleton forms the scaffold of the cell. It undergoes dynamic changes for cell motility, neurite outgrowth and other cellular responses to extracellular cues. The major components of the cytoskeleton are: actin, myosin, microtubules, and intermediate filaments. Also important in the control of the cytoskeleton are the actin binding proteins.

1.1.1.1: Actin

Actin exists in two forms, the globular monomeric form known as G-actin, or the filamentous F-actin form. F-actin consists of G-actin monomers forming a uniform, polar structure. Each actin filament possesses a relatively inert minus or pointed end and a plus or barbed end at which the filament is fast growing.

It is believed that a dynamic equilibrium exists between the monomeric G-actin and filamentous F-actin. Where there are no control factors, a process known as treadmilling occurs, in which addition of G-actin to the barbed or plus end is matched by its dissociation from the pointed or minus end (Wegner, 1976).

Actin networks can form three general arrays: parallel bundles, contractile bundles and open or loose arrangement. In parallel bundles, the F-actin is arranged so that the filaments have the same polarity resulting in the formation of structures such as microspikes or filopodia. Contractile bundles are the result of the filaments being organised with opposite polarities. Actin in this formation gives rise to stress fibres and is associated with the motor protein myosin. An open array is found in lamellipodia
structures. The F-actin forms a meshwork of many interconnecting filaments resulting in these veil like structures at the cell edge (Matsudaira, 1991).

1.1.1.2: Microtubules:

The protein tubulin when arranged in a noncovalent polymer forms physically robust structures, called microtubules. Microtubules form the mitotic spindle during cell division, whereby the chromosomes are physically segregated and the plane of cleavage is orientated by this microtubule array.

Microtubules consist of heterodimers of \(\alpha\)- and \(\beta\)-tubulin, which associate in a head to tail fashion forming a polar structure (Burns 1991). Microtubules, like actin, have a fast growing plus end and a slower growing minus end. The \(\beta\)-tubulin monomer is orientated so that it faces the plus end, whereas the \(\alpha\)-tubulin monomer is exposed at the minus end. This polarity of the microtubule structure is important in the function of the motor protein families’, kinesin (Vale and Fletterick, 1997) and dynein (Hyams and Lloyd, 1994). These proteins use the energy from the hydrolysis of ATP to move unidirectionally along microtubules (Desai & Mitchison, 1997).

Biochemical studies by Weisenberg et al. have shown that during polymerisation \(\beta\)-tubulin hydrolyses GTP (Weisenberg et al., 1968). The resulting energy allows dynamic instability to occur. Prolonged phases of polymerisation and depolymerisation occur at the ends of individual microtubules during this nonequilibrium dynamic state (Mitchison & Kirschner 1984).

Microtubules play a role in non-dividing as well as dividing eukaryotic cells. In non-dividing cells they are important in organising the cytoplasm, nucleus and organelle position as well as forming structures such as flagella and cilia (Desai & Mitchison, 1997). They also play an important role in axon formation and axonal transport (Stevens
et al., 1988; Hirokawa et al., 1996). Microtubules are stabilised by microtubule associated proteins (MAPs). In neuronal cells, these proteins have been shown to increase the polymerisation of tubulin, depress catastrophe and promote rescue (Drechsel et al., 1992; Trinczek et al., 1995), thereby increasing the amount of polymerised, steady state tubulin in the cell.

1.1.1.3: Intermediate Filaments:

Intermediate filaments, whose expression is cell-specific, are highly diverse and can account for up to 85% of total protein in differentiated cells such as keratinocytes and neuronal cells (Fuchs and Cleveland, 1998). These proteins are found as dimers, which are composed of two α-helical chains that are parallel and intertwined in a coiled-coil rod. The ends of the rods are highly conserved and associate head to tail. The dimers associate to form linear arrays, four of these in an antiparallel, half staggered manner form protofibrils. Three or four protofibrils when intertwined produce an apolar intermediate filament of 10nm in diameter. In neuronal cells the intermediate filaments consist of three proteins, the neurofilaments NF-L (67kD), NF-M (150kD) and NF-H (200kD). NF-L forms the backbone to which NF-M and NF-H integrate forming peripheral dimer arrays. In this formation, the tails of NF-H and NF-M are left protruding away from the backbone enabling them to associate with other neurofilaments and microtubules in the axoplasm. In neurones possessing stable synapses, neurofilaments accumulate and the axon diameter is increased. Neurofilaments surround the microtubules and cross bridge to each other, to microtubules and actin via a plectin (Svitkina et al., 1996), which is essential for protection against mechanical stresses (Fuchs and Cleveland, 1998).
1.1.4: Myosin:
There are multitudes of myosin proteins, which all possess a head region of approximately 80kDa of conserved sequence. Below this a neck or regulatory region is found. These are of variable length and bind between one and six light chains of calmodulin/EF-hand family proteins. The head and neck region comprise the motor domain which is responsible for the hydrolysis of ATP and hence the conversion of chemical energy into a unidirectional force along the actin filament (Bahler, 1996). The myosin head is conserved between all myosins but the tail is highly variable, hence it can possess a membrane binding site and/or a site that allows binding to a second actin filament. The tail region can therefore determine the function of the protein: attachment to plasma membrane, vesicle trafficking or alignment of actin filaments relative to each other (Alberts et al., 1994). 15 classes of myosin motors have been identified (Mermall et al., 1998), the best studied being those of Myosin I and V for which compelling evidence links these proteins to vesicle transport (Depina et al., 1999).

1.1.2: Actin Polymerisation:
Actin polymerisation is required for many forms of cell motility, nerve growth cone movement, neurite extension and cell spreading. Actin is rapidly cycled between monomeric (G-actin) and filamentous (F-actin) forms. The rate of cycling is determined by the actin binding proteins (ABPs) of which essentially there are two classes; sequestering proteins and capping proteins. Sequestering proteins act to inhibit actin polymerisation by binding the monomeric G-actin. Capping proteins bind to the barbed or plus end of the actin filament thus preventing its continued growth (Barkalow et al., 1996).
Either elongation of existing filaments or de novo nucleation of monomeric G-actin followed by elongation can produce new actin filaments (Mitchison and Cramer, 1996). Either severing creating new barbed ends or uncapping of existing barbed ends can elongate existing filaments (Higgs and Pollard, 1999).

The Rho GTPases have been shown to play a role in actin polymerisation. Rac in its GTP bound activated form causes uncapping of filaments resulting in further actin polymerisation (Hartwig et al., 1995). WASP, the Cdc42 effector molecule implicated in Wiskott Aldrich Syndrome, has also been shown to cause ectopic actin polymerisation at sites rich in WASP. This actin reorganisation is Cdc42 dependent.

As well as actin polymerisation, actin depolymerisation also occurs via severing proteins at the pointed or minus end of the filament. This process supplies the subunits for the fast growing, plus end of the actin filament and allows cytoskeletal rearrangement in the motile cell. An example of such a severing protein is actin-depolymerising factor (ADF). ADF is involved in the rapid turnover of barbed ends of actin filaments driving the forward movements of the leading edge (Carlier et al., 1999).

1.1.3: The Arp 2/3 Complex:
The Arp2/3 complex consists of seven polypeptides including the actin related proteins: Arp 2 and Arp 3, which form two major components (Machesky et al., 1999). This complex interacts with a WASP/Scar family member via a C-terminal region consisting of one or two WASp homology 2 (WH2) motifs a central linking region and an acidic region (Higgs et al., 2001) to which actin monomers are recruited and added to existing actin filaments (Rohatgi et al., 1999). This leads to the branching of an existing actin filament or the growth of a new filament by the addition of G-actin monomers to the barbed end (Blanchoin et al., 2000). In isolation, the Arp2/3 complex is intrinsically
inactive and requires actin filaments, ATP and activating proteins such as N-WASP. A conformational change occurs upon binding of ATP and the activating protein whereby the two Arps come into close proximity forming a structure favourable for actin polymerisation (Robinson et al., 2001). Currently two models for actin polymerisation by the Arp2/3 complex exist: 1) dendritic actin-nucleation model (Mullins et al., 1998; Pollard et al., 2000); 2) barbed-end nucleation model (Pantaloni et al., 2000). The Arp2/3 complex has been implicated in lamellipodia formation and protrusion in fibroblast cells, actin comet tails in *Listeria monocytogenes* (Welch, 1997), ring canal formation in oogenesis, formation of the central nervous system and morphogenesis of the eye and sensory bristles in *Drosophila* (Hudson et al., 2002; Zallen et al., 2002). The exact mechanism and actions of the Arp2/3 complex is still unrefined (Higgs et al., 2001).

**1.1.4: Actin Binding Proteins:**

The cross-linked continuous matrix of actin filaments accounts for the elasticity and strength seen in the leading edge of a motile cell. In the process of actin filament formation, bundle formation, attachment to the membrane and strengthening, many proteins are involved.

*Tropomyosin* is a widely distributed protein that is comprised of two alpha-helical chains in a coiled coil conformation, forming a chain of units polymerised end to end. It is found ubiquitously expressed and is associated with actin along the two grooves of the F-actin filament giving both structural stability and function modulation (Perry, 2001).

*Fimbrin and α-actinin* are widely distributed bundling proteins. Parallel filament bundles found at the leading edge and in microspikes or filopodia are enriched
with fimbrin, where as α-actinin is responsible for the looser cross-linking of actin filaments in stress fibres (Albert et al., 1994).

**Filamin** organises F-actin into networks and stress fibres. These proteins form dimers in a tail-to-tail manner and anchor transmembrane proteins to the actin cytoskeleton providing a scaffold for cytoplasmic signalling proteins (reviewed by van der Flier et al., 2001).

**Gelsolin**, an actin filament severing and capping protein, is regulated by calcium and PIP2 (Kwiatkowski et al., 1999; Robinson et al., 1999). The Gelsolin binds to the actin filament rapidly and causes a conformational change in the actin filament (McGough et al., 1998). Once the filament has been severed, gelsolin remains attached to the filament as a capping protein preventing short filament re-annealing or elongation at their barbed ends (Sun et al., 1999). Severing results in an increased number of actin filaments and the uncapping of gelsolin produces many barbed ends to which actin monomers can be added, allowing the cell to rebuild the actin cytoskeleton in response to external cues (Yin et al., 1999).

**Myosin**, of which 15 classes of motors have been identified (Mermall et al., 1998), most investigated being those of Myosin I and V for which compelling evidence links these proteins to vesicle transport (DePina et al., 1999). These proteins function to either attach the cytoskeleton to the plasma membrane, traffic vesicles or align actin filaments relative to each other (Alberts et al., 1994).

**Spectrin** forms a heterodimers or heterotetramers via interchain binding at the ‘head’ end between α and β chains. The ‘tail’ end possesses sites which associate the spectrin tetramers and to other proteins such as actin forming a network between spectrin and the cytoskeleton (Viel et al., 1996).
**Cofilin** causes enhanced turnover of actin filaments by increasing the depolymerisation rate from the pointed end (Carlier *et al.*, 1997) and severing the actin filaments directly (Du *et al.*, 1998). Cofilin binds to both monomeric, G-actin units and filamentous actin, showing a greater affinity for the ADP bound forms (reviewed by Bamburg, 1999)

1.2: The Development of the Mammalian Brain:

In the developing brain, neurons are formed in the ventricular zone. These neuroblasts attach themselves to radial glial cell fibres, which span the thickness of the neural tube (early brain form in developing embryo). There is a systematic relationship between the time of birth of a neuron and its migratory distant. The later a neuron leaves the cell cycle the further they migrate, past earlier born neurons forming the outer most layer, creating an inside-out laminar organisation (Hatten, 1999).

The control of the attachment of neuroblasts to glial radial cell fibres is under the control of proteins such as reelin, which promotes the dissociation of these neuronal cells from glial fibres (Ogawa *et al.*, 1995). Proteins such as doublecortin, filamin 1, cdk5 and p35 are believed to promote the interaction of neuroblasts with glial cell fibres and hence their migration to target destinations (Gleeson and Walsh, 2000).

As well as cell migration, axon guidance is a major part of neural development. Various proteins control axon guidance at either short or long range. Neurite outgrowth promoting factors include proteins such as laminins, N-cadherin, netrins and immunoglobulin cell adhesion molecules. Long/short range repulsive guidance cues include ephrins and semaphorins (Jessell and Sanes, 2000). Upon an axon reaching dendrites of a neighbouring neuronal cell or a more distal target cell, a synapse must be formed for signal transduction to occur. For example, the formation of the
neuromuscular junction involves the protein agrin, which activates the effector rapsyn and the dystrophin-glycoprotein complex, via its tyrosine kinase receptor MuSK. This modulates the maturation and maintenance of the postsynaptic apparatus (Sanes and Lichtman, 1999). PDZ-domain proteins, such as PSD-95, Grip and Homer have been implicated in the formation of the postsynaptic apparatus at excitatory synapses. At inhibitory synapses, gephyrin, which itself binds an exchange factor protein, has been shown to be involved in this process (Lee and Sheng, 2000).

1.3: Structure of a Developing Neurite:

Extensive work carried out by Wilhelm His and Santiago Ramon y Cajal resulted in the understanding that the fibres of the neural network form from a neuronal soma and make connections with neighbouring neuronal cells (Bartlett and Banker, 1984). A growing neurone develops two types of process, a single axon by which information is transferred to surrounding neurones and multiple dendrites to collect information from its neighbours (Craig et al., 1994; Bradke et al., 2000). The growth cone is the structure responsible for the forward movement of the axon to its target (Eisen, 1988). It possesses both lamellipodia and filopodia, which sense environmental cues instructing changes to the neuronal cytoskeleton and resulting in guidance decisions as to the trajectory of the growing neurite (Lin et al., 1994; Mueller et al., 1999). The periphery of the growth cone is rich in actin microfilaments, whereas the central regions have an abundance of the microtubules (Bridgman and Dailey, 1989; Tanaka and Kirschner, 1991), and myosin IIIB arranged in mini filaments in specific compartments (Bridgman et al., 2001). The actin cytoskeleton forms a meshwork of actin filaments that controls the elongation and invasion of microtubules into the growth cone. In the growing axon,
actin filaments depolymerise causing the influx of microtubules that form a meshwork to replace the former actin filament framework (Forscher et al., 1988).

The mechanism by which the cell decides which one of the growing neurites will form the axon or go on to become the dendrites is still not known. One model proposes that each of the neurites send out growth discouraging signals to one another and each possesses a self-promoting growth activity. Then one neurite is marked to become the axon, which then increases its growth discouraging signals to the surrounding neurites and promotes its own growth activity (Andersen et al., 2000). The rate of actin filament turnover in the axonal growth cone is higher than in minor neurites and hence probably forms a less stable network (Bradke et al., 2000).

In the extending axon microtubules form short, tight, continuous bundles which act as scaffold structures and tracks to transport materials from the cell body to the growth cone and vice versa (Dent et al., 1999). Microtubules are polar filaments that orientate themselves with their plus-ends distal to the cell body (Heidemann et al., 1981) at which the dynamic assembly and disassembly events occur (Baas et al., 1992).

1.4 Signal Transduction:
1.4.1: Principles of Cell Signalling:

The cell responds to extracellular cues and other stimulatory factors via a cascade of events. Cellular events such as neuronal guidance, cytokinesis and immune response are all initiated in response to specific signals, which the cell receives, from its environment or neighbouring cells. In neuronal guidance, it is the growth cone and its filopodia, which sense the matrix of proteins (laminin, fibronectin, CSPG (chondroitin sulphate proteoglycan) and myelin) or soluble factors (neurotrophins, semaphorins, netrins and others) that determines its path of growth. These factors signal through various receptors
found on the growth cone of the growing axon of the neuron, which lead to the initiation of various signalling cascades resulting in the response of the cell in either a positive or negative manner towards the guidance cue e.g. laminin being positive and CSPG being repulsive (Song and Poo, 1999).

Signal transduction can be mediated by a variety of signalling events: Paracrine, in which neighbouring cells signal to one another; endocrine, is mediated by secreted hormones; and synaptic signalling, where neurotransmitters transmit messages from one cell to another at the synaptic junction (Snyder, 1985).

1.4.2: Machinery of Cell Signalling:

Extracellular signals/cues from the matrix (e.g. laminin and fibronectin), soluble factors such as semaphorins and netrins, and cell mediated signals like slit and robo are transmitted to the intracellular compartments of the cell via surface receptor proteins to elicit a cell response. These receptor proteins consist of three main classes:

**Ion channel linked receptors** are responsible for the rapid signalling between electrically excitable cells. The release of acetylcholine (ACh) at the neuromuscular junction is triggered by the influx of calcium ions into the synaptic junction. This results in the exocytosis of ACh from the presynapse, which binds to its postsynaptic receptor, a ligand-gated cation channel, relaying the nerve impulse from the nerve cell to the muscle cell, resulting in a contraction signal being transmitted. Ion channel linked receptors such as the Ca^{2+} channel, which mediates the release of ACh, is the target of many neurotoxins such as the black widow spider venom, α-latrotoxin (causing a massive release of ACh) and the Clostridium botulinum, botulinus toxin (inhibitory effect on release) (Voet and Voet, 1995).
**G-protein coupled receptors** respond to a large variety of signalling molecules such as hormones, neurotransmitters, and local mediators. Trimeric GTP-binding proteins (G-proteins) link these receptors to second messenger enzymes and ion channels. These proteins, like the monomeric GTPases (discussed later), are molecular switches, which are active when GTP is bound and inactive in the GDP bound state. In the GTP bound state the second messenger protein can diffuse away from its receptor to which it is associated at the plasma membrane and initiates a signalling cascade through the cell in response to the extracellular stimulus. One of the most frequently used second messenger is cyclic AMP (cAMP), synthesised from ATP by the plasma membrane bound enzyme adenylyl cyclase, and rapidly destroyed in the cytoplasm by cyclic AMP phosphodiesterases.

Trimeric G proteins are composed of three different polypeptide chains, $\alpha$, $\beta$, $\gamma$. The $\alpha$ chain is responsible for the binding and hydrolysis of GTP and the activation of adenylyl cyclase. The $\beta$ and $\gamma$ chains form a tight complex, responsible for anchoring the G-protein to the plasma membrane. The protein exists as a trimer when GDP is bound, upon binding of the ligand to its receptor, GDP is exchanged for GTP and the $\alpha$ chain dissociates from the trimeric complex and binds to adenylyl cyclase thereby activating it and causing the production of cAMP (Alberts et al., 1994).

**Enzyme-linked receptors** are transmembranous receptors that also have a ligand binding receptor located on the outer plasma membrane surface and their cytosolic domain posses either intrinsic enzyme activity or the ability to directly associate with enzymes. There are five classes of enzyme-linked receptors:

1) *receptor guanylyl cyclases* catalyse the production of cGMP and use this as a messenger,
2) **receptor tyrosine kinases** transfer a phosphate group from ATP to tyrosine residues on both the receptor and intracellular signalling proteins;

3) **tyrosine-kinase-associated receptors** associate with proteins possessing tyrosine kinase activity, such as the non-receptor tyrosine kinases *Src* family proteins;

4) **receptor tyrosine phosphatases** which remove phosphate groups from tyrosine residues on specific intracellular signalling proteins;

5) **receptor serine/threonine kinases** phosphorylated serine/threonine residues on some intracellular proteins, such as the members of the *TGF-β* Superfamily (Alberts *et al.*, 1994).

**1.4.3: Receptor Tyrosine Kinases:**

These receptors are a large family of transmembrane proteins possessing intrinsic protein tyrosine kinase activity. Members include epidermal growth factor receptor (EGFR), insulin receptor (IR), platelet derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR) (Alberts *et al.*, 1994). With the exception of the insulin receptor, they are all monomeric proteins in the cell membrane, which upon binding of their relevant ligand, undergo dimerisation (van der Geer, 1994). This leads to the autophosphorylation of the receptor at the cytoplasmic domain that contains a conserved protein tyrosine kinase (PTK) core and regulatory sequences, which are the targets of autophosphorylation and phosphorylation by heterologous protein kinases (Jiang and Hunter, 1999). Most of these autophosphorylation sites are located in the noncatalytic regions of the receptor molecule, which function as binding sites for Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains of various signalling proteins (Pawson *et al.*, 1993). All of the members of the RTK family have an extracellular ligand binding domain that is mostly glycosylated, this domain is connected to the cytoplasmic domain...
via a single transmembrane helix, which anchors the receptor to the membrane (Schlessinger, 2000).

The dimerisation of RTKs leads to their activation, however, specificity is achieved by the ligand inducing this event in different manners. Ligands such as growth hormone and erythropoietin are bivalent cytokines and bind two of their relevant receptors simultaneously forming one ligand: two receptors complex (Kossiakoff et al., 1998). As a result of dimerisation and autophosphorylation of the RTK, tyrosine residues of intracellular signalling protein targets are phosphorylated by the transfer of the \( \gamma \) phosphate group from ATP. This initiates a number of signalling cascades, one of the most important being the activation of Ras (Li et al., 1993; Gale et al., 1993; Skolnik et al., 1993; Olivier et al., 1993). This leads to the activation of the mitogen activated protein kinase (MAPK) (Franklin et al., 1994), which can modulate the activity of various transcription factors (Xing et al., 1996; Simon, 2000). RTKs play an important role in cellular processes including the cell cycle, cell migration, cell metabolism and survival and in cell proliferation and differentiation (Schlessinger, 2000)

1.4.4: Kinase Signalling Cascades:

1.4.4.1: Mitogen Activated Protein Kinase:
Mitogen activated protein (MAP) kinases also known as extracellular-signal regulated kinases (ERKs) are activated by a wide variety of extracellular proliferation- and differentiation-inducing signals some of which activate RTKs (see fig. 1.4.4). All RTKs stimulate the exchange of GDP to GTP on Ras via activation of the guanine nucleotide exchange factor (GEF) Sos (Egan et al., 1993; Buday et al., 1994). An adaptor protein Grb2 forms a complex with Sos via its SH3 domain (Reif et al., 1994; Terasawa et al., 1994). This complex is recruited to the activated RTK, thus translocating Sos to the
plasma membrane where it can exchange GDP for GTP on Ras and activate the small G protein (Aronheim et al., 1994). Active Ras can interact with downstream effectors such as Raf (Jelinek et al., 1996) and PI-3 kinase (Valius and Kazlauskas, 1993; Rodriguez-Viciana et al., 1996). The activated form of Raf is able to stimulate MAP kinase kinase (MAPKK) by phosphorylation of a Ser residue in the activation loop. In turn, MAPKK phosphorylates MAPK (ERK) on specific Thr and Tyr residues (Hunter, 2000), which are separated by one amino acid residue in the activation loop. This dual residue phosphorylation ensures specific activation by MAPKK. Activated MAPK relays signals to downstream proteins by phosphorylation of various cytoplasmic proteins, including other protein kinases and gene regulatory proteins. MAPK is also rapidly translocated to the nucleus upon its activation, where it phosphorylates and activates transcription factors (Karin and Hunter, 1995). Within minutes of stimulation by a growth factor via the RTKs, immediate early gene transcription is switched on. One important protein complex in this transcriptional activation is formed by the serum response factor (SRF) and Elk-1. This complex is bound constitutively to the serum response element of the fos gene, phosphorylation of the Elk-1 component by MAPK results in the activation of this complex and subsequent transcription of the fos gene (Alberts et al., 1994).

The MAPKKK, MAPKK and MAPK proteins comprise a signalling cassette, which is highly conserved through evolution and several MAPK cascades exist in yeast, invertebrates and vertebrates (Waskiewicz and Cooper, 1995; Madhani and Fink, 1998; Garrington and Johnson, 1999). These cascades have been shown to play an important role in the control of many metabolic processes, cell cycle, cell migration, cell shape and cell proliferation and differentiation (Davis, 2000).
Figure 1.4.4: MAP-kinase cascade: Ligand binding to a RTK initiates recruitment of the RasGEF, Sos, via the adaptor protein Grb2. This leads to the activation of Ras, which can cause the activation of MAPKKK, which in turn phosphorylates MAPKK and finally to MAPK which can activate various signalling cascades. This pathway can alternatively be activated by PKC, which can activate various signalling pathways directly or via the MAP-kinase cascade.
1.4.4.2: JNK/SAPK:

Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signalling pathway is a member of the MAPK superfamily. The transcription factor c-jun is a specific phosphorylation target of JNK, whose expression is increased in response mostly to inflammatory cytokines (Binetruy et al., 1991). A subsequent stress-activated MAP kinase subfamily known as p38 has also been identified (Lee et al., 1994). Only JNK phosphorylates c-Jun at residues Ser63/73, resulting in its transcriptional activity whereas stimulation of p38 or ERK does not (Raingeaud et al., 1996; Minden et al., 1994 respectively).

JNK activity and its activation of c-Jun has been implicated in various cellular functions including proliferation, tumourigenesis, mouse embryonic development (Johnson et al., 1993), apoptosis (Xia et al., 1995) and dorsal closure in Drosophila (Hou et al., 1997).

1.4.5: Inositol Phospholipid Signalling:

The most important of the inositol phospholipids in signal transduction are phosphatidylinositol phosphate (PIP) and phosphatidylinositol biphosphate (PIP$_2$), both of which are located on the inner half of the plasma membrane. PIP$_2$, which has been shown to be required for the full activation of N-WASP by the Rho GTPase Cdc42, is of a lower concentration than phosphatidylinositol (PI). PIP$_2$ is implicated in many pathways of control including regulation of the actin cytoskeleton. In the presence of PIP$_2$, gelsolin acts to remodel the actin cytoskeleton in response to agonists (Janmey and Stossel, 1989). Cofilin is negatively regulated by PIP$_2$, in its absence acts to depolymerise actin filaments (Yonezawa et al., 1990). PIP$_2$ is able to bind many
cytoskeletal proteins and is believed to be involved in controlling the adhesion between the actin cytoskeleton and plasma membrane (Raucher et al., 2000).

The binding of a ligand to its G-protein coupled receptor causes the activation of the trimeric G protein G_q, which in turn stimulates phospholipase C-β, an inositide-specific phospholipase C. PIP_2 undergoes a cleavage by this enzyme producing the products inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is a water soluble molecule, which diffuses away from the plasma membrane into the cytosol where it binds to IP_3-gated-Ca^{2+}-channels positioned in the endoplasmic reticulum (ER) membrane or calcium release channels in the sarcoplastic reticulum (SR) of muscle cells, causing muscle contraction. Both types of channels are regulated by a positive feedback loop, where the calcium released is able to bind back to the channels resulting in an increase in calcium release. This response can be terminated by two mechanisms: 1) rapid dephosphorylation of IP_3; 2) calcium that enters the cytosol from the ER or SR is rapidly pumped out of the cell.

The other cleavage product of PIP_2, DAG has two signalling roles. Further cleavage results in the formation of arachidonic acid, which can act as a messenger or be used in the synthesis of eicosanoids, or activate the C1 (cysteine rich) domain, containing proteins such as protein kinase C (PKC), a calcium dependent kinase (Alberts et al., 1994), and the chimaerin family members (Kazanietz, 2002). The initial increase in calcium in the cytosol induced by IP_3 is believed to result in PKC translocation to the cytoplasmic face of the plasma membrane. Here it is activated by calcium, DAG and the negatively charged membrane phospholipid phosphatidylserine. PKC phosphorylates specific Ser/Thr residues on target proteins. In neuronal cells, PKC phosphorylates ion channels, thereby changing their properties and subsequently altering the excitability of the nerve cell plasma membrane. In many cell types, PKC increases the transcription of
specific genes, by activation of transcription regulatory protein cascades (Alberts et al., 1994). There are several other targets of DAG including Chimaerin, myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) and RasGRP (Ras guanine exchange factor) to name just a few (Kazanietz, 2002).

1.4.6: Important Protein Domains of Signal Transduction:

Certain conserved protein domains are known to be targets of signalling cascades and mediate the protein-protein interactions that occur. These include the Src homology 2 and 3 (SH2 and SH3) domains, phosphotyrosine-binding (PTB) domains, PDZ and WW domains. Each domain possesses a unique three-dimensional structure that is complementary to specific motifs in ligands (Kuriyan and Cowburn, 1997). Certain characteristics for these proteins have been identified. They are composed of 40-150 amino acids, folded to form one or more ligand-binding sites, termed 'recognition pockets'. Conserved residues within these pockets determine the specificity of binding to ligands and maintaining structure of the domain. Ligand interaction occurs via short complementary sequences, known as core motifs, composed of 3-6 amino acids, which determine the domain targets of ligand (Cantley and Songyang, 1997). The flanking amino acids surrounding the core sequence determines the specificity of the interaction within a family of domains (Rickles et al., 1995).

1.4.6.1: SH2 Domains:

The Src homology 2 (SH2) domain is found in many cytoplasmic proteins (Sadowski et al., 1986). SH2 domains are involved in the regulation of a variety of cellular events, such as enzyme activity, substrate recruitment and protein localisation (Pawson, 1995). Protein ligands containing the consensus sequence \( p-Yxx\psi \) (where \( p-Y \) is a
phosphotyrosine, x is any amino acid and ψ represent a hydrophobic amino acid) where the phosphotyrosine residue is bound with high affinity (Cantley and Songyang, 1997) and is the main target of SH2 domain proteins (Pawson, 1995).

The SH2 domains recognize specific phosphotyrosine residues and enable proteins containing these domains to bind activated RTKs and other intracellular signalling molecules whose tyrosine residues have been transiently phosphorylated (Alberts et al., 1994). Examples of such SH2 domain containing proteins are PI3-kinase, GTPase-activating protein (GAP) of Ras and phospholipase C-γ (PLC-γ) (Waksman et al., 1993).

1.4.6.2: SH3 Domains:

The Src homology 3 (SH3) domain is a member of the family of modules that recognise proline rich motifs in ligands (Mayer et al., 1988; Sudol, 1998). SH3 domains are implicated in regulating protein localisation, enzymatic activity and assembly of multicomponent signalling complexes (Schlessinger, 1994; Mayer and Eck, 1995). Minimal consensus sequence of SH3 domain ligands is PxxP (where P is a proline residue and x represents any other amino acid residue) (Ren et al., 1993), however the consensus sequence observed in SH3 domain ligands is ψPxψP (Mayer and Eck, 1995). SH3 domains exist in two pseudosymmetrical orientations, known as class I and class II, it is to one of these classes a SH3 domain ligand will bind, which is determined by the flanking residues at the amino side of the consensus sequence in class I domains and at the carboxyl terminus in class II domains. This is a unique feature of SH3 domain-ligand interactions, which determines specificity of interaction and allows discrimination between various SH3 domains (Lim et al., 1994; Feng et al., 1994).
1.4.6.3: PH Domains:
The pleckstrin homology (PH) domain was initially identified as a 100-120 amino acid stretch, which is found twice in pleckstrin (Haslam et al., 1993). This sequence is found in many proteins involved in cellular signalling (Mayer et al., 1993), of which over 100 have been identified to date. PH domains target their host protein to the membrane by interaction with phosphoinositides. In some cases the PH domains interact with PI(4,5)P₂ (Lemmon et al., 1997), other ligands are the products of the PI3-kinases, PIP₃ and PI(3,4)P₂ (Rameh and Cantley, 1999). Other binding modules, which possess no significant sequence homology to the PH domain, have been shown to adopt a similar core structure, such as the phosphotyrosine binding (PTB) domains (Eck et al., 1996), Ran-binding domain (Vetter et al., 1999) and the Ena/VASP homology 1/WASP homology 1 (EVH1/WH1) domain found in WASP family proteins (Prehoda et al., 1999). Nearly every PH domain binds phosphoinositides or inositol phosphates to some extent (Rameh et al., 1997). Phospholipase C-δ (PLC-δ) possesses a PH domain at its N-terminus, which is a binding site for IP₃ and PI(4,5)P₂ (Lemmon et al., 1995) and is responsible for the targeting of this protein to the plasma membrane (Paterson et al., 1995).

There are no general protein targets for PH domains like that of SH2 and SH3 domain proteins. However, it is suggested that proteins other than phosphoinositides are involved in PH domain interactions (Lemmon and Ferguson et al., 2000).

1.4.6.4: LIM Domains:
Lim domains form a double zinc-finger motif, comprised of 50-60 residues, with the consensus sequence \((C-X_2-C-X_{16-23}-H-X_2-C)-X_2-(C-X_2-C-X_{16-21}-C-X_2-H/D/C)\), (where, C is cysteine, H is histidine, D is aspartic acid and X is any residue). The name LIM
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originates from the three proteins in which it was initially identified: lin-11, isl-1 and mec-3. Two tetrahedral zinc-binding pockets are formed by the conserved cysteine, histidine and aspartic acid residues, stabilising the structure of the protein. LIM domains are similar but distinct from other zinc binding proteins (Mackay and Crossley, 1998; Schmeichel and Beckerle, 1998). Most LIM proteins posses more than one LIM domain, enabling the potential coordination of interaction between several proteins (Brown et al., 2001).

LIM domain proteins are classified into three groups, determined by the sequence homology (Dawid et al., 1998). Group 1 LIM proteins include the LIM-kinase 1 and those containing a LIM-homeodomain and are generally located in the nucleus, with the exception of LIM kinase-1. During embryonic development and tissue differentiation, the LIM-homeodomain proteins are essential, as shown by LIM-1 knockout mice whose embryo's lack a head (Shawlot and Behringer, 1995). LIM-kinase 1 phosphorylates cofilin thereby inactivating it, hence stabilising the actin cytoskeleton by inhibition of actin depolymerisation (Arber et al., 1998; Yang et al., 1998).

Group 2 LIM proteins include the muscle LIM protein MLP/CRP3. This protein plays an important role in maintaining the structural integrity of the cardiac cytoskeleton by localising to the Z lines in cardiac muscle and binding actin-binding proteins, α-actinin and β-spectrin, deletion of this protein resulted in dilated cardiomyopathy in mice (Arber et al., 1997).

Group 3 LIM proteins include the focal adhesion proteins paxillin and zyxin and other cytoskeletal interacting proteins (Brown et al., 2001), members of the skeletal muscle LIM proteins (SLIMS) which localize to the nucleus, where they interact with transcription factors, and the cytoskeleton (Brown et al., 1999).
LIM domain proteins act as a docking site for signalling proteins allowing the amplification/integration of signalling pathways and regulation of their localisation. Phosphorylation of serine residues on LIM domains in paxillin promotes its localisation to focal adhesions and promotes cell spreading (Brown et al., 1998).

1.4.6.5: Other Domains:

Other domains, WW, PDZ and PTB, are also known to be motifs of signal transduction proteins.

**WW domains** are functionally related to SH3 domains as they too can bind proline rich ligands (Chen and Sudol, 1995). These domains, posses two highly conserved tryptophan residues, located 20-22 amino acids apart (Sudol et al., 1996) and two groups have been identified. Group I WW domains interact with ligands possessing a PPxY (where P is a proline residue, x is any amino acid and Y represents a tyrosine residue) (Chen and Sudol, 1995). The flanking amino terminal residues of these group I WW domains usually contain proline, lysine, cysteine or tyrosine residues and the carboxyl terminus flanking residues include prolines or lysine and/or arginine (Chen et al., 1997). Group II WW domains target proteins containing PPLP cores with at least three additional prolines in the amino or carboxyl flanking sequences (Bedford et al., 1997). These domains have been implicated in several human diseases including Liddle's syndrome of hypertension, muscular dystrophy and Alzheimer's disease (Sudol, 1996).

**PDZ domains** are found in cytosolic proteins many of which are located at cell-to-cell contact specialised regions. These domains are found in proteins: post synaptic density protein 95 (PSD-95); disc large tumor suppressor (Dlg) and a tight junction protein (ZO1), hence the name PDZ (Ponting and Phillips, 1997). Three PDZ domain
groups have been identified, which all recognize unique carboxyterminal motifs and require a hydrophobic amino acid in this motif (Sudol et al., 1998).

**Phosphotyrosine-binding (PTB) domains** play a similar role to the SH2 domains (Gustafson et al., 1995). There are two groups of PTB domains, group I and group II, determined by their ligand recognition. Group I, PTB domains recognise ligands possessing the consensus sequence NPxp-Y (N representing asparagine, P-proline, x-any amino acid and p-Y a phosphotyrosine residue). Src homologous and collagen (Shc) and insulin receptor substrate-I (IRS-I) PTB domains belong to the group I PTBs (Van der Geer and Pawson, 1995). The group II PTBs bind ligands with the core sequence NpxY (Y represents a tyrosine residue), where phosphorylation of the tyrosine residue is not required (Zambrano et al., 1997).

### 1.4.7: Integrin Signalling:

The integrins comprise a large family of cell adhesion molecules, mediating interactions between the cytoplasm and extracellular environment. They regulate many cellular events such as cell death, proliferation, migration and differentiation (Hynes, 1992). They achieve their effects by providing a link between the extracellular matrix (ECM) and cytoskeleton, as well as transducing bi-directional signals across the cell membrane. Integrins are heterodimers of α and β subunits, which are non-covalently linked and expressed on the cell surface. To date 16α and 8β mammalian subunits that associate to form 22 αβ heterodimers have been identified. These heterodimers are divided into three groups: β1, β2 and αv, each of which recognise specific ligands (Milner and Campbell, 2002).

In the central nervous system, the β1 and αv are expressed on a variety of cells, which is determined by location and stage of development (Pinkstaff et al., 1999). β1
integrins are implicated in neuronal migration in CNS development, as inhibition of α3β1 integrin reduces neuronal migration along radial glial cells (Anton et al., 1999) and is a cell surface receptor for Reelin, an ECM protein, essential for normal cortical development (Rice and Curran, 2001). Integrins are also fundamental in synaptogenesis in the developing CNS (Benson et al., 2000). Long term potentiation (LTP) is subject to modulation by integrins; inhibition of integrin-ECM interactions results in LTP decay (Bahr et al., 1997). Studies using Drosophila implicate integrins in learning and memory formation by affecting synaptic structure and transmission of signals across the synapse (Grotewiel et al., 1998; Rohrbough et al., 2000). Infection or injury within the CNS stimulates microglia to transform from their resting ramified state to an activated amoeboid form, in which there is an increased expression of the integrins α4β1 and lymphocyte function associated antigen (LFA-1) (Hailer et al., 1996). This increased expression is observed in activated microglia in Alzheimer's disease (Akiyama and McGeer, 1990), following lipopolysaccharide (LPS) infusion (Kloss et al., 2001) and multiple sclerosis lesions (Bo et al., 1996).

Various protein tyrosine kinases are activated by integrins, such as the focal adhesion kinase (FAK). The exact mechanism of activation of this kinase is not known but it is coupled to the assembly of focal adhesions. FAK interacts with the cytoplasmic tails of integrin β subunits directly or via cytoskeletal proteins talin and paxillin, leading to its localisation to focal complexes (Chen et al., 1995; Lewis and Schwartz, 1995). Activation of FAK leads to its autophosphorylation, creating a binding site for Src or Fyn via their SH2 domains (Schaller et al., 1994; Schlaepfer et al., 1994). Src kinase is then able to phosphorylate various focal adhesion proteins (Schlaepfer et al., 1997).

FAK is a potential activator of PI3-kinase, either directly or via Src kinase (Chen et al., 1996), which phosphorylates FAK, forming a binding site for the adaptor protein
Grb2 and the RasGEF mSOS (Schlaepfer et al., 1994), linking FAK to various signalling cascades including the MAPK pathway.

Integrins belonging to the β1 and αv groups have been shown to activate the tyrosine kinase Fyn, which in turn activates the adaptor protein Shc (Wary et al., 1996). The α integrin subunit is coupled in this pathway by the membrane adaptor protein caveolin-1. Integrin binding to Fyn results in its activation enabling its SH3 domain to interact with a proline rich sequence in Shc, leading to its phosphorylation and subsequent interaction with the Grb2-mSOS complex (Wary et al., 1998). Both FAK and Shc are believed to contribute to the activation of the MAPK cascade upon binding of Shc-linked integrins to the ECM (Schlaepfer et al., 1997).

1.5: The Ras Superfamily:

The Ras Superfamily consists of more than 60 members (identified to date) in mammals. This family can be sub divided, based on sequence homology into: Ras, Rho, Ran/TCA, Rab, Arf, Rap and Rag (Herrmann et al., 1996).

The Ras sub family consists of Ras, Rap, R-Ras, TC21 and Ral. All the members contain a core effector binding region (residues 32-40) to which their specific effectors bind, except Ral, which does not share this homologous domain. Ras has three isoforms H-, K- and N-Ras, which are important in cell proliferation and differentiation. The N-terminal sequences of these proteins are identical, with divergence occurring largely at the C-terminus. However, all isoforms contain a C-terminal farnesylation (CAAX-box) that is responsible for the targeting of Ras to the membrane after translation. It is here that Ras becomes activated and is able to interact with its downstream effectors in response to extracellular stimuli (Herrmann et al., 1996).
1.5.1: The Molecular Switch and Activation of Ras:
Ras like other GTPases cycles between an inactive (GDP bound) and active (GTP bound) state. This cycling is facilitated by guanine nucleotide exchange (GEFs) and GTPase activating proteins (GAPs). GEFs such as Sos1/2, RasGRF1, RasGRP, CNRasGEF, are recruited to the membrane upon the receipt of an extracellular mitogenic signal via the relevant activated receptor protein tyrosine kinase (RPTK) and facilitate the exchange of GDP for GTP, hence activating Ras. Ras is then able to interact with its downstream effector proteins relaying the extracellular stimuli through the cell (Boguski and McCormick, 1993).

Once the stimuli has ceased, GAP proteins such as p120 GAP and NF1-GAP act to increase the intrinsic GTPase activity of Ras thereby hydrolysing GTP to GDP resulting in Ras going to its inactive state. In this inactive conformation, Ras is unable to bind its downstream effectors and the signal is not relayed (Milburn et al., 1990)

1.5.2: Oncogenic Ras:
Oncogenic Ras breaks the usual cycle between active and inactive states by having a lower intrinsic GTPase activity and is insensitive to GAP stimulation. This highly active isoform of Ras is generated by the mutation of residues 12, 13 and 61 (Barbacid, 1987). These mutations give Ras the ability to continue signalling to downstream effectors even in the absence of extracellular stimuli (Barbacid, 1987).

Oncogenic Ras has the ability to induce full transformation of cells, characterised by a mesenchymal phenotype, in which there is a decrease in cell-cell adhesion and an increase in stress fibre and focal complex formation (Zhong et al., 1997). This phenotype is possibly due to the activation of Rho. In MDCK cells, Ras activation leads to the downregulation of Rac, which increases Rho activity. This reduction of Rac
activity results from the stimulation of the Raf/ERK pathway that represses the transcription of the Rac GEF Tiam1 (Bar-Sagi et al., 2000).

Oncogenic Ras acts on the survival machinery of the cell, by activating the NF-κB-dependent transcription pathway (Finco et al., 1997), which results in the suppression of oncogenic-induced apoptosis (Mayo et al., 1997). Ras has the potential to induce uncontrolled cell proliferation and is upregulated in many mammalian tumours (Walter et al., 1986).

1.5.3: Ras Effectors:
Ras has many effectors, the most characterised being the Raf serine/threonine kinases (A-Raf, B-Raf, c-Raf1). Raf activation results from its interaction with the GTP-bound form of Ras at its core effector binding region, this leads to the activation of the mitogen-activated kinase kinases (MEK1/2). This in turn activates the extracellular-signal-regulated kinase (ERK) mitogen-activated protein kinases (Erk MAPKs) cascade (Bar-Sagi et al., 2000; Pruitt et al., 2001).

Another of the extensively investigated effectors of Ras is phosphoinositide 3-phosphate lipid kinase (PI3K) (Downward, 1998). Ras binds to PI3K thereby activating this lipid kinase and facilitating the conversion of phosphatidylinositol 4,5-phosphate (PIP2) to phosphatidylinositol 3,4,5-phosphate (PIP3). This elevation results in the activation of the Akt/PKB serine/threonine kinase (Franke et al., 1995). Rac GEFs activation has also been linked to an increase in the levels of PIP3, thereby linking Ras to the small Rho GTPases (Hawkins, 1995).

A family of GEFs (RalGDS, RGL and Rlf/RGL2) have also been identified as Ras effector proteins. These GEFs are known to activate the Ral family of small GTPases. This interaction only occurs with the GTP-bound form of Ras and is abolished
by mutations in the effector binding region. The RalGEF/RalGDS competes with Raf for interaction with active Ras (Herrmann et al., 1996).

1.5.4: Ras and the Cell Cycle:

Cells, which have been deprived of serum, remain in Go phase of the cell cycle until growth factor stimulated. At this time, the cells undergo an intense signalling phase that lasts between 30 and 60 minutes, after which the receptors are internalised and degraded reducing the signalling events (Pardee, 1989). For completion of a cell cycle, the cells must be in the presence of growth factors for the transition between Go and G1 and in mid G1 or for 8-10 hours continuously (Jones et al., 2001).

Ras plays an important role in the progression and control of cells through the cell cycle. The Ras effectors have been shown to be involved in the transition from various states in the cell cycle. PI3K is important in the progression from Go to S phase via its activity in mid G1 phase (Brennan et al., 2002). Ras is active throughout the cell cycle but its stimulation of cyclin D1 in G2 is essential for the cells to not only undergo that cycle’s mitosis but the subsequent full cycle as well (reviewed by Stacey et al., 2002).
The cell remains in the inactive state known as $G_0$ until it is stimulated by either mitogens or growth factors, upon which it enters the cell cycle of division and progresses to $G_1$. In $G_1$, Ras acts in the early stages to stimulate the MEK pathway which leads to ERK activation and the increase in cyclin D1. In late $G_1$, Ras signals to its downstream effector PI3K causing activation of Akt and the increase in the expression of $p21^{cip}$ and the downregulation of $p27^{kip}$. During S phase DNA synthesis occurs in preparation for the cell division. In $G_2$, the cell has a time lapse between DNA synthesis and mitosis. Ras acts in $G_2$ to increase cyclin D1, this activity is essential for the cell to undergo the present cycle of mitosis and the subsequent cycle of cell division. M phase is the final stage of the cell cycle where division occurs and then the cell enters into $G_0$ where it can remain for many hours, days or even years until mitogen signals are removed.
1.6: The Rho Family GTPases:

The Rho family are a member of the Ras superfamily. These proteins are a family of small GTPases also known as p21’s. There are 17 members of this family; the most characterised members of this family are RhoA (Ras homologous member A), Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42). They act as intracellular molecular switches in the cell, which are responsible for the transduction of extracellular stimuli to the actin cytoskeleton and nucleus of the cell. These proteins cycle between an active and inactive state, active when GTP is bound and inactive upon the hydrolyses of GTP to GDP. This hydrolyses step is facilitated by GTPase activating proteins (GAPs) which enhance the internal GTPase activity of the protein. The exchange of GDP for GTP and resultant activation of the Rho GTPases is controlled by proteins collectively known as Guanine nucleotide exchange factors (GEFs) (Luo, 2000). In the active GTP bound state these proteins interact with down stream effectors transducing signals through the cell. One such pathway is the interaction with effector proteins responsible for the reorganisation of the actin cytoskeleton (Bishop et al., 2000; Luo, 2000).

1.6.1: Rho GTPase Regulatory Proteins:

Rho GTPases are regulated by control of the intrinsic GTPase activity, activation by exchange of GDP for GTP and rate of dissociation from GDP or GTP, by GAPs, GEFs and GDIs respectively.
1.6.1.1: Rho GAPs:

The Rho GAPs are responsible for the increase in the internal GTPase activity of the p21 proteins. This catalyses of GTP hydrolysis is achieved by two mechanisms: 1) direct contribution in which an arginine residue (Arg finger) is inserted into the active site to neutralise negative charge in the transition state; 2) stabilisation of the GTPase switch regions (Donovan et al., 2002). More than 16 Rho-GAPs have been shown to activate the intrinsic GTPase activity of the Rho-GTPases (Scita et al., 2000). These proteins all share a highly related GAP domain (Ridley et al., 1993) which consists of ~170 amino acids, displaying large α helical content and similar topologies. The first Rho family GAP to be identified was a Rac1, Rac2 and Cdc42 specific GAP known as Bcr (Diekmann et al., 1991).

1.6.1.2: Rho GEFs:

These proteins are responsible for the exchange of GDP for GTP, thereby activating the Rho GTPase enabling interaction with downstream effectors. Approximately 60 GEFs of varying sizes and complexity have been identified to date in the genome (Feig, 1994) whose activities have been shown for Rho-GTPases. All Rho-GEFS, posses a Dbl homology domain-pleckstrin homology domain (DH-PH) signature. Within the DH domain the enzymatic core of the Rho-GEF is encrypted and the associated PH domain encodes a lipid-binding region and possibly a protein-protein interaction site (Cerione and Zheng, 1996).
1.6.1.3: Rho GDIs:

Rho guanine nucleotide dissociation inhibitors (GDIs) are responsible for the inhibition of the dissociation of GDP from Rho-GTPases (Fukumoto et al., 1990). Association between Rho-GDI and active (GTP bound) and inactive (GDP bound) Rho-GTPases is equal. They act to interfere with the hydrolysis of GTP as well as the dissociation from GDP, thus regulating both exchange and hydrolysis of guanine nucleotides (Hart et al., 1992; Chuang et al., 1993). Hence, GDI’s are responsible for slowing the cycling rate of GTPases between their active and inactive states.
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**Figure 1.6: Rho GTPase Cycle:**

A model of action for GDIs, GEFs and GAPs in controlling the Rho GTPases. These proteins control the rate of cycling of the Rho GTPases between their GTP bound (active) and GDP bound (inactive) states. GDIs bind when GDP or GTP is bound, hindering the exchange of GDP for GTP and the hydrolysis of GTP to GDP, respectively. GAPs increase the internal GTPase activity of the Rho family members causing the hydrolysis of GTP to GDP. GEFs facilitate the exchange of GDP for GTP thereby activating the Rho GTPase.
1.6.2: Rho Family Functions:

1.6.2.1: Functions of Cdc42:

The Rho GTPase Cdc42 is implicated in a variety of cellular functions, including receptor-mediated signal transduction leading to initiation of transcription, cell cycle progression, actin rearrangement and apoptosis. The Cdc42 family comprises of 11 members that range in size from 190 to 192 amino acids with high homology to one another. All Cdc42 proteins possess a C-terminal membrane targeting consensus sequence C-X-X-L, with the exceptions of two brain specific members, Cdc42Mmb and G25K which have a phenylalanine in place of the leucine residue. Cdc42 comprises many domains of which four, located at the N-terminus, are responsible for the binding and hydrolysis of GTP to GDP. The switch I domain or effector binding region (residues 26-50) has the capability to possibly bind more than one effector simultaneously and also perhaps possesses regions which are specific for certain effectors to bind. The Rho GTPases have an insert domain of ~13 residues unique to the Rho GTPases and not present in other Ras Superfamily members. GEF interaction is believed to occur primarily at a central region comprised of the β4-α3 strand region (residues 82-100) (Johnson, 1999).

Transcriptional activation: Cdc42 acts to couple cell surface receptors to the MAP kinases, relaying extracellular cues to intracellular events, such as stress response, mitogenesis, cell growth, prostaglandin biosynthesis and immunity gene expression. During stress response, initiated by changes in the cells environment the stress-activated protein kinases (SAPKs) and c-Jun kinases (JNKs) are activated. Cdc42 activates JNK and p38 (Bagrodia et al., 1995), which causes an elevation in the c-Jun AP-1 transcriptional activity (Minden et al., 1995). This stimulation of the JNK/SAPK and
p38 kinases, results in their translocation to the nucleus, where various transcriptional activators are phosphorylated and gene expression is initiated (Johnson, 1999).

The stress activated programmed cell death response known as apoptosis has been shown to involve Cdc42, Pak isoforms, and JNK/SAPK and p38 protein kinase cascades. In the immune system, apoptosis is mediated via coupling activated Fas receptors to the caspase protease cascade, which leads to the proteolytic cleavage of Pak2 (Rudel et al., 1998), and components of the JNK pathway (Juo et al., 1997). Expression of activated Cdc42 in Jurkat T lymphocytes and rat sympathetic neurones results in the induction of apoptosis via activation of the JNK pathway (Chuang et al., 1997; Bazenet et al., 1998). Nef-associated kinase (NAK) activation is mediated by Cdc42, which leads to serum response element-dependent transcription and expression of dominant negative Cdc42 in COS cells results in the reduction of HIV-1 production (Lu et al., 1996).

In response to bacterial invasion, Cdc42 causes the activation of various pathways, which cause rearrangement of the actin cytoskeleton for Salmonella or Shigella infection (Dramsi et al., 1998) or activation of the JNK pathway-dependent induction of proinflammatory cytokines for the invasion of Neisseria gonorrhoeae in epithelial cells (Naumann et al., 1998).

**Actin rearrangements:** Cdc42 has been shown to interact with various downstream effectors, which results in the rearrangement of the actin cytoskeleton in response to extracellular cues. Microinjection of constitutively active Cdc42 into Swiss 3T3 fibroblast cells causes the formation of peripheral actin microspikes, vinculin containing focal complexes and a reduction in Rho induced stress fibres (Kozma et al., 1995; Nobes et al., 1995). Cdc42 also plays a role in the mediation of the actin cytoskeleton rearrangements that results form cell-cell and cell-substratum adhesion (Kuroda et al., 1998). Integrin-dependent adhesion and cell spreading in NIH 3T3
fibroblasts are Cdc42 mediated (Clark et al., 1998). In neuroblastoma cells, N1E-115s, dominant negative Cdc42 expression inhibits neurite outgrowth and acetylcholine-dependent filopodia formation. During differentiation of cultured cortical rat neurons, Cdc42 appears to play a role in the control of pyramidal to nonpyrimidal cell transformation (Threadgill et al., 1997). These findings show a role for Cdc42 in dendrite and axon formation in neuronal cells probably via its effects on the actin cytoskeleton.

**Cell polarity:** Cdc42 overexpression in HeLa cells is localised to focal complexes and peripheral actin microspikes found at regions of polarised growth in the cell (Dutartre et al., 1996). Cdc42 is polarised in its location in elongated *Drosophila* wing disc epithelial cells, where it is restricted to the apical and basal membranes (Eaton et al., 1996). Cdc42 is found generally to be membrane bound, either the plasma or internal membranes, in regions of polarisation within the cell (Johnson, 1999).

### 1.6.2.2: Functions of Rac:

The Rho GTPase Rac has been implicated in various mechanisms of control of the actin cytoskeleton and processes such as oxidative destruction during phagocytosis of bacterial invaders, interaction and regulation of inositol lipid kinases.

**NADPH oxidase:** The NADPH oxidase enzyme generates superoxide, which consequently forms hydrogen peroxide leading to the production of hydroxyl free radicals and hypochlorous acid, which are effective destructive agents utilised by leukocytes for phagocytosis. Rac and its effector p67phox have been shown to be components of the NADPH oxidase complex (Diekmann et al., 1994) of which Rac is a crucial component (Knaus et al., 1991). The activity of NADPH oxidase is regulated by
the state of Rac, in the GDP bound state inhibition of this enzyme is observed, whereas in the GTP bound state activity output is increased (Heyworth et al., 1993).

**Inositol lipid kinases:** Phosphatidylinositol lipids have long been implicated in the regulation of actin dynamics. Rac has been shown to regulate the activity of the type-I phosphatidylinositol 4-phosphate 5-kinase (PIP5K). PIP5K produces the enzymatic product PIP$_2$, which has been shown to be involved in the regulation of the interaction of profilin, α-actinin, vinculin, talin and actin-capping proteins (Ren et al., 1998). In platelets, the addition of recombinant Rac protein resulted in the activation of PIP5K causing an increase in PIP$_2$ synthesis, leading to the uncapping and polymerisation of actin filaments (Hartwig et al., 1995). Rac has also been implicated in the activation of PI3K in a GTP-dependent manner (Bokoch et al., 1996), which mediates the interleukin-2 activation of protein kinase C (Gómez et al., 1997).

**Actin reorganisation:** Overexpression of Rac induces the formation of membrane ruffles, in which the actin filaments form a lattice structure. Treatment of fibroblast cells with PDGF results in the formation membrane ruffles, which is due to an upregulation of Rac activity (Ridley et al., 1992). Rac is also involved in the migration of border cells in the developing embryo of Drosophila. Inhibition of Rac causes the migration of these cells to be blocked during oogenesis (Murphy et al., 1996).

1.6.2.3: Functions of Rho:

Rho too has been implicated in the reorganisation of the actin cytoskeleton (neurite/growth cone collapse, formation of stress fibres) as well as in other cellular events such as oncogenic transformation.

**Oncogenic transformation:** progression to malignancy is a multi-stage process, which results in the uncontrolled ability of cells to leave their normal environment and
invade the surrounding tissues. Overexpression of RhoC results in induced metastasis while its inhibition diminishes the ability of cells to invade and metastase (Clark et al., 2000). RhoA and its downstream effector Rho Kinase (ROK) are also implicated in tumour-cell invasion. Inhibition of ROK in LPA induced tumour cells their invasive abilities are suppressed (Itoh et al., 1999).

**Endothelial cell permeability:** The endothelial cell layer acts as a barrier of protection from potential harmful bacteria in the bloodstream. The permeability of the endothelial cell layer is determined by cell shape. Exposure to inflammatory agents such as thrombin and the Gram-negative bacteria outer membrane component lipopolysaccharide (LPS) up regulates the Rho/ROK pathway (Alexander et al., 2000; Aepfelbacher et al., 1997, respectively). This upregulation of Rho signalling to its effector ROK results in the inhibition of myosin light chain II phosphatase (MLC-phosphatase) (Matsui et al., 1996) leading to the phosphorylation of myosin light chain at residues Thr-18 and Ser-19, increasing the actin-activated Mg-ATPase activity of myosin, inducing actomyosin contraction (Adelstein et al., 1982).

**Actin cytoskeleton rearrangements:** Overexpression of RhoA has been shown to induce stress fibre formation in fibroblast cells (Paterson et al., 1990). The activation of the Rho pathway by LPA induces both stress fibre and focal adhesion formation in fibroblast cells (Ridley et al., 1992). It is cooperation of the Rho downstream effectors ROK and mDia that results in the formation of bundled actin stress fibres and their alignment with microtubules (Watanabe et al., 1997; Tominaga et al., 2000; Ishizaki et al., 2001). Further discussion of ROK and mDiaphanous can be found in sections 1.6.2 below.
1.6.3: Combined Actions of the Rho GTPases on the Cytoskeleton:

The Rho family GTPases play a role in many activities including smooth muscle contraction, stress fibre and focal adhesion formation, lamellipodia and filopodia production, cell motility and migration, neurite extension and retraction, cell-cell adhesion (Hall, 1998).

**Stress Fibre and Focal Adhesion Formation:** Stress fibres comprise of actin filaments and associated myosin filaments. Vinculin, α-actinin, and talin complex with integrin to form the stress fibre anchors, focal adhesions (Ridley et al., 1992). Serum starvation of Swiss 3t3 cells, removal of LPA or treatment with the Clostridium botulinum C3 exoenzyme reduces their formation (Chardin et al., 1989). Focal adhesion and stress fibre formations are controlled by the action of Rho via ROK (Leung et al., 1995 and 1996) and the activation of myosin II (Leung et al., 1998; Kaibuchi et al., 1999).

**Lamellipodia and Filopodia:** Both lamellipodia and filopodia are peripheral actin structures that respond to extracellular cues. Rac activation downstream of growth factor stimulation e.g. PDGF leads to the formation of lamellipodia and membrane ruffling (Ridley et al., 1992), whereas Cdc42 characteristically causes filopodia production in response to bradykinin (Kozma et al., 1995). The mechanism by which filopodia and lamellipodia are produced by the p21’s is still largely unknown, however increasing knowledge of the downstream effectors to the Rho GTPases and the effect on the actin cytoskeleton is rapidly expanding.

**Neurite Retraction and Extension:** Neurites respond to extracellular cues either from a source or in a matrix, which act as either chemoattractants or repellents, causing the neurite to reorganise its cytoskeletal structure accordingly (Zheng et al., 1996; Defilippi et al., 1999). In neuroblastoma cells Rho induces neurite collapse by the
contraction of the cortical actin-myosin system (Jalink et al., 1994), activation of Rac and Cdc42, however, lead to filopodia and lamellipodia formation in the growth cone and neurite shaft (Kozma et al., 1997). Acetylcholine, when released from a needle, has been shown to independently activate Rac/Cdc42 inducing neurite outgrowth with filopodia and lamellipodia along the neurite shaft and on the growth cone (Kozma et al., 1996). Stimulation of the Rho pathway with LPA causes neurite collapse (Tigyi et al., 1996) whose effects can be blocked by the use of C3 exoenzyme (Jalink et al., 1994) or ROK inhibition (Hirose et al., 1998) and can result in the switch to Cdc42/Rac signalling and hence neurite formation (Kozma et al., 1996).

1.7: Rho GTPase Effector Proteins:

The Rho family GTPases cause changes in the actin cytoskeleton in response to extracellular cues via their interaction with their effector molecules. These effector proteins are the link between the p21's and proteins such as the Arp2/3 complex, which has been shown to be involved in actin dynamics (Welch, 1999).

1.7.1: Cdc42/Rac Effectors:

1.7.1.1: WASp Family:

The Wiskott Aldrich Syndrome protein (WASp) family comprises of several members: WASp found expressed solely in hematopoietic cells, Neural WASp (N-WASp) which is expressed highly in neuronal cells but is more ubiquitously expressed than WASp and WAVE/Scar of which there are three isoforms (WAVE/Scar1, Scar 2 and 3), and the yeast homologues Las17p or Bee1p (Higgs et al., 1999).
1.7.1.1: WASp:

Mutations of WASp are responsible for the rare X-linked immunodeficiency disease first identified by Wiskott in 1936 (reviewed by Snapper et al., 1999). The T cells of WAS patients display both signalling and cytoskeletal abnormalities (reviewed by Remold-O'Donnell et al., 1996). They display surface blebbing, decreased microvilli, and abnormal actin filament patterns (Molina et al., 1992) as well as having an inability to migrate and polarise their cytoskeleton in response to chemoattractants (Badolato et al., 1998; Zicha et al., 1998).

WASp is a 502 amino acid protein of which more than 15% is polyproline residues (Derry et al., 1994). There are many SH3 binding domains amongst the polyproline stretches with which proteins such as Grb2, PLCγ, Nck and PI3K interact (Yu et al., 1994). WASp has been shown to interact with Cdc42, (and Rac but with a far lower affinity) (Fukuoka et al., 1997; Kolluri et al., 1996). WASp contains two regions known as WASP homology 1 and WASP homology 2 (WH1 and WH2) domains, found in the N- and C-terminus of the protein. These WH domains have been found in other cytoskeletal associated proteins that are also polyproline rich (Ponting et al., 1997).

The WH1 domain overlaps with the N-terminal pleckstrin homology domain, which is responsible for the targeting of WASp and N-WASp to the membrane upon activation by the binding of Cdc42 to GBD domain and of PIP2 to the WH1 region (Miki et al., 1996). The WH2 domain shows homology to verprolin, which is responsible for cytoskeletal organisation in yeast (Vaduva et al., 1997) and mediates actin binding (Miki et al., 1998). As in N-WASp, WASp contains a cofilin homology domain located at the C-terminus, which is believed to be responsible for the binding of the Arp2/3 complex and controlling actin dynamics in the cell (Miki et al., 1998; Hufner et al., 2001).
**Introduction**

**Figure 1.7.1.1.1A: Structure of WASP:**

WASP in its autoinhibited state. The cofilin homology region (residues 461-479) (coloured red) interacts with the GBD domain (residues 250-276) (coloured yellow) (Kim et al., 2000). This interaction between the GBD domain and the cofilin homology (CH) region masks the VCA region preventing the Arp2/3 complex being able to bind. Upon binding of Cdc42 to the GBD a conformation not favourable to the autoinhibited structure is formed. This destabilises the GBD-CH interaction, allowing a conformational change to occur, unmasking the VCA region and hence allowing binding of the Arp2/3 complex to this region (Abul-Manan et al., 1999; Kim et al., 2000).

Taken from Buck et al., 2001

**Figure 1.7.1.1.1B: Cdc42 Binding to GBD Domain of WASP:**

Schematic of the interaction between the β2/β3 hairpin and α5 helix of Cdc42 and the N-terminus of the GBD domain of WASP. WASP is displayed as the yellow ball and stick structure with red side chains. Cdc42 as the blue ribbon structure with green ball and stick side chains. Intramolecular hydrogen bonds existing between residues 234 and 237 of the GBD domain of WASP are shown as dashed lines (Abul-Manan et al., 1999).

Taken from Abdul-Manan et al., 1999
1.7.1.1.2: N-WASp:

Neural Wiskott Aldrich Syndrome protein (N-WASp) is 50% homologous to WASp and is ubiquitously expressed with high levels of expression in brain, heart and lung (Miki et al., 1996). It is a 505 amino acid protein consisting of nine functional motifs: pleckstrin homology domain, IQ motif, basic region, GTPase binding domain, poly-proline region, two verprolin homology domains, cofilin homology domain and acidic region. All of these regions are conserved from human to rat proteins suggesting N-WASp plays the same important role in each species (Fukuoka et al., 1997).

As with WASP the pleckstrin homology (PH) domain is believed to be responsible for the targeting of N-WASp to the membrane upon its activation by Cdc42 (Miki et al., 1996). Cdc42 in its active GTP bound state, binds to the GTPase binding domain (GBD) of N-WASp (Symons et al., 1996; Kolluri et al., 1996). The poly-proline region of N-WASp has numerous sequences matching the Src-homolgy-3 (SH3) binding core consensus sequence, P-p-X-P, where P's are proline residues, X is usually hydrophobic residues and p, proline residues (Yu et al., 1994). Upon binding of Cdc42 and PIP2 to the GBD and basic region respectively, N-WASp undergoes a conformational change by which the protein becomes active (Rohatgi et al., 2000). The verprolin-cofilin-acidic (VCA) region that in the closed conformation is masked and interacting with the GBD region becomes exposed allowing interaction with proteins such as the Arp2/3 complex (Kim et al., 2000; Rohatgi et al., 2000).

The VCA region has been shown to be the binding site for the Arp2/3 complex and filamentous actin (Miki et al., 1998; Rohatgi et al., 1999). Of the WASp family proteins, only N-WASp possesses two verprolin homology domains (Yamaguchi et al., 2000). It is at these VH domains that monomeric actin units interact (Miki et al., 1998) and the Arp2/3 complex binds to the cofilin homology domain (Hufner et al., 2001).
The presence of two VH domains has been shown to cause an increase in the rate of actin polymerisation by N-WASp and its binding partner the Arp2/3 complex (Yamaguchi et al., 2000). A study of N-WASp activation revealed that the coflin homology/central region (CH) interacts with the GBD domain, forming a closed and inhibited conformation. Upon binding of Cdc42 to the GBD domain the CH domain interaction is lost and a conformational change occurs in the protein (Kim et al., 2000) thereby activating N-WASp and unmasking the VH and acidic regions (Miki et al., 1998; Rohatgi et al., 1999) to which monomeric G-actin and the Arp2/3 complex interact, respectively (Yamaguchi et al., 2002). The verprolin homology domains of N-WASP are essential for the depolymerisation of actin (Miki and Takenawa, 1998), bringing G-actin units in close proximity to the Arp2/3 complex, resulting in actin nucleation events. These domains are also essential for the binding and activation of the Arp2/3 complex by WASP (Hufner et al., 2001).

N-WASp is directly involved with the reorganisation of the actin cytoskeleton via its interaction with the Arp2/3 complex (Miki et al., 1998; Snapper et al., 1999; Rohatgi et al., 1999; Higgs et al., 1999; Zalevsky et al., 2001; Weaver et al., 2002). It is located at the leading edge of cells and has been suggested to be fundamental in the formation of peripheral filopodia (Miki et al., 1998). N-WASp, unlike its closely related family member WASp contains an IQ motif located between the PH and GBD domain at the N-terminus of the protein. IQ motifs bind calcium/calmodulin suggesting that N-WASp may be under calcium control (Rhoads et al., 1997).

The N-terminal WH1 domain of N-WASP has also been shown to have a weak affinity for the Arp2/3 complex and could possibly be another means by which N-WASP binds and activates this protein complex, resulting in actin nucleation events (Suetsugu et al., 2001).
1.7.1.1.3: WAVE/Scar:

WASp family verprolin-homologous protein (WAVE)/Scar proteins differ somewhat to that of WASp and N-WASp in their structure, however, they too are strong potentiators of the Arp2/3 complex and actin dynamics of the cell (Higgs et al., 2001). The N-terminus of WAVE/Scar proteins is known as the Scar homology (SH) domain and is highly conserved amongst Scar proteins but is different to that of the WH1/EVH1 domain found in WASp and N-WASp (Prehoda et al., 1999). This region of WASp/N-WASp has high homology to a region in the Drosophila protein Enabled (Ena) and the mammalian protein vasodilator-stimulated phosphoprotein (VASP), which are implicated in the spatial control of actin dynamics (Prehoda et al., 1999; Higgs et al., 2001). The analogous region in WAVE/Scar to the GBD domain of WASp and N-WASp does not contain the consensus CRIB motif and does not bind Rho GTPases (Higgs et al., 2001). All WASp family proteins possess a verprolin-cofilin-acidic (VCA) region at their C-terminus to which the Arp2/3 complex binds and is activated, causing the induction of actin dynamics required for the formation of peripheral protrusions at the leading edge of motile cells (Takenawa et al., 2001). WAVE/Scars have been implicated in the formation of membrane ruffles downstream of Rac, but no direct binding has been shown (Miki et al., 1998; Machesky et al., 1998). It has been suggested that the interaction between Rac and WAVE2 is mediated by IRSp53, another Rho GTPase effector (Miki et al., 2002).
1.7.1.1.4: WASP Interacting Proteins:

WASp proteins have many interactors that either modulate their function or mediate their interaction with other proteins such as those associated with the cytoskeleton.

1.7.1.1.4.1: WIP:

Wiskott Aldrich Syndrome Interacting Protein is a ubiquitously expressed, 503 amino acid protein which displays homology to the yeast protein verprolin at its amino terminus (Ramesh et al., 1997). Overexpression of this protein causes an increase in polymerised actin in human lymphoid cells and induces actin-rich peripheral structures (Ramesh et al., 1997). WIP has a high proline content and contains potential SH3 domain binding sequences. WIP interacts with Nck, profilin and WASp. The Nck interaction is via residues 321-415 and to profilin via two consensus sequences located at the N- and C-terminus (residues 8-13 and 427-432 respectively) (Antón et al., 1998), thereby possibly acting as a mediator for Nck binding to profilin. The C-terminus of WIP (residues 377-503) has been shown to interact with WASp via an undefined region in the first 170 amino acids of the protein, of which both the PH and polyproline regions are required (Ramesh et al., 1997). This interaction of WASp and WIP could be a further way of linking WASp proteins to the actin cytoskeleton.

1.7.1.1.4.2: WISH:

WASp Interacting SH3 protein (WISH) is a 711 amino acid protein with a SH3 domain at its N-terminus and three class II-type SH3 binding motifs, a leucine zipper-like motif, overlapping proline rich sequences and a serine-rich sequence (Fukuoka et al., 2001). WISH interacts specifically with the polyproline region of N-WASp via its SH3 domain. The binding of WISH to N-WASp increased its Arp2/3 activation and actin
polymerisation equivalent to a level of the N-WASp activated by PIP$_2$. This activity increase induced by WISH is independent of Cdc42. WISH is unable to directly bind or activate the Arp2/3 complex. Coexpression of these two proteins even in the absence of Cdc42 results in the formation of microspikes in Cos7 cells. WISH is a 90kDa protein that can directly interact with and activate N-WASp in a Cdc42 independent manner. WISH also interacts via its proline rich sequences with the SH3 domains of upstream adaptor proteins such as Ash/Grb2 as well as other SH3 domain proteins such as PLC$\gamma$, Fyn, Nck and PI3-Kinase (Fukuoka et al., 2001).

1.7.1.4.3: WIRE:

WIP Related protein (WIRE) is a 440 amino acid protein with a 27% proline content that interacts with WASp, N-WASp and $\beta$Nck the PDGF-β receptor substrate (Aspenström, 2002). WIRE has a 40% sequence homology to the other recently identified N-WASp interacting protein WIP and is a WASp/N-WASp specific protein as no interaction occurs with any of the three WAVE/Scar proteins. There is a WH2 domain found at the N-terminus, a core KLKK motif that implicates monomeric actin binding (Martinez-Quiles et al., 2001) and several potential profilin binding motives. WIRE is found ubiquitously expressed and is implicated in the regulation of actin dynamics downstream of the PDGF-β receptor. It is found in filamentous actin bundles and its overexpression induces a reduction in polymerised actin and the formation of lamellipodia and filopodia in the presence of PDGF (Aspenström, 2002), which normally induces only ruffling (Ridley et al., 1992).
1.7.1.1: WASP Family Proteins and Their Interactors:

The WASp family members WAVE/Scar have a diverse N-terminus from that of WASp and N-WASp by possessing a Scar homology domain (SH) in place of a pleckstrin homology (PH) domain or WASp homology 1 (WH1) region. It is to this PH/WH1 domain that WIP has been shown to interact as well as target the protein to the membrane upon its activation by binding of active GTP-bound Cdc42. The poly-proline region and VCA region is a common feature of all the WASp family members, allowing the interaction with SH3 domain containing proteins and control of actin dynamics of the cell via the Arp2/3 complex, respectively.
1.7.1.2: IRS p53/58:

Insulin Receptor Substrate (IRS) has many splice variants, two of which encode a 53kDa (IRSp53) (Oda et al., 1999) and a larger 58kDa isoform (Govind et al., 2001). The two isoforms were initially identified in hamster with the main region of divergence being at the C-terminus of the protein (Yeh et al., 1996). The isoform IRS-58 used in this study was isolated in a yeast two-hybrid screen using a brain cDNA library. IRS-58 was identified as being the human isoform of the mouse IRSp53/58 insulin receptor tyrosine kinase substrate (Govind et al., 2001). Two other variants were later identified from human library screens; Brain-specific Angiogenesis Inhibitor Associated Protein 2α (BAIAP2α) and BAIAP2β (Oda et al., 1999). These proteins show almost 100% sequence identity apart from divergence at the C-terminus (Oda et al., 1999).

IRS-58 is a brain enriched 58kDa protein that contains four domains: SH3 binding domain, a partial CRIB motif, SH3 domain and a tryptophan, tryptophan (WW) binding domain. IRS-58 has been shown to be a Cdc42 effector, which is implicated in both neurite outgrowth and filopodia formation in N1E-115 cells and filopodia formation in Swiss 3T3 cells (Govind et al., 2001). IRSp53 interacts with Cdc42 (Krugmann et al., 2001), however Miki and co-workers reported it also interacts with Rac (Miki et al., 2000). IRSp53 is believed to be the intermediate between Rac and WAVE2 forming a complex resulting in the formation of membrane ruffles (Miki et al., 2000; Miki et al., 2002). Miki and Takenawa propose that the N-terminus of IRSp53 forms an intramolecular interaction with the rest of the protein causing the protein to be autoinhibited. They suggest that the binding of WAVE2 to the SH3 domain of IRSp53 inhibits this intramolecular interaction and allows the interaction with Rac to occur (Miki et al., 2002). The direct interaction of Rac with IRS-58 however has been disputed (Krugmann et al., 2001; Govind et al., 2001). IRSp53 has also been shown to interact
with Mena at its SH3 domain, acting synergistically to promote filopodia formation. The N-terminal region of IRSp53 also inhibits the interaction between IRSp53 and Mena (Krugmann et al., 2001).

In this thesis, the human isoform of IRS-58 isolated by S. Govind in the yeast two hybrid screen of brain cDNA libraries was used in all experiments.
1.7.1.2: IRSp53/58 and its Interactors:

IRS isoforms have almost 100% sequence identity with divergence only at the C-terminus where splice variants IRSp53 and IRS-58 differ. IRS has been shown to interact directly with Cdc42 at its partial CRIB motif, which overlaps with a polyproline region. The SH3 domain of IRS is the interaction site for proteins such as WAVE and Mena. IRS is believed to form an autoinhibited structure in which the N-terminus of the protein interacts with another region of the protein, thereby masking the SH3 domain and preventing interaction with its binding partners.
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1.7.1.3: PAK:

p21 Activated Kinases (PAK) are serine threonine kinases of which to date two groups have been identified each possessing three members (Jaffer et al., 2002). The first of the Pak proteins (Pak1/αPak) was identified by Manser et al., in 1994 through a screen for binding partners of the Rho GTPases Rac and Cdc42 (Manser et al., 1994). The three members of the Group I Paks (Pak1/αPak, Pak2/γPak, Pak3/βPak) are structurally highly homologous, but their tissues of expression differ greatly. αPak is found in brain, muscle and spleen (Manser et al., 1994); γPak is ubiquitously expressed (Teo et al., 1995) and βPak is unique to brain (Bagrodia et al., 1995; Manser et al., 1995). The Group II Paks are; Pak4, found ubiquitously expressed (Abo et al., 1998); the recently discovered Pak 5, which is brain specific (Dan et al., 2002) and Pak6 whose expression is high in testis and prostate (Yang et al., 2001). The Group I Paks are highly homologous in the kinase region of the protein located at the C-terminus as well as at the N-terminus where a regulatory domain is found. The N-terminal regulatory region contains three established conserved proline rich regions (Manser et al., 1998), however other possible proline rich regions have been suggested (Sells et al., 1997; Bagrodia and Cerione, 1999). This is followed by a classic CRIB motif that is 95% homologous between members. Located between the CRIB motif and the kinase domain is an acidic region. Nck has been shown to interact with the initial proline rich region at the N-terminus of the protein and acts to localise Pak to the membrane for activation (Lu et al., 1997). Another Pak interactor, PIX (Pak Interacting Exchange factor) interacts with the third, atypical, SH3 binding domain (residues 182-203 on αPak) (Manser et al., 1998).

Pak activity is controlled by the regulatory domain, its interaction with the Rho GTPases Rac or Cdc42 and its autophosphorylation. Binding of Rac or Cdc42 to the CRIB motif results in a conformational change in the protein causing the C-terminal
kinase domain to become exposed. The protein is then autophosphorylated and in an active state (Bagrodia et al., 1999), enabling interaction with substrates such as myelin basic protein, NADPH oxidase component p47^{phox} (Benna et al., 1994; Knaus et al., 1995). Pak is autophosphorylated at seven sites (S21, S57, S144, S149, S199, S204 and T423) (Manser et al., 1997).

The construction of a cDNA encoding a CAAX box fused to the C-terminus of αPak causes this protein to be translocated to the membrane and hence activated. This protein has been shown previously to induce neurite outgrowth in PC12 cells (Daniels et al., 1998) and here in N1E-115 neuroblastoma cells. Actions of the PAKs on cytoskeletal components is largely dependent on the proline rich SH3 binding domains located at the N-terminus of the protein and independent of the kinase domain at the C-terminus of the protein. Certain myosin isoforms are phosphorylated and their activity modulated by the Paks phosphorylating the heavy and/or light chains leading to an increase in their actin-dependent ATPase activity (Wu et al., 1996; Brzeska et al., 1997).

The Group I Paks have been shown to play a role in apoptosis having both positive and negative effects. γPak is cleaved during apoptosis, which causes its activation by the release of the kinase domain, this results in aiding the morphological changes seen in cells during apoptosis (Rudel et al., 1997). αPak also has an inhibitory effect on apoptosis by phosphorylating and inhibiting Bad (Schurmann et al., 2000).

The c-Jun amino terminal kinase (JNK) and p38 that modulate transcription in response to environmental cues, are stimulated by the Rho GTPases and Pak family members act to stimulate this pathway by phosphorylation of upstream components (Zhang et al., 1995).
Paks play a role in many processes in the cell, such as actin cytoskeletal rearrangement and stimulation of the MAP kinase (p38) cascade, which enables the cell to respond to extracellular stimuli (Bagrodia et al., 1999).

1.7.1.3.1: PAK Interactors:

Pak has been shown to interact with several proteins, some of which have been further investigated in this project.

1.7.1.3.1.1: PIX:

Two isoforms of the Pak Interacting Exchange factors (PIX) have been identified, αPix a 78kDa protein and βPIX of 85kDa. PIX family proteins are Rac1 GEFS which possess a SH3 domain at their N-terminus and this is responsible for the interaction with the third proline rich motif of the mammalian Paks. The targeting of Pak to focal complexes is dependent on the interaction of Pak with PIX via this proline rich region in Pak previously identified as a focal complex targeting sequence (Manser et al., 1997).

Both α- and β-PIX posses an N-terminal SH3 domain, a Dbl homology domain and a pleckstrin homology domain, followed by a C-terminal myosin like domain. αPIX contains one extra domain located at its very N-terminus, a calponin homology domain.

PIX is a possible candidate for the GEF (Manser et al., 1998), which mediates the cross talk that occurs between Cdc42 and Rac1 (Ridley et al., 1992; Kozma et al., 1995). Pak has high affinity for GTP-Cdc42 resulting in its activation and the unmasking of its the proline rich region to which PIX binds, which in turn activates Rac, resulting in a positive feedback loop being established independent of upstream signals (Manser et al., 1998).
1.7.1.3.1.2: GIT1:

G-protein-coupled receptor kinase (GRK)-interacting targets (GIT) are binding partners of PIX (Premont et al., 1998), which allows the localisation of Pak with focal complexes (Manser et al., 1998). GIT proteins are 90kDa proteins, which contain an Arf GAP domain at their N-terminus and have the capability of binding to paxillin (Turner et al., 1999). Pak is linked to focal complexes (integrin-dependent sites that link the actin cytoskeleton to the extracellular matrix) via GIT1. GIT1 increases the turnover of focal complexes in a PIX and C-terminal paxillin binding domain dependent manner. GIT1 also links the focal adhesion kinase (FAK) to Pak signalling, as FAK possesses a functionally similar C-terminus that targets paxillin and binds directly to GIT1 (Zhao et al., 2000).

1.7.1.3.1.3: POPX:

Partner of PIX (POPX) has two isoforms (POPX1 and POPX2). POPX1 is a 757 amino acid protein and POPX2 is a shorter protein of 454 amino acids. Both proteins have a core phosphatase region, which are 66% identical, homologous flanking sequences and largely divergent N- and C-terminal regions (235 and 270 residue extensions respectively). POPX1 expression like Pak is high in brain and testis, POPX2, however is ubiquitously expressed. POPX interacts with Pak in the presence of PIX, forming a trimeric POPX-PIX-Pak complex. Activation of αPak is antagonised by POPX by dephosphorylating and hence down regulating its kinase domain via the interaction with PIX (Koh et al., 2002).
Pak consists of many domains to which various proteins can interact. It is a Cdc42/Rac effector, but primarily binds Cdc42. PIX binds to the third proline-rich region (residues 182-203) of Pak, which then interacts with GIT1 causing the targeting of Pak to focal complexes. This leads to the dissociation of paxillin, resulting in the turnover of focal complexes and cell motility. PIX has also been identified as a GEF for Rac1, which can act to activate Rac and therefore establish a positive feedback loop to upstream effectors and possibly mediating cross talk between Cdc42 and Rac1. Pak also forms a complex via POPX, which acts to downregulate Pak activation by dephosphorylation of its kinase domain. The synthetic attachment of a CAAX box to the C-terminus of αPak enables it to induce neurite outgrowth in PC12 and N1E-115 cells. Pak can interact with Nck via its N-terminal proline-rich region.
1.7.2: Rho Effectors:

Rho has been shown to have opposite effects to that of Cdc42 and Rac in control of changes to the actin cytoskeleton. Unlike the GTPases Cdc42 and Rac, which via their effectors can induce outgrowth and the formation of filopodia and lamellipodia, Rho, is implicated in growth cone collapse (Tigyi et al., 1996; Kozma et al., 1997) and the formation of stress fibres and focal complexes (Ridley et al., 1992).

1.7.2.1: ROK:

RhoA-binding Kinase (ROK) exists in two isoforms, ROKα and ROKβ, which show 90% homology in the N-terminal kinase domain. Both isoforms are 160kDa proteins with a N-terminal serine/threonine kinase domain, a coiled coil region, a Rho-binding domain and a C-terminal cysteine/histidine-rich/pleckstrin homology domain. The N-terminal kinase domain contains a highly conserved 30 amino acid stretch in both isoforms of which 20 are identical between the two proteins and essential for the binding of RhoA (Leung et al., 1996). The ROK kinase domain displays high homology to the myotonic dystrophy kinase (Brook et al., 1992). ROK is translocated to the plasma membrane upon its association with Rho resulting in stress fibre and focal adhesion complex formation (Leung et al., 1995). This cytoskeletal rearrangement is dependent on the N-terminal kinase domain, removal or mutation of this domain leads to the disassembly of stress fibres and focal complexes (Leung et al., 1996).

The catalytic activity of ROK is controlled by an intramolecular interaction of the C-terminus with the kinase domain as well as formation of multimeric complexes resulting in the trans-autophosphorylation of the kinase domain. The inhibitory interaction of the C-terminus with the kinase domain is disrupted upon binding of active Rho to the Rho binding domain, causing its activation (Amano et al., 1999). However, it
has been documented that the catalytic activity of ROK is dependent on dimerisation/trans-autophosphorylation event, in which multiple intermolecular interactions occur between the coiled-coil domains (Chen et al., 2002).

ROK has also been shown to regulate the activity of collapsin response mediator protein (CRMP) by phosphorylation of the threonine 555 residue, resulting in the induction of growth cone collapse (Arimura et al., 2000).

The regulatory light chain of myosin II and the regulatory subunit of myosin light chain phosphatase are phosphorylated by ROK. This results in the combined increased phosphorylation of the myosin light chain (MLC) promoting the ATPase and motor activities of myosin II leading to neurite retraction (Kimura et al., 1996).

1.7.2.2: mDiaphanous:

The mammalian homologue of the *Drosophila* diaphanous (Castrillon et al., 1994) is a 140kDa protein, belonging to the formin-related protein family. p140mDia contains an N-terminal Rho specific binding domain, a polyproline stretch and a C-terminal formin homology 2 (FH2) domain (Watanabe et al., 1997) and a C-terminal intramolecular interaction domain (CIID) (Watanabe et al., 1999). The CIID and N-terminus Rho binding domain interact to form a closed, autoinhibited conformation, which is released upon binding of the active GTP-bound form of RhoA (Watanabe et al., 1999; Alberts, 2001). Active Rho bound p140mDia associates with profilin forming a complex that is localised to membrane ruffles and their tips in motile cells, thereby linking Rho and profilin and the actin cytoskeleton. This complex formation is dependent upon RhoA as treatment with C3 exoenzyme or Rho-GDI abolishes any colocalisation of the three proteins (Watanabe et al., 1997). mDia has been shown to be involved in stimulating actin reorganisation to promote stress fibre formation in cooperation with ROK via its
FH domain (Nakano et al., 1999). The FH domain of mDia allows the interaction of the protein with various SH3-domain containing proteins thereby linking mDia to tyrosine kinase signalling and serum response factor (SRF)-dependent transcriptional activity (Satoh and Tominaga, 2001). mDia has also been shown to bind IRSp53 through its SH3 domain (Fujiwara et al., 2000).

1.7.3: Other Rho GTPase Effectors/Interactors:

There are many Rho GTPases identified to date, some of which are specific to Cdc42, Rac or Rho and a small proportion which are able to interact with more than one effector or even all three.

n-Chimaerin, initially identified as a Rac GAP (Hall et al., 1990; Manser et al., 1992, Ahmed et al., 1993) was later discovered to have effector function for both Rac and Cdc42 (Kozma et al., 1996).

p67^phox is a Rac specific effector (Diekmann et al., 1994) and is phosphorylated (Dusi et al., 1993) in a Pak mediated manner (Ahmed et al., 1998). It has been proposed that this interaction between p67^phox and Pak is one means of NADPH oxidase regulation (Ahmed et al., 1998)

ACK (Activated Cdc42 associated Kinase) is a non-receptor tyrosine kinase that specifically binds Cdc42 in its activated form (Manser et al., 1993). This protein is believed to be involved in regulating cell adhesion (Yang and Cerione, 1997).

POR1 (Partner Of Rac) as the name implies is a Rac specific interactor and is believed to synergise with RasV12 in inducing membrane ruffles (Van-Aelst et al., 1996).
p140Sra-1 (Specifically Rac1 associated protein) is another Rac specific interactor and colocalises with RacV12 and the cortical actin of membrane ruffles (Kobayashi et al., 1998).

p35 is a specific interactor of Rac and regulates Cdk5 activity upon binding. It is specific to neurones and is therefore often used as a neuronal marker (Nikolic et al., 1998). Cdk5/p35 are involved in neuronal cell migration, neurite outgrowth and regulation of adhesion as well as being important in neuronal development (Paglini and Cáceres, 2001).

Borgs (Binders Of Rho GTPases) are a five-member family of Cdc42 and TC10 GTPase interacting proteins. Borgs-1 and 3 have been proposed as being involved in cell spreading (Joberty et al., 1999). Borg-5 (also known as MSE55) has been shown to be involved in Cdc42 mediated actin cytoskeleton reorganisation events (Burbelo et al., 1999).

PKN is a Rho specific target protein. It is a serine/threonine kinase activated upon binding of Rho in its GTP bound form (Watanabe et al., 1996). PKN is believed to be involved in Rho mediated actin cytoskeleton reorganisation events such as stress fibre formation (Amano et al., 1996).

Citron binds both Rho and Cdc42. It has been reported that citron kinases is involved in cytokinesis (Madaule et al., 1998).

Partitioning-defective (PAR) proteins, initially identified in the nematode Caenorhabditis elegans, have six family members which are implicated in the asymmetric cell divisions in the early development of C. elegans (Kim, 1997), of which PAR6 and PAR3 have been shown to interact directly (Joberty et al., 2000) forming a complex with Rac/Cdc42 and PKCζ. This complex is believed to be responsible for the induction of cell polarity (Lin et al., 2000; Qui et al., 2001).
Two GTPases activating proteins (GAPs) have been identified which show GAP activity \textit{in vitro} towards Rac, Rho and Cdc42, known as Grit and Exo T. Grit, is a GAP that is involved in the regulation of neurite outgrowth via its interaction with the TrkA receptor (Nakamura et al., 2002). Exo T is a \textit{Pseudomonas aeruginosa} bacterial protein, which has been shown \textit{in vivo} to act as a GAP for Rac, Rho and Cdc42, and has the ability to cause stress fibre disassembly in epithelial cells (Kazmierczak and Engel, 2002).

1.8: Cross Talk between the Rho GTPases:
Rho, Rac and Cdc42 were initially described as functioning in a hierarchal cascade following experiments using serum starved Swiss 3T3 cells microinjected with protein or agonists for these GTPases. Stimulation of Cdc42 leads to the rapid activation of Rac, which in turn activates Rho but in a much slower and weaker manner than that of Cdc42 to Rac signalling (Kozma et al., 1995; Nobes et al., 1995). This Rac mediated Rho activation is believed to involve lipid intermediates (Peppelenbosch et al., 1995). The cross talk between Cdc42 and Rac is still under investigation; one possible mechanism is via the Cdc42/Rac effector Pak interactor p85-Cool/βPIX. PIX is a RacGEF as well as an interactor of Pak (Manser et al., 1998) and since Pak is preferably a Cdc42 effector this is a possible mechanism of cross talk between Cdc42 and Rac. Therefore, Pak is located upstream of Rac (Obermeier et al., 1998). There is evidence that Cdc42/Rac and Rho can act antagonistically. In N1E-115 neuroblastoma cells, C3 treatment, which inhibits Rho activity, or expression of constitutively active Cdc42/Rac results in neurite outgrowth. Expression of constitutively active Rho or inhibition of Cdc42/Rac causes neurite collapse (Kozma et al., 1997). BHK cells transfected with dominant negative
Rho GTPases, displayed morphologies characteristic of the noninhibited GTPase(s) (Moorman et al., 1999).

The activities of the Rho GTPases and their effects on the actin cytoskeleton are determined primarily by the expression levels of downstream effectors. Actomyosin dynamics provides evidence for convergence of the Rac/Cdc42 and Rho pathways. p160Rho kinase (ROK) can phosphorylate myosin light chain (MLC) phosphatase and potentially MLC directly (reviewed in Narumiya et al., 1997), which is believed to promote actomyosin filament assembly. Rac however, has been reported to promote actomyosin filament disassembly, by inducing phosphorylation of myosin heavy chain and MLC kinase in a Pak dependent manner (van Leeuwen et al., 1999).

RhoG has been shown to be able to activate Rac and Cdc42 independently (Gauthier-Rouviere et al., 1998) in the presence of microtubules. Microtubules and the actin cytoskeleton clearly coordinate in some way, depolymerisation of microtubules leads to Rho activation, conversely Rac activation is induced by microtubule repolymerisation (Zhang et al., 1997; Waterman-Storer et al., 1999).

Other small GTPases such as Ras have been shown to affect the Rho GTPases. The Ras target Ral, interacts with the Cdc42/Rac GAP RalBP1 (Cantor et al., 1995; Jullien-Flores et al., 1995). Expression of dominant negative Cdc42/Rac inhibits NGF-induced neurite outgrowth in PC12 cells a process which can be mimicked by the constitutively active Ral GEF. In the presence of constitutively active Cdc42/Rac, Ral GEF can rescue neurite outgrowth (Goi et al., 1999). The Ras GAP, p120RasGAP, has been shown to associate with the Rho GAP, p190RhoGAP, which inhibits Rho-mediated stress fibre formation, this complex formation coincides with the downregulation of stress fibres (McGlade et al., 1993; Fincham et al., 1999).
ARF6 and Rab5 are linked to the Rho GTPase-dependent cytoskeletal reorganisations. Ruffling induced by constitutively active Rac is antagonised by dominant negative ARF6 (Radhakrishna et al., 1999). Dominant negative Rab5 inhibits the Rac and Rho actin cytoskeleton reconstruction after PMA treatment (Imamura et al., 1998). This data implicates the Rho GTPases in morphological changes required for endocytosis (reviewed in Chavrier and Goud, 1999).

1.7: Rho GTPase Family Effectors/Interactors:

A schematic of the Rho GTPases Rac, Rho, Cdc42. Illustrating some of their interactors/effectors and the downstream signalling events to the actin cytoskeleton (Figure adapted from Dickson, 2001).
Chapter Two

Materials and Methods
Materials & Methods:

2.1: Materials:

2.1.1: General Laboratory Reagents:
General laboratory chemicals were obtained from Sigma or BDH. Purified water was produced in the laboratory using an Elga Purification system. It was deionised, purified by reverse osmosis and sterilised by autoclaving. Ethanol, methanol, isopropanol, glycerol, HCl, sodium hydroxide, Tris-HCl, Tween 20, Triton-x-100, bromophenol blue, glycine and glacial-acetic acid were obtained from BDH, Hayman and Sigma. Phosphate buffered saline (PBS) tablets from Oxoid. Bovine serum albumin (BSA), ampicillin and SDS from Sigma. Bacterial media components from Difco.

2.1.2: DNA Manipulation Reagents:
DNA restriction enzymes were obtained from Boehringer Mannheim, Gibco-BRL, NEB. Wizard DNA purification mini-prep system, Magic PCR clean-up system from Promega. Qiagen midi and mini preps were also used. Agarose, Ethidium bromide, HaeIII-digested \( \phi X174 \) and HindIII-digested \( \lambda \)7 DNA markers from Gibco-BRL. *Epicurian Coli* XL1-Blue competent cells from Stratagene. DNA modifying enzymes from Promega and Stratagene and random-primed DNA labelling kit from Boehringer Mannheim.

2.1.3: Protein Manipulation Reagents:
Pre-stained molecular weight markers from Gibco-BRL. Sodium fluoride, sodium vanadate and PMSF from sigma. Complete general protease inhibitor tablets from Roche Diagnostics. PVDF transfer membrane from NEN. Acrylamide/Bis-acrylamide (30%/0.8% and 40%/2.105%) from Scotlab. DTT, TEMED, and \( \beta \)-Mercaptoethanol
from Sigma, Bio-rad and BDH. $[\alpha^{32}\text{P}]\text{dCTP (3000Ci/mmol, 10mCi/ml; 0.8mM)}$ from NEN. Hybond-N filters, Hyperfilm-ECL and ECL reagents from Amersham. Autoradiography X-Omat film from Kodak. G-50 Sephadex from Pharmacia biotech.

2.1.4: Tissue Culture and Cell Staining Reagents:

HA-rabbit, GST-rabbit, NWASP-goat, Arp2-goat polyclonal antibodies from Santa Cruz, Biotechnology inc. FLAG-rabbit polyclonal antibody from Sigma. IRS58-mouse monoclonal antibody from Terry Jowett and Sheila Govind, UCL. Secondary antibodies conjugated to FITC, TRITC or Cy5 from Jackson Immunochemicals. Phalloidin conjugated to FITC or TRITC from Sigma. Secondary antibodies conjugated to HRP from DAKO. Dulbecco’s Modified Eagle Medium (DMEM) with 0.11g/l NA PYR with pyroxide, foetal calf serum, antibiotic/antimycotic solution, trypsin, lipofectAMINE 2000 were all obtained from Gibco-BRL. Mouse laminin solution was purchased from ICN Flow. NWASP$^{\times}$ from Dr. Ralph Kuhn and Dr. Sohail Ahmed.

2.1.5: cDNA Constructs:

cDNA constructs using the mammalian expression vector pXJ40. HA-IRS-58 from Dr. Sheila Govind, HA-PAKCAAX from Dr. Edward Manser, GST-NNWASP (1-162aa), HA-NWASP, HA-Cdc42V12 and HA-Rac1N17 from Dr. Thomas Leung. GFP-actin from Dr. Dong Jing Ming.

2.1.6: Oligomer Synthesis and cDNA sequencing Service:

Oligomers were synthesised by Genosys, Sigma. cDNA constructs sequenced by Cytomyx.
2.2: Methods:

2.2.1: DNA Manipulations:

2.2.1.1: Cloning of Domains of NWASP into a Eukaryotic Expression Vector:

Various domains of NWASP produced by PCR were cloned into the polylinker region of the FLAG tagged pXJ40 eukaryotic expression vector for transient transfection into neuroblastoma N1E-115 cells. The pXJ40 vector contains a cytomegalovirus (CMV) enhancer and a Kozak initiation sequence. At the 5' end of the polylinker region a FLAG epitope is encoded, which can be used for antibody detection of the expression of transiently transfected plasmid DNA in cells.

Domains of NWASP were amplified using the PCR technique. The oligonucleotides were designed with a 5' in frame Hind III restriction site and 3' Pst I restriction site so that the fragments could be directionally cloned into the polylinker region of the pXJ40 vector. The following oligonucleotides were used to clone the various domains of NWASP.

VH1 (1197-1250) 5' GGA AAC AAA GCA GCT CTT TTG
                   G N K A A L L
                   3' TTT TTT TAG CTG AGC
                   K K L Q A
VH2 (1284-1334) 5' GGA AGG GAT GCA CTT CTA GAC
                    G R D A L L D
                    3' GGA TTT CAA CTG AAT GCC
                    S K L Q I G
VH1-COF (1197-1454) 5' GGA AAC AAA GCA GCT CTT TTG
                      G N K A A L L
                      3' TTC ATC TTC ATC TGA GGA ATG ATG GCT
                      E D E D S S H I A
2.2.1.2: Primer Design:

The required DNA sequence for mouse NWASP was acquired (accession: O08816). The sequence was inserted into the Editseq program and a restriction map was created using Mapdraw. Primer oligonucleotides were between 24 and 30 bp in length.

2.2.1.3: Polymerase Chain Reaction (PCR):

PCR uses single stranded DNA as a template to amplify required DNA sequences. This \textit{in vitro} technique amplifies a required DNA sequence by the use of two oligonucleotides, which are complementary to opposite strands of the DNA template at either end of the DNA sequence to be amplified. The TaqPlus Long polymerase (Stratagene) enzyme (a mixture of Taq2000 DNA polymerase and cloned Pfu DNA polymerase) extends these oligonucleotides by incorporating complementary nucleotides to the template DNA between the two primers. The DNA is heat denatured and the whole process of annealing and synthesis is repeated. This technique proceeds to produce the required DNA sequence in an exponential manner as both the original DNA template and subsequently formed DNA sequences can act as a template for the oligonucleotides to anneal to, to form complementary DNA sequences.

The full length mouse NWASP construct cloned into the Hind III and Bgl II sites of the PXJ40 expression vector was used as a PCR template. To each PCR reaction tube the following was added:

- 10µl 10x TaqPlus Long low salt reaction buffer for template lengths 0-10Kb (200mM Tris-Cl, pH 8.75; 100mM KCl, 100mM (NH₄)₂SO₄, 20mM MgSO₄, 1% Triton X-100, 1mg/ml nuclease free BSA)
- 50ng template DNA
- 2.5µl of 5'→3' oligomer (150ng)
2.5μl of 3'→5' oligomer (150ng)
2μl dNTP mix (25mM of ATP, TTP, CTP, GTP)
ddH₂O making a final reaction volume of 100μl.

To each tube 2μl Taq Plus long polymerase (5U/μl) mix was added.

Tubes were placed into a Perkin Elmer PCR machine for the following cycles:
94°C for 5 minutes to denature template DNA.
95°C for 30 seconds to denature template DNA in later amplification cycles
X°C for 1 minute to allow annealing of oligomers to template DNA
72°C for 5 minutes to allow extension of oligomers so as to copy template DNA

This part of the cycle was repeated 25 times in order to amplify the required section of the template DNA.

72°C for 5 minutes to allow final extensions of oligomers
7°C to store PCR products overnight and to end reaction

The value of X is based upon the Tm value of the oligonucleotides used in the reaction.

\[ Tm = 2(A+T) + 4(G+C) \]

where A, T, G and C refer to the nucleotide base content.

PCR products were analysed by running 10μl on either a 1% agarose gel or a 16% acrylamide gel depending on the size of the products.
2.2.2: Enzymatic Modifications of DNA:

2.2.2.1: Restriction Digestion of DNA:

A variety of endonucleases were used to digest vectors, fragments of DNA and plasmid DNA using the buffer of the relevant salt concentration provided by the manufacturer. Restriction digests were typically carried out in a 10μl reaction volume with the reaction buffer at a tenfold dilution. This volume was generally used for diagnostic purposes, however a larger volume of 100-200μl was required for preparation of vectors or fragments for ligation or probe synthesis. Units of enzyme used in the reaction were determined by the amount of DNA present. It was ensured that an excessive amount of enzyme was not used in order to prevent "star activity" (non specific cutting) which results from glycerol present in the enzyme samples (>5% v/v contributes to star activity). Digests were carried out at 37°C for 1-2 hours. Double digests were carried out in the same way, using the buffer with the lowest salt concentration when a compatible buffer was not available.

2.2.2.2: DNA Ligation:

Restriction digested PCR fragments and expression vectors were mixed with the enzyme T4 DNA ligase (Promega). This enzyme catalyses the formation of phosphodiester bonds between the 5'phosphate and 3'hydroxyl groups of adjacent nucleotides in either cohesive-ended or blunt-ended fragments. In a DNA ligation reaction the following components are found:

20ng insert DNA

100ng linearised vector
2μl of 10 x T4 reaction buffer (30mM Tris-Cl, pH 7.8; 10mM MgCl₂, 10mM DTT, 1mM ATP)

ddH₂O making a final reaction volume of 20μl

To this 1μg of T4 DNA ligase enzyme (3U/μg) was added. The reaction mixture was incubated at 23°C in a water bath for 4-5 hours. 10μl of this ligation mixed was used to transform *Epicurian Coli* XL1-Blue competent cells.

### 2.2.2.3: Inactivation/Removal of Enzymes:

To stop a restriction digest reaction the enzymes were either heat inactivated by heating to 65°C for 20 minutes or removed from the sample by phenol/chloroform extraction as described in section 2.2.8 if stable beyond this temperature.

### 2.2.3: Transformation of *Epicurian Coli* (XL1 Blue Competent Cells):

*Epicurian Coli* XL1 Blue competent cells (Stratagene) were thawed on ice. 100μl of cells were aliquoted into 1.5ml eppendorf tubes. To each aliquot 1.7μl of β-mercaptoethanol (14.2M stock solution) was added giving a final concentration of 25mM. The tube was swirled gently and then incubated on ice for 10 minutes, swirling every 2 minutes. To each tube of competent cells 50ng of DNA was added. Cells were again incubated on ice for 30 minutes. Cells were heat shocked for 45 seconds at 42°C in a water bath, to allow the transformation of the competent cells with the plasmid DNA. Samples were incubated for 2 minutes on ice. To each tube of cells 900μl of prewarmed L-Broth (2% (w/v) tryptone, 1% (w/v) yeast extract, 2% (w/v) NaCl) containing 50μg/ml ampicillin was added. The cells were then incubated at 37°C for 1 hour in a shaker. If a retransformation was carried out 50μl of competent cells were used and the 1 hour
incubation step was omitted. Various amounts of the cells were spread onto LB/agar plates (2% (w/v) tryptone, 1% (w/v) yeast extract, 2% (w/v) NaCl, 1.5% agar) containing 50μg/ml ampicillin using a sterile glass spreader. Plates were placed inverted in a 37°C incubator for 12-16 hours to allow colony growth.

2.2.4: Identification of Transformed Bacterial Cells:

2.2.4.1: Colony Lift, Lysis and DNA Fixation:

Bacterial colonies transformed with the required DNA sequence were identified using a radiolabeled probe in a hybridisation screen. In situ lysis of the colonies and the subsequent release of plasmid DNA immobilised onto nitrocellulose filters were undertaken as described by Grunstein and Hogness (Grunstein and Hogness, 1975). The filters were screened with a complementary radiolabeled probe (prepared as described in 2.2.4.2), which hybridises to the DNA of interest.

Epicurian Coli XL1-Blue competent cells were transformed with the PXJ40 vector containing the DNA insert of interest and plated out onto LB agar plates (see section 2.2.3). These plates containing approximately 50-100 colonies were replica plated onto Hybond-N (nylon) membranes of 0.45 microns. An ink loaded needle was used to jab the filters and plates to mark their orientation for identification of the bacterial colonies containing the DNA of interest. Filters were placed colony side up on fresh LB agar, ampicillin-containing plates and incubated at 37°C for 1-2 hours to allow colony growth. The master plates were incubated for 4-5 hours to allow the disturbed colonies to regrow after which time they were stored at 4°C for up to 1 month.

Filters were treated as follows on 3MM Whatmann filter paper soaked in the relevant solution colony side up:

10% SDS for 3 minutes for lysis of bacterial cell walls
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Denaturation buffer (1.5M NaCl, 0.5M NaOH) for 7 minutes to denature the plasmid DNA
Neutralisation buffer (1M Tris-Cl, pH 7.4; 1.5M NaCl) for 3 minutes to neutralise the filters

2 x SSC (20 x stock solution: 3M NaCl, 0.3M Na_2 citrate)

2 x SSC/0.1%(w/v) SDS to remove bacterial cell debris

The filters were then left to air dry on 3MM Whatmann filter paper. Once dry they were covered in saran wrap and placed on an UV transluminator (312nm-wavelength) DNA side down to fix the DNA to the filters. Filters were then screened using a radiolabeled probe.

2.2.4.2: Hybridisation Probe Synthesis:

A [³²P]-labelled probe complementary to DNA sequence of the insert was synthesised by random primed DNA labelling. This technique is based on hybridisation of a mixture of random hexanucleotides (dATP, dGTP, dCTP and dTTP) to complementary DNA to be labelled. The Klenow enzyme synthesises a complementary strand to the template by extending a random hexonucleotide primer at the 3’ OH terminal. Unlabelled dCTP was replaced with the modified [α³²P]dCTP so that this radiolabelled hexonucleotide was incorporated into the synthesised DNA strand. The resulting radiolabelled DNA strand can be used to identify colonies containing the DNA insert required.

50-100ng of linearised DNA (>100bp) containing the sequence of the insert DNA, was heat denatured at 100°C for 10 minutes in an eppendorf tube, then cooled on ice. This DNA sample acts as a template for the random primed labelling procedure. To the DNA template sample the following was added:

1µl of 0.5mM dATP, dTTP, and dGTP (final concentration 0.05mM in ratio 1:1:1)
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2µl 10 x reaction buffer

5µl/50µCi [α\(^{32}\)P]dCTP, 3000Ci/mmol

1µl Klenow enzyme (2U/µl)

ddH\(_2\)O making a final reaction volume of 20µl.

Reaction was incubated at 37°C for 30 minutes. The reaction was stopped by heat inactivation of the Klenow enzyme by heating to 65°C for 15 minutes.

Unincorporated [α\(^{32}\)P]dCTP was removed by passing probe through a G-50 Sephadex column. The column was prepared by piercing a hole in the bottom of an eppendorf tube with a needle. A piece of glass wool was packed into the bottom of the eppendorf tube and a slurry of G-50 Sephadex beads was added to the tube. An eppendorf tube was placed beneath the column to act as a collecting tube. The column was briefly centrifuged in a bench top microcentrifuge. The addition of G-50 Sephadex beads was repeated until the column was tightly packed.

The [\(^{32}\)P]-labelled probe volume was increased to 200µl and a clean eppendorf placed beneath the column. The probe was applied to the column and briefly centrifuged.

The radiolabelled probe DNA is excluded from the gel causing it to pass through the column first leaving the free label behind in the column. The probe was then either stored at -20°C until required or denatured by heating to 100°C for 10 minutes followed by chilling on ice before being used in a hybridisation screen.

2.2.4.3: In-situ Hybridisation:

Filters were moistened in 6 x SSC buffer and placed into hybrid tubes. To each tube 25ml of prehybridisation buffer (5 x SSPE (20 x stock solution: 3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA, pH 7.7), 5 x Denhardt’s solution (50 x stock solution:
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5g Ficoll, 5g polyvinylpyrrolidone, 5g BSA in 500ml of ddH₂O, filter sterilised, aliquoted and stored at -20°C), 0.5%(w/v) SDS, 10mg/ml salmon sperm DNA). Filters were incubated with the prehybridisation solution for 1 hour at 65°C in a rotating oven. The prepared probe was added to the hybridisation solution and the filters were incubated overnight at 45°C.

After hybridisation the filters are washed in 2 x SSPE/0.1% (w/v) SDS at room temperature for 10 minutes rotating. This is replaced with 1 x SSPE/0.1% (w/v) SDS and the filters are incubated at 65°C for 15 minutes rotating. A final wash with 0.1 x SSPE/0.1% (w/v) SDS at 65°C for 10 minutes is carried out when a high stringency wash is required. The filters are then covered in saran wrap and placed in a X-ray film cassette where the filters are exposed to X-ray film (Kodak XAR-2) in a -70°C freezer for 5-24 hours. Colonies containing the required DNA insert to which the radiolabelled probe has hybridised appear as black dots on the autoradiographic film.

2.2.5: Isolation of DNA Fragments from Agarose Gels:

A preparative 1.2% agarose gel was placed into an electrophoresis tank containing 1xTBE buffer. 20µl of the DNA sample was loaded into the small well, and 180µl was loaded into the large well. Electrophoresis tank was at a constant voltage of 120V for 1 hour or until the dye front had migrated 75% along the gel. A scalpel was used to cut the small well lane from the rest of the gel. This was placed in a tank containing ethidium bromide (40µg/ml) for 15 minutes. This section of the gel was aligned with the rest of the gel and visualised in a UV transluminator of wavelength 312nm to visualise the band required for excision.
The required band was excised from the preparative gel, was then finely chopped and split into four eppendorf tubes. To each tube 1ml of Promega wizard DNA mini prep clean up resin was added. Samples were incubated on a rotator at room temperature for 30 minutes or until the gel had dissolved. Samples were then transferred to 2ml syringes that were attached to mini prep columns. The samples were passed through the columns and the flow through was discarded. The columns were placed into eppendorf tubes and centrifuged in a microcentrifuge at 14,000rpm for 2 minutes. Columns were washed with 2ml of 80% isopropanol by centrifugation, left to air dry for 5 minutes at room temperature to ensure no transfer of the isopropanol solution. 50µl of preheated 1xTE buffer (heated to 80°C in a dry heating block) was applied to each column to elute DNA. The DNA was eluted into clean eppendorf tubes by centrifugation at 14,000rpm for 2 minutes and the concentration was estimated (refer to section 2.2.6.3). A diagnostic restriction digest was carried out and a 5µl sample was run on a 1.5% agarose gel to ensure required DNA present.

2.2.6: Hot-tip PCR:
Potential positive colonies (identified by in situ hybridisation screening) were picked with a sterile toothpick and placed into 5ml of prewarmed L-Broth containing 50µg/ml ampicillin. The cultures were incubated, shaking at 37°C for 16-20 hours. A heat sterilised streaking wire was dipped into the culture and streaked across a LB/amp plate. Plates were incubated at 37°C for 16 hours. Several single colonies were picked with sterile tooth picks and crossed onto a grided LB/amp plate. Plates were again incubated for 16 hours at 37°C. Several colonies were picked with sterile toothpicks and touched
into the bottom of clean PCR tubes, then into 5ml prewarmed L-Broth containing 50µg/ml ampicillin. To each of the PCR tubes the following components were added:

10µl 10x TaqPlus Long low salt reaction buffer for template lengths 0-10Kb

2.5µl of 5'→3' oligomer (150ng)

2.5µl of 3'→5' oligomer (150ng)

2µl dNTP mix (25mM of ATP, TTP, CTP, GTP)

ddH2O making a final reaction volume of 100µl.

To each tube 2µl Taq Plus long polymerse (5U/µl) mix was added.

Tubes were placed into a Perkin Elmer PCR machine and cycled as described in section 2.2.1.3.

5µl of each sample was run on a 16% acrylamide gel to determine if required insert is present in the picked colony and whether the multiple or single inserts are present in the vector. Gel was stained in ethidium bromide (40µg/ml) solution for 15 minutes, visualised on a UV transluminator and photographed.

Colonies that produced single bands of the required size on the acrylamide gel were selected and DNA purification prep was carried out as detailed in section 2.2.8.

2.2.7: Gel Electrophoresis of DNA:

2.2.7.1: Agarose Gel Electrophoresis:

10µl of PCR products/linearised DNA was mixed with 2µl of 6 x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and loaded into wells of the agarose gel using a P20 Gilson pipette. 5µl of Hae III digested φX174 DNA (0.6µg/µl 6 x loading buffer) and Hind III digested λ DNA (0.6µg/µl 6 x loading buffer) of sizes 23.13kb to 125bp and 1353bp to 72bp respectively, were mixed and
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loaded into one of the wells on the agarose gel. Electrophoresed at a constant voltage of 140V for 45 minutes.

2.2.7.2: Acrylamide Gel Electrophoresis:

To resolve PCR products smaller than 200 base pairs in length a 16% acrylamide gel was prepared as follows:

2.5ml 10X Tris Borate EDTA (TBE) (0.045M tris borate, 1mM EDTA)
10ml 40:2 acrylamide:bisacrylamide (sequencing grade)
12.5ml ddH₂O
125μl 10% APS
12.5μl Temed

Gel was prepared in a round bottom flask and cast into SDS PAGE plates. The gel was allowed to set at room temperature for 30 minutes or until remainder of mix was set. Gel was placed into a tank containing enough 1 x TBE buffer to cover the surface. 10μl of PCR products were mixed with 2μl 6x DNA loading buffer and loaded into the wells of the acrylamide gel. Gel was run at a constant voltage of 120V until dye had run to the bottom edge of the gel.

2.2.7.3: Visualisation of DNA with Ethidium Bromide:

The gel was removed from the tank and placed into a tank of distilled water containing 40μg/ml of ethidium bromide for 15 minutes. Ethidium bromide is an intercalating agent which is incorporated into the DNA samples. Upon excitation with ultra violet (UV) light (312nm wavelength) the ethidium bromide fluoresces making it visible. Data was recorded onto polaroid film.
2.2.8: Switching of Mammalian Expression Vector Tag of Existing Constructs:
NWASP domain constructs VH1, VH2 and VH1-Cof which were cloned into the pXJ40-FLAG vector were digested with restrictive enzymes EcoR1 and BamH1 as described in section 2.2.2.1 to remove the FLAG tag. The digest products were run on a 1% low melt agarose gel which was prepared as described in section 2.2.6.1. The vector portion containing the insert, with the FLAG tag excised was cut from the gel. The empty pXJ40-GST vector was digested with in the same way as the NWASP domain constructs. This time however the GST tag was excised from the gel. To each of the gel excised fragments 100μl of ddH2O was added and heated to 65°C for 5 minutes. The GST tag fragment and the pXJ40 vector/NWASP domain fragment were ligated as described previously in section 2.2.2.2. to form a pXJ40-GST-NWASP domain construct.

2.2.9: Plasmid DNA Preparation:

2.2.9.1: Promega “Mini Preps”
A single colony of transformed XL1-Blue competent cells containing the required plasmid DNA was picked using a sterile toothpick. The toothpick was placed into 10ml of prewarmed L-Broth containing 50μg/ml ampicillin in a 50 ml falcon tube. The culture was incubated typically at 37°C in a shaker for 16 hours. A 1ml sample was mixed with 10% glycerol and stored at -70°C. The remaining samples were centrifuged at 10,000rpm in a bench top microcentrifuge for 5 minutes at room temperature. The supernatant was decanted and the pellet was resuspended in 250μl cell resuspension buffer (50mM Tris-Cl, pH8; 10mM EDTA, 100μg/ml RNase A). Samples were vortexed to ensure full resuspension of the cell pellet. To each sample 250μl cell lysis buffer (200mM NaOH, 1% SDS) was added and mixed by inversion. Samples were then incubated at room
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temperature for 5 minutes. 10μl alkaline protease solution was added and mixed by inversion. Samples were incubated at room temperature for a further 5 minutes. 350μl of neutralising buffer (3m potassium acetate, pH5.5) was added mixed immediately by inversion. Samples were centrifuged at 14,000rpm for 10 minutes to remove bacterial cell debris. The lysates were transferred to mini prep columns which were placed into 2ml collection tubes. Columns were centrifuged at 14,000rpm for 1 minute. Flow through was discarded. Columns were washed by the application of 250μl of column wash solution (1M NaCl, 50mM MOPS, pH7; 15% isopropanol) followed by centrifugation at 14,000rpm for 2 minutes at room temperature. Columns were transferred to clean eppendorf tubes. Plasmid DNA was eluted by the addition of 100μl of nuclease free water onto the mini prep columns followed by centrifugation at 14,000rpm for 1 minute. Samples were cleaned by phenol/chloroform extraction.

2.2.9.2: Qiagen "Midi Preps":

A single colony was picked from a freshly streaked L-Broth agar plate containing 50μg/ml ampicillin with a sterile toothpick and used to inoculate 10ml of prewarmed L-Broth. The starter culture was incubated for 8 hours, diluted 1/100 into 250ml prewarmed L-Broth containing 50μg/ml ampicillin in a 2 litre conical flask and incubated for a further 16 hours vigourously shaking at 37°C.

The bacterial cells were harvested by centrifugation at 3,500rpm for 15 minutes at 4°C in a Beckman J6-HC centrifuge. Supernatant was removed and pellets were either stored in the -20°C until a later date or treated so as to recover the plasmid DNA.

Bacterial pellets were resuspended in 6ml buffer P1 (50mM Tris-Cl, pH8; 10mM EDTA, 100μg/ml RNase A) by vortexing. 6ml buffer P2 (200mM NaOH, 1% SDS) was added
to each sample to lyse the cells. Samples were inverted 4 times and incubated at room
temperature for 5 minutes during which time the QIAfilter cartridge was prepared. 10ml
of chilled buffer P3(3M potassium acetate, pH5.5) was added to the lysate to stop further
lysis occurring by neutralising the acid. Samples were mixed immediately by inversion.
The lysate was poured into the barrel of a QIAfilter cartridge and incubated at room
temperature for 10 minutes. A precipitate containing the genomic DNA, proteins and
detergent forms and floats to the top of the solution in the cartridge. During this
incubation period a HiSpeed Midi tip was equilibrated by application of 4ml buffer QBT
(750mM NaCl, 50mM MOPS, pH7; 15% isopropanol, 0.15% Triton X-100) which was
allowed to pass through by gravity flow.

The cell lysate was applied to the equilibrated tip by being passed through the
QIAfilter with the use of a plunger. The cleared lysate entered the resin by gravity flow.
The tip was washed with 20ml of buffer QC (1M NaCl, 50mM MOPS, pH 7; 15%
isopropanol) to remove any contaminants in the plasmid preparation. DNA was eluted
with 5ml of buffer QF (1.25M NaCl, 50mM Tris, Tris-Cl, pH8.5; 15% isopropanol).
Eluted DNA was precipitated by addition of 3.5ml (0.7 volumes) of room temperature
isopropanol followed by incubation period of 5 minutes at room temperature. During this
time a QIAprecipitator was attached to a 20ml syringe with the plunger removed. The
eluate/isopropanol mix was poured into the 20ml syringe and pushed through the
precipitator. The precipitator was attached to a 5ml syringe 1ml of TE buffer (10mM
Tris-Cl, pH 8; 1mM EDTA) was added to the syringe to elute the DNA. The eluate was
reapplied to the 5ml syringe and passed through the precipitator again to ensure full
recovery of the plasmid DNA. The eluated DNA was then phenol/chloroform extracted.
2.2.10: DNA Purification:

2.2.10.1: Phenol/Chloroform Extraction:

1.5ml phase light tubes (Sigma) were prepared by centrifuging at 14,000rpm for 2 minutes. DNA samples were placed into the prepared tubes along with an equal volume of phenol. The samples were inverted several times until a white emulsion was formed followed by centrifuging at 14,000rpm for 2 minutes. The gel in the phase light tubes formed an interface between the aqueous DNA containing layer and the phenol/RNA and protein containing layer. The upper aqueous DNA containing layer was removed and placed into fresh prepared phase light tubes with an equal volume of water saturated chloroform. Procedure for phenol step was repeated for chloroform. Upper aqueous DNA containing layer was removed and the DNA was recovered by ethanol precipitation.

2.2.10.2: Ethanol Precipitation of Small DNA Fragments:

DNA samples were adjusted to pH5.2 by addition of 0.1 volumes of 3M sodium acetate pH 5.2 and two volumes of ice cold absolute ethanol and 1μl of GenElute lipopolyacrylamide (LPA) was added to each sample. The GenElute LPA binds and helps to precipitate DNA fragments of 20 bp or larger. Samples were centrifuged at 14,000rpm for 5 minutes to pellet the DNA. Pellets were resuspended in 50 or 100μl 1x TE (10mM Tris-Cl, pH 8; 1mM EDTA) buffer.
2.2.11: Quantitation of DNA in Solution:

5μl of purified plasmid DNA was diluted in 995μl of TE buffer. The optical density of each sample was measured in an UV cuvette at wavelengths of 260nm and 280nm using a deuterium lamp spectrophotometer. The concentration of DNA was estimated using the following equation:

\[ [\text{DNA}] \mu g/\mu l = 50 \times (\text{total volume/volume DNA}) \times (1/1000) \times A_{260nm} \]

2.2.12: Protein Expression and Purification:

2.2.12.1: Overexpression of pXJ40 Constructs in COS 7 Cells:

A sub confluent 90mm plate of COS 7 cells were serum starved for 1 hour in DMEM only. 1ml DMEM media was placed into eppendorf tubes to which 10μg of the DNA construct and 30μl of lipofectamine 2000 (Gibco, Life technologies) was added. Transfection mix was incubated at room temperature for 45 minutes. Transfection mix was applied to the plate of COS 7 cells and incubated for 4 hours to allow the take up of the DNA constructs into the cells. Transfection mix media was removed. Cells were rinsed using DMEM containing 10%FCS and 1% antibiotic/antimycotic. 10mls of this media was applied to each plate. Protein expression was allowed to occur in the cells for 16 hours.

2.2.12.2: Preparation of Cell Lysates:

The transfected COS 7 cells were rinsed briefly in PBS. 2 x 10mls PBS was applied to each plate and cells were removed from the dish by scraping. Cell suspensions were centrifuged at 1500rpm for 7 minutes. The cell pellet was resuspended in 1ml PBS and
transferred to sterile eppendorf tubes. Samples were centrifuged in a microfuge for 1 minute. Supernatant was removed and pellet was resuspended in 200µl of lysis buffer (20mM Tris/HCl, 5mM EDTA, 150mM NaCl, 10% glycerol, 1% triton-x-100, 1mM PMSF, 1mM DTT, 1mM NaF, 1mM NaVO₄, 1mM PI and 1 x protease inhibitor cocktail (Sigma)). Cells were incubated on ice for 5 minutes to allow lysis to occur. Samples were sonicated in a sonication ice bath for 3 minutes using a program of 20 seconds on 5 seconds off, at level 6. Samples were centrifuged at 20,000g for 20 minutes at 4°C. Supernatant was removed (soluble fraction) and pellet was washed in lysis buffer (insoluble fraction).

2.2.12.3: Preparation of SDS-Polyacrylamide Gels:

SDS polyacrylamide gel electrophoresis was used to separate proteins on the basis of their molecular mass. This was performed using polyacrylamide gels in a discontinuous buffer system. The final acrylamide concentration in the resolving gel is determined by the molecular weight of the proteins that require separation. The composition of a 22% acrylamide resolving gel for separation of proteins typically ranging from 20KDa to 1KDa is as follows:

- 7.3ml 30% acrylamide/bis-acrylamide
- 2.5ml 1.5M Tris/HCl pH8.8
- 100µl 10% (w/v) SDS
- 50µl 10% APS
- 7µl TEMED

Approximately 7.5mls of this mix was pipetted into a mini gel apparatus (Biorad) allowing space for a staking gel. ddH₂O was applied to the top of the acrylamide mix and left to
polymerise at room temperature for 45 minutes. Once polymerised the ddH₂O was poured off and any residual fluid removed using filter paper. A stacking mix was prepared as follows:

1. 1.67ml 30% acrylamide/bis-acrylamide
2. 1.25ml 0.5M Tris/HCl pH6.8
3. 7ml ddH₂O
4. 100µl 10% (w/v) SDS
5. 50µl 10% APS
6. 12µl TEMED

The stacking gel was pipetted onto the polymerised resolving gel, into which a teflon comb was inserted. The stacking gel was left to polymerise at room temperature for 30 minutes. Once polymerised the comb was removed and residual non-polymerised acrylamide was removed by washing the wells with ddH₂O.

**2.2.12.4: Separation of Proteins by SDS PAGE:**

Protein samples were separated according to their molecular weight. Protein markers of known size were run in parallel to samples to estimate the molecular weight of proteins separated on the acrylamide gel. Samples were mixed with 5 x SDS-gel loading buffer (10% SDS, 50% glycerol, 0.3M Tris/HCl pH6.8, 0.125mls β-mercaptoethanol, bromophenol blue, ddH₂O to 5mls) and heat denatured at 100°C for 5 minutes before being loaded onto the denaturing SDS polyacrylamide gels. A 22% SDS-gel was typically used to separate proteins ranging from 20kDa to 4kDa. 10% gels were used to separate proteins of 16kDa to 68kDa molecular weight. Proteins were separated using a Biorad vertical gel discontinuous buffer system. Gel was run at 140V for typically 1 ½
hours in 1 x electrode buffer (10 x electrode buffer: 0.25mM Tris, 0.5M Glycine, 1% SDS).

2.2.13: Western Transfer of Proteins onto Nitrocellulose Filters (Semi-dry blotting):
Proteins separated by electrophoresis on denaturing SDS-acrylamide gels were transferred onto nitrocellulose for probing with antibodies (western blotting). The SDS-gels were equilibrated in 1 x transfer buffer (100ml of 10 x stock (48mM Tris, 39mM glycine, 1.3mM (0.037%) SDS, 20% (v/v) methanol made up to 1 litre with ddH₂O). Two pieces of extra thick Biorad filter paper, two pieces of Whatman filter paper and one piece of PVDF membrane were cut to a size of 10cm x 7cm. The PVDF membrane was soaked in methanol for 30 minutes, rinsed in ddH₂O and then equilibrated in 1 x transfer buffer along with the Biorad filter paper and the Whatman filter paper for 30 minutes. A sandwich was prepared as follows on a Biorad Transfer-Blot SD semi-dry transfer system from bottom to top: Thick Biorad filter paper, Whatman filter paper, PVDF membrane, SDS-acrylamide gel, Whatman filter paper, thick Biorad filter paper. Proteins were transferred overnight at a constant voltage of 8 volts.

2.2.14: Immunoanalysis of Nitrocellulose Immobilised Proteins:
Proteins immobilised onto nitrocellulose filters by semi-dry blotting were stained for 3 minutes in coomasie blue (0.5g coomasie brilliant blue, 45% methanol, 10% acetic acid, 45% ddH₂O) and then destained in destain (40% methanol, 50% ddH₂O, 10% acetic acid) for 20 minutes followed by washing for 3 x 10minutes in PBS. Filters were then blocked for 1 hour at room temperature or overnight at 4°C in 5% marvel in PBS. Filters were washed for 5 x 5 minutes in PBS/0.1% Tween 20. Filters were incubated with primary antibodies used at typically 2μg/ml in 1% marvel in PBS for 1 ½ hours at room
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temperature. Filters were washed for 5 x 5 minutes in PBS/0.1% Tween 20 prior to incubation with a Horseradish peroxidase (HRP) conjugated secondary antibody (Dako) used at 1:1000 dilution of stock in 1% marvel in PBS. Filters were incubated for 1 hour at room temperature. Again filters were washed for 5 x 5 minutes in PBS/0.1% Tween 20 before probing the filters with ECL western blotting detection reagents (Amersham). The ECL system is a non-radioactive, illuminescent reagent used for detection of antibodies conjugated with horseradish peroxidase. Equal volumes of ECL reagents 1 and 2 were mixed briefly before being applied to the filters for one minute. Excess liquid was removed by blotting on Whatman filter paper. Filters were wrapped in saran wrap before being placed into a film cassette protein side up and exposed to ECL film. A series of varying time length exposures were performed to obtain an optimum signal.

2.2.15: Cell Culture and Manipulations:

2.2.15.1.1: Cell Culture of N1E-115 Cells:

N1E-115 neuroblastoma cells are a clone derived from a C-1300 mouse neuroblastoma (Amano et al., 1971). They are adrenergic neurones with a high level of acetylcholine esterase enzyme present. This cell line also has a relatively high production rate of tyrosine hydroxylase, which is involved in the noradrenaline synthesis pathway (enzyme responsible for the conversion of tyrosine to DOPA).

Cells were cultured in Dulbecco’s Modified Eagle Medium with 0.11g/1 NA PYR with pyridoxine (DMEM) containing 10% foetal calf serum (FCS) and 1% antibiotic/antimycotic solution (penicillin, streptomycin and amphotericin) in 90mm tissue culture dishes (Nunc). Cell cultures were grown in a Heraus, Hera Cell incubator at 37°C in a humid environment and 5% CO₂.
2.2.15.1.2: Cell Culture of NWASP<sup>−/−</sup> Cell Lines:

NWASP knock out cells (supplied by Ralf Kuhn, Artemis Pharmaceuticals GmbH, Neurather Ring1, 51063 Koln, Germany) were prepared by immortalising NWASP<sup>flox/flox</sup> E14 primary fibroblasts with retroviruses followed by subcloning. Various NWASP<sup>del/del</sup> cell lines were selected after transient expression of Cre. Cells were cultured in DMEM and 10% FCS and 1% antibiotic/antimycotic solution at 32°C in a humid environment and 5% CO₂.

2.2.15.2: Cell Maintenance:

Cells were grown to sub-confluence before being split and plated onto fresh dishes typically at 1/5, 1/10 or 1/20 densities. Cells were fed every other day and on average split twice a week.

Media was removed from the dish using a suction line and sterile glass pipette in a Hepair tissue culture hood. Cells were detached by pipetting 10ml of fresh media and placed into a 50ml falcon tube. Spinning at 750rpm in a Beckman GP centrifuge for 7 minutes pelleted cells. The supernatant was removed and cell pellet was resuspended in 10ml of fresh media by gentle pipetting. Cells were then passed through a green needle syringe (G21) twice to reduce cell clumping. Cells were plated out onto 90mm dishes at the required density in fresh media.

COS-7 and NWASP cells were treated in the same manner as N1E-115 cells, except for the use of 3mls of prewarmed trypsin for 2 minutes or until cells appear rounded, in order to remove cells from the plate. This is due to COS-7 and NWASP cells attaching to the tissue culture dish much more strongly than the N1E-115 cells. 7mls of prewarmed media was then added to the dish to prevent further actions of the trypsin and cells were placed in a falcon tube and treated as described above.
2.2.15.3: Freezing Down of Cell Stocks:

A sub-confluent plate was harvested in 10 ml of fresh media and placed in a 50ml falcon tube. Centrifuging at 750rpm for 7 minutes at room temperature pelleted the Cells. The media was aspirated and the cell pellet resuspended in 4ml of 90% FCS and 10% DMSO. 1.8ml of cell suspension is transferred to screw cap tubes. The tubes are wrapped well in tissue paper and placed in a polystyrene box before being stored in the -20°C freezer for 2 hours. This box is transferred immediately to the -70°C freezer for 16-48 hours. Tubes are then transferred to liquid nitrogen storage, where they can be stored until required.

2.2.15.4: Bringing Up of Cell Stocks from Liquid Nitrogen Storage:

Vials of cell stocks are thawed rapidly at 37°C in a water bath. Cells are transferred to a 50ml falcon tube containing 10ml of prewarmed DMEM containing 10% FCS and 1% antibiotic/antimycotic solution. Cells are pelleted by centrifuging at 750rpm for 7 minutes. Media was aspirated and cell pellet is resuspended in 10ml of fresh media. Cells are plated out in 90mm dishes at either 1/3 and 2/3 or 1/4 and 3/4 densities. Cells are incubated at 37°C in a humid environment and 5% CO₂.

2.2.15.5: Preparation of Coverslips:

Round glass coverslips of 16mm diameter were washed briefly in 40% HCl and 60% ethanol and then rinsed in ddH₂O followed by heat sterilisation for 4 hours at 121°C. These coverslips were then placed in a tissue culture dish and coated with mouse laminin (10μg/ml) for 16 hours at 4°C. The laminin solution was removed and the coverslips were left to air dry in the tissue culture hood for 10 minutes. They were then rinsed briefly in ddH₂O. The coverslips were then either placed into 35mm dishes with 3ml of
DMEM containing 5% FCS and 1% antibiotic/antimycotic solution ready for plating out of cells for transfection or stored at 4°C for up to one week.

2.2.15.6: Transient Transfection of N1E-115 Cells:

A sub-confluent plate was harvested and cells were pelleted by centrifugation at 750rpm for 7 minutes. Supernatant was removed and the cell pellet was resuspended in DMEM containing 5% FCS and 1% antibiotic/antimycotic. A 100μl sample of the cell suspension was removed and placed in an eppendorf tube to which 10μl of 1% trypan blue was added. Cells were counted using a haemocytometer and an estimation of the number of cells per ml was calculated. Between 1 x 10⁴ and 1.5 x 10⁵ cells were seeded onto the prepared coverslips in 35mm dishes. Cells were then incubated over night at 37°C in a humid environment and 5% CO₂ to allow the cells to sit down ready for transfection.

Media was removed and replaced with 1ml of DMEM. Cells were returned to the incubator for 1 hour. 200μl of DMEM was placed into eppendorf tubes. To each tube 1μg of the plasmid DNA of interest in a eukaryotic expression vector (PXJ40) was added, along with 6μl of lipofectamine 2000 (Gibco). Samples were incubated for 45 minutes at room temperature. DNA samples were pipetted onto each of the coverslips in a drop wise manner. Cells were returned to the incubator for 4 hours to allow the DNA to be taken up into the cells passively. After this time the transfection mix was aspirated and replaced with 3ml of DMEM containing 5%FCS and 1% antibiotic/antimycotic solution. Cells were returned to the incubator for 16-20 hours to allow transcription and translation of the transfected DNA into protein. The media was then removed and cells were fixed and stained. For a short expression time experiment the cells were fixed after 6-7 hours.
expression time. For reduced serum experiments, transfection mix was replaced with DMEM containing 1% FCS and 1% antibiotic/antimycotic solution.

2.2.15.7: Transient Transfection of COS-7 Cells:
COS-7 cells were grown on 90mm petri dishes until subconfluent in DMEM containing 10% FCS and 1% antibiotic/antimycotic. Media was removed and cells were serum starved in 4mls DMEM only for 1 hour. Transfection mixes were set up as follows: 1ml DMEM, 5μg plasmid DNA and 20μl Lipofectamine reagent. Samples were incubated at room temperature for 45 minutes. Transfection mix was applied to the cells and incubated at 37°C for 5 hours. Transfection mix was removed and 10mls DMEM containing 10% FCS and 1% antibiotic/antimycotic was added to the cells. Cells were incubated at 37°C for 16-40 hours. Transfected COS-7 cell lysates were prepared as described in section 2.2.11.2 above.

2.2.15.8: Immunostaining of N1E-115 Cells:
Media was removed and coverslips were washed in PBS. Cells were fixed in 4% paraformaldehyde in PBS for 10 minutes in a class 2 tissue culture hood. Cells were then washed twice in PBS for 10 minutes. 0.5% Triton X-100 in PBS was added to each petri dish to permeabilise cells for 1 minute. Cells were again washed in PBS twice for 10 minutes. Cells were blocked using 3% BSA in PBS solution for 15 minutes. Cells were incubated with the primary antibody used typically at 5μg/ml concentration or as detailed by supplier in 1% BSA in PBS solution. 20μl was dropped onto a parafilm covered 90mm petri dish inverted lid. Coverslips were placed onto the antibody solution cell side down using tweezers. Cells were incubated with the primary antibody solution at 37°C for 2 hours in a humid chamber. Coverslips were rinsed in PBS/0.1% Tween 20 by
floating them cell side down on the wells of 24 well tissue culture plates for 2 x 10 minutes. Cells were then incubated with secondary antibodies, which have a fluorescence tag (FITC, TRITC or Cy5) attached. Secondary antibodies were used at 15μg/ml concentration in 1% BSA in PBS solution. Cells could also be stained with phalloidin (FITC or TRITC labelled), which was used at a concentration of 10ng/ml to stain F-actin structures. Cells were incubated at 37°C for 1 hour in a humid chamber. Cells were rinsed as before in PBS/0.1% Tween 20 for 2 x 10 minutes. When phalloidin was used, this work was carried out in a designated fume hood. Coverslips were left to air dry for 5 minutes and then mounted onto glass slides using one drop of immunofluor mountant. Cells were then visualised and analysed on a fluorescence microscope (Zeiss). A dual laser confocal microscope (Zeiss) using LSM software version 4 was used to obtain images of stained cells.

2.2.15.9: Bioporter Delivery of Antibodies:

Cells were seeded onto laminin coated coverslips as for transient transfections. Bioporter reagent was prepared in 250μl of methanol and allowed to dry to a film in eppendorf tubes in a tissue culture hood for 4 hours. The film was solublised in 900μl DMEM and antibodies were prepared as 10μg in 100μl PBS and mixed with the bioporter reagent, vortexed and left to stand at room temperature for 5 minutes. Mix was applied to cells in serum free DMEM. Mix was incubated with cells for 4 hours after which mix removed and replaced with 2ml DMEM + 1% FCS and incubated for 2 hours cell at 37°C, before being fixed using 4% PFA (see section 2.214.8).
2.2.16: Live Imaging Studies:

2.2.16.1: Filopodia Dynamics of N1E-115 Cells:

3 cm petri dishes with a hole cut in the base over which a coverslip was placed were coated with mouse laminin as described in 2.2.14.5. 1 x 10^5 cells were seeded onto these imaging dishes in DMEM and 5% FCS and 1% antibiotic/antimycotic and incubated overnight at 37°C and 5% CO₂ to attach to dishes. Cells were transiently transfected with required construct and pXJ40-GFP-actin (provided by Dr. Dong Jing-Ming, IMCB, Singapore) as described in section 2.2.14.6.

To each imaging dish 36μl of 1M Hepes was added into the 2ml media in order to maintain carbon dioxide levels in the media. Cells were imaged using the FITC laser on Biorad, Radiance 2000, dual laser confocal microscope with laser sharp 2000 software. Images were taken over a period of 40 minutes at 10 second intervals. Movies were compiled using Adobe premiere software.

2.2.16.2: Morphological Studies of NWASP⁺ Cells:

Cells were seeded onto imaging dishes at 1 x 10⁴ density in 2mls DMEM and 10% FCS and 1% antibiotic/antimycotic. Cells were incubated overnight at 32°C and 5% CO₂ to adhere to dishes.

cDNA of required constructs were prepared at 50ng/μl in ddH₂O and centrifuged at 14,000rpm for 30 minutes. 3μl of samples were loaded into a microinjection needle. The nuclei of around 100 cells were injected using a pressure of 240hPa with a constant pressure of 56hPa for 0.5 seconds. Cells were imaged over a period of 40 minutes at 10 second intervals using settings as described for N1E-115 cell imaging.
Chapter Three

Results I
3: Results

The family of Rho GTPases are involved in cytoskeletal reorganization events (Aspenstrom, 1999). Both Rac and Cdc42 are known to induce neurite outgrowth, inhibition of these Rho GTPases inhibits this process due to the upregulation of the Rho pathway, which has been shown to be involved in neurite collapse (Hall, 1998; Mueller, 1999; Luo, 2000). The Rho GTPases interact with effector proteins, which can result in a cytoskeletal response. Many of these proteins specific to Rac and Cdc42 have been identified, some of which have been linked to the reorganisation of the actin cytoskeleton (Bishop et al, 2000). The Cdc42 effector IRS-58 induces neurite outgrowth in N1E-115 cells (Govind et al, 2001), similarly N-WASP (Banzai et al, 2000) and the synthetically membrane targeted form of αPak, αPakCAAX (Daniels et al, 1998), have been shown to cause neurite outgrowth in PC12 cells.

The N1E-115 cell line is a clone derived from a C-1300 mouse neuroblastoma. They are adrenergic neurones with a high level of acetylcholine esterase present. This cell line also has a relatively high production rate of tyrosine hydroxylase (the enzyme responsible for the conversion of tyrosine to dihydroxyphenylalanine (DOPA)), which is involved in the noradrenaline synthesis pathway (Amano et al, 1971). This cell line has been used for many morphological studies, as it is relatively well characterised (Kozma et al, 1995, 1997; van Leeuwen et al, 1997; Govind et al, 2001; Hall et al, 2001; Ishii et al, 2001). Neurite formation can be induced by serum deprival. This cell line has the advantage of being easy to maintain and that high levels of transient transfection can be achieved.
The aim of this study was to investigate the role of the three Cdc42 effectors IRS-58, N-WASP and αPakCAAX in the N1E-115 neuroblastoma cell line and their morphological effects in relation to one another; to determine the morphological changes that result from overexpression of these proteins and to determine whether they promote neurite outgrowth, in a synergistic or independent manner.

3.1: IRS-58, N-WASP & Pak CAAX Promote Neurite Outgrowth in N1E-115 Cells:
In order to investigate neurite outgrowth in response to the Cdc42 effectors N-WASP, IRS-58 and PakCAAX the neuroblastoma cell line N1E-115 cells were employed. These cells were seeded onto laminin coated coverslips in the presence of 5% serum containing media, transiently transfected with N-WASP, IRS-58 or αPakCAAX, and then allowed to express for 16 hours.

Cells transfected with N-WASP possessed processes, which were in excess of two cell diameters (fig. 2). These processes could be classified as neurites due to their positive staining with antibodies against neurite markers, such as neurofilaments and p35 (Sarner et al., 2000; Hall et al., 2001). These neurites were generally very thin in appearance possessing filopodia like structures along their length, as well flattened areas along the neurite shaft, referred to as varicosities (see arrow fig. 2A). These cells also displayed relatively well developed growth cones with filopodia like structures present.

The anti HA antibody staining (which shows overexpressed N-WASP distribution) appears to be strongest in the cytoplasm of the cell body, the varicosities of the neurites and the growth cones. Colocalisation studies of N-WASP and F-actin (stained with phalloidin) show that this occurs in the cytoplasm of the cell body and to a much lesser extent in the neurites or extremities of the cell.
Fig. 1: N1E-115 cells transiently transfected with empty pXJ40-HA vector on laminin coated coverslips in the absence of serum (A) and the presence of 5% serum (B) containing media. F-actin stained using TRITC labelled phalloidin. Scale bar equivalent to 40μm.
Fig.2: N1E-115 cells transiently transfected with N-WASP in the PXJ40-HA tagged eucaryotic expression vector on laminin coated coverslips in the presence of 5% serum. Scale bar equivalent to 40µm.
Cells that have been transfected with IRS-58 also induce neurite outgrowth (fig. 3). Their morphologies are however somewhat different to that observed with N-WASP transfected cells (fig. 2) and serum starved control cells (fig. 1). The neurites are thick and possess well developed growth cones. Robust, actin-rich (as shown by phalloidin staining) filopodia-like structures are found on these growth cones (arrow fig. 3A) and along the length of the neurites. The cells generally have multiple neurites with quite extensive branching.

Overexpressed IRS-58 appears to be localised (as shown by anti HA antibody staining) to the cell body, neurites and filopodia-like structures found on the growth cones. Colocalisation studies of IRS-58 with F-actin suggest that the two are localising in the filopodia-like structures found on the growth cones and along the neurites of the cell.
Fig. 3: N1E-115 cells transiently transfected with IRS-58 in the PXJ40-HA eucaryotic expression vector on laminin coated coverslips in the presence of 5% serum containing media. Scale bar equivalent to 40μm.
Transient transfection with αPak does not induce neurite outgrowth. However, if a farnesylation motif (CAAX) is attached to the C-terminus of this molecule, resulting in its localisation at the membrane, neurite outgrowth is observed (Daniels et al, 1998). Following transfection with αPakCAAX on laminin coated coverslips in the presence of 5% serum containing media (fig. 4), N1E-115 cells possess long, thin, smooth and quite highly branched neurites. The neurites don’t appear to have developed growth cones or many filopodia like structures present along their length. As observed in cells transfected with IRS-58, under the same conditions, these cells too appear multipolar. The neurites are of equal diameter along their length and possess no varicosities as those observed in the neurites of N-WASP transfected cells.

Anti HA staining shows that overexpressed αPakCAAX is mostly localised to the cell body and neurite shaft with a lower level of expression at the far extremities of the neurite. Colocalisation of the F-actin and αPakCAAX appears to occur mainly at the periphery of the cell body with only a slight amount in the neurites.
Fig. 4: N1E-115 cells transiently transfected with Pak CAAX in the PXJ40-HA eucaryotic expression vector on laminin coated coverslips in the presence of 5% serum containing media. Scale bar equivalent to 40μm.
Neuroblastoma cells transiently transfected with empty pXJ40-HA vector under the same conditions as those transfected with IRS-58, N-WASP and αPakCAAX, failed to induce neurite outgrowth (fig. 1B). The cells generally appeared rounded and slightly flattened, with few peripheral actin structures. This is indicative that the processes are not formed simply in response to the conditions of transfection, stimulation of the laminin via the integrins or the pXJ40 mammalian expression vector but are a specific result of the over expression of effector proteins IRS-58, N-WASP and αPakCAAX.
To further analyse the ability of the above-mentioned effector molecules to induce neurite outgrowth, number of neurites and branches per neurite were quantitated. The data statistical analyses of these counts are shown in figures 5A and B below, respectively. Neurites were classified as processes if they were greater than or equal to 2 cell diameters. An average of 200 cells were analysed over three separate experiments for each effector. This shows that N-WASP, IRS-58 and αPakCAAX all induce neurite outgrowth significantly ($p<0.01$) above the control cells that have been transfected with the empty pXJ40 mammalian expression vector (see fig. 5A). The average number of neurites per cell however does not differ greatly for each of the effectors or from the control, GFP transfected cells (see fig. 5B). This indicates none of these effectors increases the number of neurites produced per cell.

The formation of more branched neurites by the effectors N-WASP, IRS-58 or PakCAAX in alone were not statistically different to that of control GFP expressing cells (fig. 5B).

Collectively the data suggests that IRS-58, N-WASP and αPakCAAX all induce neurite outgrowth in N1E-115 cells on a laminin matrix in the presence of 5% serum. None of these effectors individually increases the number of neither neurites per cell nor branches per neurite above that of control, GFP expressing cells.
Fig. 5A:  

**Neurite Outgrowth Induced By Effectors**

<table>
<thead>
<tr>
<th>DNA Used In Transfection</th>
<th>% Cells With Neurites</th>
</tr>
</thead>
<tbody>
<tr>
<td>NWASP</td>
<td>*</td>
</tr>
<tr>
<td>IRS58</td>
<td>*</td>
</tr>
<tr>
<td>PakCAAX</td>
<td>*</td>
</tr>
<tr>
<td>Empty Vector</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5B:  

**Morphology of Neurites**

<table>
<thead>
<tr>
<th>DNA Transfected</th>
<th>Average Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-WASP</td>
<td>1.8</td>
</tr>
<tr>
<td>IRS-58</td>
<td>1.6</td>
</tr>
<tr>
<td>PakCAAX</td>
<td>1.4</td>
</tr>
<tr>
<td>GFP</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Fig. 5A + 5B:  Counts of the number of transfected N1E-115 cells with neurites >2 cell diameters. Approximately 200 cells were counted per experiment and the experiment was repeated 3 times. Error bars represent SEM values across 3 experiments.

* p<0.01 level of confidence for cells transfected with N-WASP, IRS-58 or PakCAAX compared to those transfected with empty vector.
3.2: Investigation of Co-operative Effects Between Cdc42 Targets:

The effectors IRS-58, N-WASP and αPakCAAX have been shown in this study (and by others during the course of this project (Banzai et al., 2000; Govind et al., 2001) to induce neurite outgrowth although there are subtle differences in the cell morphology induced. All these proteins are targets of Cdc42 (an inducer of outgrowth) and their mechanism of action or interaction between them in Cdc42-pathways in neurite outgrowth is unknown. Each of the effectors in this study produced an apparent distinctive morphology of the neurites resulting from the overexpression in the N1E-115 cells. IRS-58 cells were generally multipolar in their outgrowth, with robust actin rich filopodia found on developed growth cones and along the neurite shaft (fig. 3) N-WASP overexpression resulted in a somewhat different morphology, the neurites were branched with varicosities along their length with very thin filopodia like structures (fig. 2). Finally, transient transfections of αPakCAAX into N1E-115 cells caused neurites to be produced that were very thin and branched with no developed growth cone and few filopodia-like structures (fig. 4). None of these morphologies are equivalent to the control serum deprived cells (fig. 1A).

To determine if the effectors work synergistically in neurite formation combinations of N-WASP, IRS-58 and αPakCAAX were transiently transfected into N1E-115 cells seeded on a laminin matrix in 5% serum containing media.
Transient transfections of N1E-115 cells (on a laminin matrix in 5% serum containing media) with various dual combinations of the effector molecules N-WASP, IRS-58 and PakCAAX did not result in a synergistic effect on the length of neurites produced. Instead, new varied morphologies were observed as a result of the overexpression of two of these Cdc42 effectors in N1E-115 cells (fig. 6). Dual transfections with IRS-58 and N-WASP resulted in quite thin, long neurites with varicosities along their length (fig. 6B). The neurites also posses filopodia like structures along their length, characteristic of IRS-58 transfected cells but with a low actin content, as observed in cells overexpressing N-WASP. However, the well developed growth cone typical of cells transfected with either IRS-58 or N-WASP is absent from these dually transfected cells.

Overexpression of IRS-58 and αPakCAAX together results in a morphology that could be classified as more characteristic of that of αPakCAAX expressing cells (fig. 6D). The cells have long, thin, highly branched processes, which is typical of αPakCAAX transfected cells (fig. 4) but with small hair like structures along their length. These filopodia-like structures are frequently observed in IRS-58 expressing cells (fig. 3) but are absent from those expressing αPakCAAX. The characteristic developed growth cones observed in IRS-58 overexpressing cells are absent in these dual transfected cells (compare fig. 3 and fig. 6D).

Dendrites characteristically branch at irregular intervals and the neurites are thicker at the cell body, tapering to the ends of the process. Axons, however are much more regular in diameter and branch mainly at the very ends of the process (Jan and Jan, 2001). The sporadic branching and tapering of the processes at their extremities in cells expressing the effectors IRS-58 and αPakCAAX are similar characteristics to those described for dendritic branches (fig. 6D).
N1E-115 cells on a laminin matrix transiently transfected with N-WASP and PakCAAX in the presence of 5% serum containing media have a quite extravagant morphology (fig. 6F). The cells produced a highly branched network of neurites with flattened areas at branching sites. An array of processes around the cell body was observed, giving a "spider web" like appearance. Flattened areas were observed in some processes at which points branches radiated (see arrow fig. 6F). These changes in neurite form are quite different to the varicosities characteristic of the neurites of N-WASP transfected cells, which appear to have a rounded structure and do not form a centre from which branches radiate. This morphology is not typical of cells transfected with either N-WASP or αPakCAAX alone (figs. 6A + E) but is similar to N1E-115 cells expressing Tiam, the Rac exchange factor (van Leeuwen et al., 1997). This observed morphology could be a result of the actions of Pak on its downstream interactor PIX (a GEF for Rac), hence upregulating Rac activity leading to outgrowth (Manser et al., 1998). N-WASP, however, is a Cdc42 effector, which has been linked directly to actin dynamics (Miki et al., 1996). Hence, the morphology of these cells overexpressing N-WASP and PakCAAX could be a result of the activation of both the Cdc42 and Rac pathways simultaneously, producing this complex outgrowth pattern. The cells transfected with PakCAAX and either IRS-58 or N-WASP, posses thin tapered neurites with a very few filopodia like structures, characteristics of the morphology of Pak expressing cells. The morphology observed in the dually transfected cells, with PakCAAX, is suggestive that Pak is acting as the predominant effector controlling morphology.
Fig. 6: N1E-115 cells transfected with combinations of Rho GTPase effector molecules all known to induce neurite outgrowth and produce varying morphologies.
Statistical analyses of the morphological effects of dual transfection of the neurite causing effectors N-WASP, IRS-58 and αPakCAAX were carried out. The percentage of neurite bearing cells was not significantly increased by the combinations of the effectors (fig. 7A), however the degree of complexity was somewhat altered. The αPakCAAX and N-WASP expressing cells produced the most complex outgrowth by having the greatest number of neurites per cell and the largest extent of branching per neurite. Cells dually expressing N-WASP with αPakCAAX have significantly more highly branched neurites than IRS-58 alone (p = 0.04) or IRS-58 in combination with N-WASP (p = 0.03) or control GFP expressing cells (p = 0.02). However, this combination does not produce more complex neurites than cells expressing either N-WASP or αPakCAAX alone. The combination of IRS-58 and αPakCAAX or N-WASP and IRS-58, does not result in neurites of high complexity, suggesting that such level of complexity requires the actions of both the effectors N-WASP and αPakCAAX. Each of these effectors may be acting downstream of Cdc42 and Rac respectively thereby activating two independent pathways known to affect the actin cytoskeleton (Kozma et al., 1997) and possibly the complexity of outgrowth. Alternatively, N-WASP may be located downstream of PakCAAX and over-expression of both effectors results in an additive effect leading to the morphology observed.
Figure 7A:

Neurite Outgrowth Induced By Effectors

Figure 7B:

Neurite Morphologies

Fig. 7A + 7B: Counts of the number of transfected N1E-115 cells with neurites >2 cell diameters. Approximately 200 cells were counted per experiment and the experiment was repeated 3 times. Error bars represent SEM values across 3 experiments.

* p<0.05 level of confidence for N-WASP + PakCAAX combination compared to IRS-58, IRS-58 + N-WASP and GFP transfected cells.
3.3: PIX and GIT1 Overexpression Block Induction of Neurite Outgrowth by N-WASP and IRS-58:

The Pak-interacting exchange factor (PIX) is a Rac GTP exchange factor that interacts with Pak via residues 182-203 (the third atypical SH3 binding region) (Manser et al., 1998). PIX specifically binds to Pak via an SH3 interaction, originally identified as a focal complex targeting sequence (Manser et al., 1997). PIX is also an interactor of the G-protein-receptor kinase-interacting protein (GIT1). It is thought to be the interaction of Cdc42 with the Pak/PIX complex that drives the association of GIT1 with focal complexes in a C-terminal paxillin binding domain dependent manner. This results in the dissociation of paxillin from the focal complex, leading to their disassembly and cell motility (Zhou et al., 2000).

The involvement of PIX and GIT1 in focal complex turnover and cell motility had been demonstrated (Zhou et al., 2000). To investigate the effect of PIX or GIT1 in these Cdc42 effector pathways and in the morphology generated by αPakCAAX they were overexpressed in combination with N-WASP, IRS-58 or αPakCAAX.
Results I

Transfection of the N1E-115 cells with either of the PIX or GIT1 constructs, alone or in combination resulted in large amounts of cell death if expression time following replacement of serum was 16-20 hours. Therefore, the expression time was reduced to 7 hours before the cells were fixed and stained.

Co-transfections of the N1E-115 cells on a laminin matrix in the presence of 5% serum containing media with either IRS-58, N-WASP or αPakCAAX in combination with either PIX or GIT1 resulted in cells producing very few neurites, but displayed extensive peripheral actin microspike structures (fig. 8). Transfection with either PIX or GIT1 alone or in combination under the same conditions does not induce neurite outgrowth (fig. 8). Generally, the cells were much more flattened and possessed many filopodia-like structures. Cells transiently transfected with either PIX or GIT1 produce a characteristic motile morphology; a leading edge with a large number of filopodia like structures and a trailing edge with very few such structures (Wadsworth, 1999).
Fig. 8: N1E-115 cells transiently transfected with combinations of Rho GTPase effector molecules and interactors. Scale bars equivalent to 40μm.
The effect of transfection of N1E-115 cells with PIX or GIT1 in combination with IRS-58, N-WASP and αPakCAAX on neurite outgrowth were quantified (fig. 9).

Figure 9:

**Effects of PIX and GIT1 on Neurite Inducing Effectors**

![Graph showing the effects of PIX and GIT1 on neurite inducing effectors.](image)

Fig.9: Counts of the number of transfected N1E-115 cells with neurites > 2 cell diameters. Approximately 200 cells were counted per experiment and the experiment was repeated 3 times. Error bars represent SEM values across 3 experiments.

* p < 0.05 for N-WASP and IRS-58 expressing cells compared to cells expressing these effectors in combination with either PIX or GIT1.
Statistical analysis shows that dual transfection of N1E-115 cells with PIX or with GIT1 together with either N-WASP, IRS-58 or αPakCAAX does not increase the number of neurite bearing cells. Conversely, a reduction of the percentage of cells possessing neurites is observed (fig. 9). This reduction appears to be greatest in dual transfections with IRS-58. Due to the toxicity of the PIX and GIT1 constructs in N1E-115 cells the expression time in 5% serum containing media following transient transfections was reduced to 7 hours. To determine the effects this reduction of expression time of the effectors IRS-58, N-WASP and αPakCAAX had on the resultant neurite outgrowth these effectors were also expressed for only 7 hours. Reduction of the expression time to 7 hours for αPakCAAX transfected cells resulted in a reduction in the number of neurite bearing cells to half that observed following 16 hours expression (compare figs .5A and 9). Dual transfections with either PIX or GIT1 in combination with N-WASP or IRS-58 results in neurite outgrowth being reduced significantly (p < 0.05) compared to transfections with N-WASP or IRS-58 alone under the same conditions. However, PIX and GIT1 do not appear to affect neurite outgrowth in cells dually transfected with αPakCAAX.

Together this data suggests that for neurite outgrowth to occur, cell motility must be inhibited. Increases levels of effectors known to induce cell motility, such as GIT1, act to increase focal complex turnover (Zhao et al., 2000), and thereby increase cell motility and inhibit neurite formation.
3.4: Summary:

Overexpression of the Cdc42 effector molecules N-WASP, IRS-58 and the membrane-targeted αPak induce neurite outgrowth in N1E-115 cells when seeded on a laminin matrix in the presence of 5% serum. Combinations of these effector molecules did not result in a synergistic effect with respect to neurite outgrowth, although different morphologies were observed. The most striking being that of the N-WASP and αPakCAAX combination, in which an intricate mesh work of branching and flattening is seen. Both N-WASP, and to a lesser extent IRS-58 generate branches in combination with αPakCAAX, whereas the combination of IRS-58 and N-WASP results in a far lower complexity of neurite morphology being observed.

Cotransfections with PIX or GIT1 with either N-WASP or IRS-58 resulted in a significant reduction of neurite outgrowth compared to the level seen produced by IRS-58 or N-WASP alone. These cells possessed a far more flattened morphology with fewer neurites. This is potentially due to PIX (Rac GEF) upregulating Rac activity (which characteristically causes cell spreading/flattening (Kozma et al., 1997)) and its potential mediation of cross talk between Rac1 and Cdc42 (Ridley et al., 1992; Kozma et al., 1995; Manser et al., 1998).

A reduction in αPakCAAX induced neurite outgrowth following 7 hours expression compared to 16 hours was observed. Coexpression of either PIX or GIT1 with αPakCAAX did not further reduce significantly the number of neurite bearing cells.
Chapter Four

Results II
4: Results II:

4.1: Investigation of Regions of N-WASP Responsible for Neuritogenesis:

The Cdc42 effector molecule NWASP consists of 9 domains. The C-terminal region of this protein consists of two verprolin homology domains (VH1 and VH2), a cofilin homology domain (Cof) and an acidic region (A) (see fig. 1.7.1.1) (Miki et al., 1998). The cofilin homology domain has been identified as being part of the region of interaction with the Arp2/3 complex (Rohatgi et al., 1999). Collectively these four domains are known as the VCA region and during the course of this project many studies investigated the function of this region in terms of how N-WASP plays a role in the actin dynamics of the Arp2/3 complex (Miki et al., 1998; Rohatgi et al., 1999; Zalevsky et al., 2001; Weaver et al., 2002). Actin filaments bind to the verprolin homology regions of N-WASP bringing them in close proximity to the Arp2/3 complex, which binds to the acidic region (Miki and Takenawa, 1998). In an effort to examine further, the role of N-WASP in actin dynamics of neurite outgrowth, the VH1 and VH2 domains were separately cloned into the mammalian expression vector pXJ40-FLAG (see materials and methods 2.2.1.1).

Transient transfections of sections of N-WASP; the N-terminal half of N-WASP (NN-WASP(1-621)), and the C-terminal sections, VH1(1196-1252), VH2(1284-1334) and VH1-Cof(1196-1453) domain were undertaken in N1E-115 cells seeded onto a laminin matrix in low serum conditions (1%). Such conditions induce neurite formation in N1E-115 cells; this neurite formation was inhibited to varying degrees by these VH constructs (fig. 10), the most significant inhibition was observed by the VH2 domain construct. These
experiments showed that the C-terminal, Arp2/3 complex/actin interactive region is required to promote neurite outgrowth.

Figure 10:

**Figure 10:** Percentage of transfected N1E-115 cells with neurites in 1%FCS

Approximately 200 cells were counted per coverslip and experiment was repeated three times. Error bars represent SEM values across these experiments.

* p = 0.04 level of confidence compared to serum starved control cells.

** p = 0.003 level of confidence compared to the verprolin homology 2 domain.

# p = 0.03 level of confidence compared to full length N-WASP
Overexpression of the C-terminal N-WASP constructs VH1, VH2 and VH1-Cof generated filopodia with a very different morphology to those produced by full length N-WASP. The filopodia produced by these C-terminal constructs appear much more robust and stain more intensely for F-actin compared to N-WASP transfected cells under the same conditions (fig. 11). Overexpressed N-WASP is located predominantly in the cytosol and in varicosities along the neurite shaft (as shown by antibody staining) (see figure 1, section 3.1, results I). The N-terminal truncation of N-WASP (N-NWASP1-621), which has the poly-proline and VCA region deleted, displays overall morphology similar to that produced by the full length protein. However, the number of filopodia like structures is reduced, and no developed growth cones were observed in N1E-115 cells, overexpressing the N-NWASP construct. The number of neurite bearing cells overexpressing this construct is also significantly reduced (p = 0.03). This suggests, the C-terminal region of N-WASP could be important for its ability to induce neurite outgrowth and filopodia like structures. Cells were plated on a laminin matrix in the presence of 1% serum containing media (compare figs. 11A and 11C).

In low serum conditions, which induces neurite outgrowth in N1E-115 cells, overexpression of the VH2 domain inhibited outgrowth to the greatest extent compared to VH1, VH1-Cof domain and N-terminal N-WASP constructs. For this reason the VH2 domain construct and its negative effects on neurite outgrowth was the focus of further investigation.
Fig. 11: N1E-115 cells on a laminin matrix, transiently transfected in presence of 1% serum with the N-terminal construct N-NWASP with a GST tag, N-WASP with a HA tag and C-terminal constructs of N-WASP VH1, VH2 and VH1-Cof with a FLAG tag in the pXJ40 mammalian expression vector. Scale bar equivalent to 20μm.
4.2: VH2 Domain Blocks Outgrowth Induced by Other Effectors:

The verprolin homology domains of N-WASP have been shown to directly bind F-actin (Miki and Takenawa, 1998). In its inactive state N-WASP forms a closed conformation in which the cofilin homology or central domain interacts with the GTPase binding domain (GBD) (Abdul-Manan et al., 1999; Kim et al., 2000). Upon binding of Cdc42 to the GBD and PIP$_2$ to the polyproline region, N-WASP becomes activated and the interaction between the cof domain and the GBD is lost, resulting in a conformational change in N-WASP. This change in conformation unmasks the VCA region of the protein allowing the interaction with the Arp2/3 complex and actin to occur (Higgs et al., 2000; Kim et al., 2000).

The GBD or Cdc42/Rac interactive binding (CRIB) motif has a minimal 16 residue consensus sequence (ISxPxxxxFxHxxHVG), which is present in most Cdc42/Rac effector molecules (Burbelo et al., 1995). It is this motif with which the cof domain interacts intramolecularly within full length N-WASP forming the autoinhibited structure (Abdul-Manan et al., 1999; Kim et al., 2000). The VH domains of WASP family proteins have a highly conserved motif (LLxxIxxGxxL) (Miki and Takenawa, 1998). Interestingly, N-WASP unlike other WASP family proteins has two VH domains to which filamentous actin has been shown to interact. This has the resultant effect of activating the Arp2/3 to a greater extent than that of the other WASP family members (Yamaguchi et al., 2002). To determine the biological function of the VH2 domain on outgrowth induced by N-WASP and other Cdc42 effectors, dual transient transfections using the VH2 domain and the Cdc42 effectors IRS58, N-WASP and $\alpha$PakCAAX were carried out in N1E-115 cells seeded on laminin in the presence of 5% serum.
Cells co-transfected with the VH2 domain and either IRS-58, N-WASP or αPakCAAX in the presence of 5% serum showed not only a significant reduction (p < 0.01) in the number of neurite bearing cells compared to controls (IRS-58, N-WASP, αPakCAAX) (fig. 12) but also changes in morphology. The cells appeared much more flattened and produced many peripheral microspikes (fig. 13). The flattened morphology is characteristic of Rac activation, however the peripheral microspikes are typical of Cdc42 activity (Kozma et al., 1996). N-WASP directly interacts with the Arp2/3 complex, implicated in actin dynamics (Rohatgi et al., 1999) and is therefore likely to be located downstream of other Cdc42 effectors such as IRS-58. The VH2 domain has been shown to interact directly with G-actin, and increase the rate of actin depolymerisation.

Figure 12:
**Figure 12:**

**Percentage of Neurite Bearing N1E-115 Cells Following Transfection**

![Percentage of Neurite Bearing N1E-115 Cells Following Transfection](image)

Fig. 12: Counts of the number of transfected N1E-115 cells with neurites >2 cell diameters. Approximately 200 cells were counted per experiment and the experiment was repeated 3 times. Error bars represent SEM values across 3 experiments.

* $p < 0.05$ level of confidence compared to Cdc42 effector alone.
N1E-115 cells overexpressing both N-WASP and the VH2 domain together produced filopodia which appeared much more robust and actin rich in structure than those transfected with N-WASP alone under the same conditions. These cells also possessed some membrane ruffles (compare fig. 2, 3.1.1 and fig. 13A), suggestive of Rac activation (Kozma et al., 1997).

Co-transfection of either IRS-58 or αPakCAAX with the VH2 domain resulted in N1E-115 cells possessing a flattened morphology with many peripheral, actin rich microspikes (fig. 13). The αPakCAAX/VH2 domain co-transfected cells also displayed greater membrane ruffling, suggesting increased Rac activity in these cells.

The overexpression of full length N-WASP in N1E-115 cells on a laminin matrix in the presence of 5% serum is shown to be predominantly cytoplasmic with extremely low levels in the peripheral structures (fig. 2B, 3.1.1). When N-WASP is coexpressed with the VH2 domain, which localises to the actin rich periphery (fig. 13A’’), staining for N-WASP suggests there is a shift in its the localisation to the periphery (fig. 13A’). It is in these peripheral structures that these two proteins appear to colocalise (fig. 13A’’’).

Coexpression of either IRS-58 or αPakCAAX with the VH2 domain also colocalise in peripheral structures of N1E-115 cells. However, unlike N-WASP, overexpressed IRS-58 and αPakCAAX themselves localize to peripheral structures in N1E-115 cells (figs. 3B and 4B respectively) and the VH2 domain does not appear to affect this distribution.
Fig. 13: N1E-115 cells seeded onto laminin coated coverslips transiently transfected with the VH2 domain and the neurite inducing effectors N-WASP, IRS-58 and PakCAAX. These cDNAs are cloned into the pXJ40-HA vector and stained with a TRITC labelled secondary antibody, the VH2 domain construct is in the pXJ40-FLAG vector and stained with the secondary FITC antibody (see methods). Cells were allowed to express for 16 hours in 5% FCS containing media.
4.3: Change in Localisation of N-WASP:

N-WASP has a pleckstrin homology (PH) domain at its N-terminus. This domain is believed to act as a targeting mechanism of the protein to the leading edge (Saraste et al., 1995; Imai et al., 1999; Lemmon et al., 2002). When the protein is in its inactive state it sustains a closed conformation in which the cofilin homology (CH) domain interacts with the GTPase binding domain of the protein, until Cdc42 and PIP2 bind and cause a conformational change to occur unmasking the VH and acidic regions of the protein previously hidden (Higgs et al., 2000; Kim et al., 2000).

Transfection of N-WASP into N1E-115 cells on a laminin matrix in the presence of serum shows it to be expressed primarily in the cytoplasm of the cell and in varicosities along the neurite shaft, with barely detectable levels in filopodia (fig. 2B, 3.1). Cotransfection of the VH2 domain with N-WASP under the same conditions results in a shift in N-WASP expression to the periphery and in the actin rich microspikes (fig. 14).
Fig. 14: N1E-115 cells seeded onto laminin coated coverslips transfected with N-WASP (A-A'') alone or with VH2 domain (B-B'') in the presence of 5% FCS containing media. Images C-C'' and D-D'' are cells stained for endogenous NWASP in low serum (1% FCS) conditions alone and with the overexpression of the VH2 domain respectively.
Coexpression of the VH2 domain construct with N-WASP results in a change in the localisation of the N-WASP expression. A much higher expression level of N-WASP is observed in the peripheral and filopodia like structures (arrow in fig. 14B’) in the presence of the VH2 domain. The filopodia like structures possess a more robust, actin rich and definite appearance compared to those observed for N1E-115 cells transfected with only N-WASP under the same conditions (compare figs. 14A and B).

The change in N-WASP localisation is not only observed for overexpressed protein but also for endogenous N-WASP found in N1E-115 cells. N1E-115 cells seeded onto a laminin matrix and caused to differentiate by the removal of serum for 16 hours were fixed and stained (as described in 2.2.14.10) using anti N-WASP antibodies. Endogenous N-WASP too is predominantly expressed in the cytosol with very low levels observed in the filopodia and periphery (fig. 14C’). Overexpression of the VH2 domain, in cells treated in the same manner results in a shift of the endogenous N-WASP expression to the periphery and filopodia of the cells (fig. 14D”). This shift in endogenous N-WASP mirrors that seen with the overexpressed N-WASP in the presence of the VH2 domain, indicating that the observed change in localisation of N-WASP was not simply due to overexpression.
Figure 15:

![Image of gel](#)

Fig. 15: Localisation of VH2 domain expression in COS7 transfected cells. Lane: 1) VH1 soluble fraction; 2) VH1 cytoskeletal fraction; 3) VH2 soluble fraction; 4) VH2 cytoskeletal fraction; 5) GST soluble fraction; 6) GST cytoskeletal fraction. Solubilised in 1% triton-x-100 containing buffer (see methods section 2.2.12.1).

The subcellular distribution was also investigated by cell fractionation. The VH2 domain expression detected by GST-tag antibody was shown to be solely in the cytoskeletal fraction of the cell (1% Triton-x-100 insoluble fraction). Cell staining of N1E-115 cells transfected with the VH2 domain shows the protein to be localised to the peripheral regions of the cell. The VH domains are part of the VCA region of N-WASP, which have been shown to be the region of interaction with/severing of actin and required for activation of the Arp2/3 complex (Miki and Takenawa, 1998; Yamaguchi et al., 2002). The VH2 domain could therefore be interacting with the triton insoluble actin cytoskeleton, an association, which agrees with its localisation in N1E-115 cell staining.
4.4: N-WASP Antibody Blocks Neurite Outgrowth:

Both N-WASP overexpression and serum reduction induces neurite outgrowth in N1E-115 neuroblastoma cells. N-WASP has been shown to interact with the Arp2/3 complex, which results in actin turnover (Miki et al., 1998; Rohatgi et al., 1999), suggesting N-WASP plays an important role in the actin dynamics of a cell. It was shown, during the course of this project, to be involved in neurite outgrowth in PC12 cells (Banzai et al., 2000) and here in N1E-115 cells. These results suggest, N-WASP could possibly be an extreme downstream effector to which other effectors signal resulting in its interaction with the Arp2/3 complex and actin turnover in the cell.

To further investigate whether or not N-WASP is required for neurite outgrowth, specific antibodies directed against N-WASP were delivered into N1E-115 cells, by use of the passive protein delivery system, bioporter.
Figure 16: Cells transfected with antibodies against N-WASP. Cell counts of number of cells with neurites > 2 cell diameters. Approximately 200 cells counted per experiment and the experiment was repeated three times. Error bars represent SEM values across 3 experiments.

* p = 0.04 level of confidence compared to bioporter + IgG FITC control cells.
N1E-115 cells seeded onto a laminin matrix in the absence of serum were treated with the bioporter agent to deliver both anti N-WASP antibodies, N15 and D15 (Santa Cruz), which are raised against the N-terminus and central regions of N-WASP, respectively. These cells displayed a significant reduction of neurite outgrowth compared to that of cells simply treated with the bioporter system and the secondary IgG antibody only. To check that the reduction in neurite outgrowth observed was due to an effect caused by the antibodies and not simply an artefact of the assay, cells were transfected with an anti actin antibody (Sigma). This resulted in the complete blockade of neurite outgrowth and actin structures of the cells were also severely compromised. The cells appeared to have no F-actin structures, which could be detected by phalloidin staining of the fixed cells. This suggests, the antibodies delivered are indeed biologically active and targeting specific proteins. The bioporter delivery system itself, as well as the secondary IgG antibody, appeared to have no significant effects on the reduction of neurite outgrowth produced by N1E-115 cells on a laminin matrix in low serum conditions (fig. 16).
4.5: Summary:

The C-terminus region of N-WASP has three C-terminal domains (VCA) previously shown to be involved in interaction with the Arp2/3 complex (Miki et al., 1998; Rohatgi et al., 1999), of which the VH (verprolin homology) domains bind directly to F-actin. Overexpression of the VH2 domain (a 19 amino acid sequence) acts to inhibit neurite outgrowth induced by low serum conditions and by the effectors IRS-58, αPakCAAX and N-WASP itself in N1E-115 cells. The VH2 domain also affects the morphology of the filopodia produced by cells, making them more robust and actin rich. This domain is localised to the membrane/cytoskeletal fraction of the cell and its overexpression results in an increase in the levels of N-WASP in the particulate fraction probably due to increased interactions with cytoskeletal actin. Delivery of N-WASP antibodies into N1E-115 cells in the absence of serum, inhibits neurite outgrowth, which suggests, N-WASP plays a key role in the actin dynamics required for this process. These results are consistent with N-WASP being involved downstream of other effectors in neurite dynamics.
Chapter Five

Results III
5: Results III:

5.1: Actin Dynamics and Filopodia Formation: Cdc42 Effectors in Live Cells:

The effector IRS-58 has also been shown to be involved in actin dynamics of a cell (Govind et al., 2001; Miki et al., 1996; Bi et al., 1999; Rohatgi et al., 1999). Whereas N-WASP interacts directly with the Arp2/3 complex via its VCA region at the C-terminus of the protein (Miki et al., 1998; Rohatgi et al., 1999), IRS-58 involvement in actin dynamics is via its association with Mena (Krugmann et al., 2001) and promotes the formation of filopodia. It is however, still under debate as to whether N-WASP is essential for the formation of filopodia or indeed whether it plays a central role in their formation (Snapper et al., 2001; Lommel et al., 2001).

Filopodia are actin based structures that are produced at the periphery of a cell and are often found in high numbers at the leading edge or at the growth cone. These actin rich, finger like projections are believed to be sensory devices for the environment of the moving cell or growing neurite. Neurites bearing growth cones and filopodia have been shown to be able to sense the environment and move towards a chemoattractant (Zheng et al., 1996). The formation and dynamics of these peripheral structures is therefore important to guidance decisions made by a growing neurite.

To understand the role of N-WASP and IRS-58 in filopodia formation and to try and determine whether N-WASP is essential in their formation, a series of time-lapse fluorescence imaging studies were undertaken. A construct encoding GFP labelled actin was transiently transfected into live cells, in combination with the effector construct of interest. This construct encoding fluorescently labelled actin made it possible to visualise the filamentous actin in live cells and observe their morphological changes in real time.
5.2: Morphology of Filopodia in N1E-115 Cells in Real Time:

N1E-115 cells on a laminin matrix in the presence of 5% serum transfected with N-WASP or IRS-58 and the GFP-actin were observed in real time for 40 minutes, 16 hours post transfection. These cells displayed neurite outgrowth and produced filopodia, which were morphologically very different from one another. Cells transfected with IRS-58 and subsequently fixed and stained, possessed filopodia like structures that appeared very robust and actin rich, with colocalisation of IRS-58 and actin in these peripheral protrusions (fig. 3, 3.1). N-WASP transfected cells, however, produced filopodia like structures that are very fine, have a low actin content (as shown by phalloidin staining) and seem to be of a far less robust structure than those observed for IRS-58. Colocalisation between N-WASP and F-actin in these peripheral structures was limited and not as clearly defined as seen with IRS-58 (fig. 2, 3.1).

To determine how the dynamics of filopodia production related to these morphological differences/differences in staining in IRS-58 and N-WASP expressing cells, fluorescent live imaging studies were undertaken using the GFP labelled actin construct. The dynamics of the filopodia like structures produced and their stability was investigated to determine whether the IRS-58 filopodia truly are more stable than the N-WASP structures or is it that they are simply more actin rich?

Cells were observed using fluorescence time-lapse imaging by transfection of a GFP-actin construct plus the construct of interest into N1E-115 cells growing on laminin. These cells were observed over 40 minutes at 10 second intervals 16 hours post transfection either in the presence of serum for IRS-58, N-WASP, Cdc42V12 and N-
WASP VH2 domain plus GFP-actin transfected cells, or absence of serum for control cells (GFP-actin only).

Cells transfected with solely the GFP-actin construct were serum deprived to induce neurite outgrowth so as to make them comparable to those cells transfected with neurite inducing effectors in this assay. These cells display the normal characteristics of serum deprived N1E-115 cells growing on a laminin matrix (fig. 1A, 3.1). The filopodia produced by these cells are quite robust and fluoresce strongly, suggestive of high actin content in these peripheral structures (fig. 17).

(See CD, Folder 1: N1E-115 GFP actin file for movie)
Fig. 17: Frames of fluorescence time lapse imaging of N1E-115 cells transiently transfected with the pXJ40 actin-GFP construct alone. Frames were taken every 10 seconds over a 40 minute time period.
Neuroblastoma cells on a laminin matrix, overexpressing IRS-58 and the GFP-actin construct in the presence of 5% serum were observed using fluorescent time-lapse imaging 16 hours post transfection (fig. 18). These cells produced pronounced filopodia like structures, which display a very high fluorescence, indicative of high actin content (red arrow, 10 seconds, fig. 18). They also appear to be more robust and longer in structure, both of these features are different to those observed in the control cells (compare figs. 17 & 18). These cells displayed no measurable motility in the 40 minute time frame in which they were observed (see CD, Folder 1: N1E-115 IRS-58 for video).
Fig. 18: Frames of fluorescence time lapse imaging of N1E-115 cells transiently transfected with the pXJ40 actin-GFP and IRS-58 constructs. Frames were taken every 10 seconds over a 40 minute time period.
Transfection with GFP-actin and N-WASP under the same conditions results in the formation of filopodia like structures that weakly fluoresced, which is suggestive of low actin content (red arrow, 10 seconds, fig.19). These cells have filopodia like structures with morphology comparable to fixed transfected cells (fig. 2, 3.1). These filopodia like structures are very dynamic in nature and extend and retract from the cell at a rapid rate. These cells, also have no measurable level of motility, but in contrast to the IRS-58 expressing cells the peripheral projections are quite dynamic (see CD, Folder 1: N1E-115 N-WASP for video).
Fig. 19: Frames of fluorescence time lapse imaging of N1E-115 cells transiently transfected with the pXJ40 actin-GFP and N-WASP constructs. Frames were taken every 10 seconds over a 40 minute time period.
Cdc42V12 and GFP-actin expressing cells possessed characteristic lamellae with rib like actin structures (red arrow, 10 seconds, fig. 20). The lamella retracted and the rib like structures protruded to form microspikes (blue arrow, 10 mins 10 secs, fig. 21). These cells appeared to reduce in size and collapse at points of contact with other cells. The microspikes produced by these cells fluoresced quite strongly, suggestive of a high actin content and did not appear to retract and reform as rapidly as those peripheral filopodia like structures produced by the cells transfected with N-WASP (see CD, Folder 1: N1E-115 Cdc42V12 for video).
Fig. 20: Frames of fluorescence time lapse imaging of N1E-115 cells transiently transfected with the pXJ40 actin-GFP and Cdc42V12 constructs. Frames were taken every 10 seconds over a 40 minute time period.
The VH2 domain of N-WASP co-transfected with GFP-actin into N1E-115 cells produces a phenotype different to those observed with cells overexpressing N-WASP itself, IRS-58 or the constitutively active GTPase Cdc42V12. The cells have robust, highly fluorescent filopodia like structures (see green arrow, 10 seconds, fig. 21), suggestive of high actin content. These cells formed a leading edge in which a high actin density was observed (green arrow, 10 mins 10 secs, fig. 21) and a trailing edge (yellow arrow) which displayed lower actin content (based upon levels of fluorescence observed). The cells progressed forwards from the leading edge across the field of view over the 40 minute time period of observation. Cells overexpressing the VH2 domain of N-WASP appeared to be motile unlike the other proteins used in this assay. The distribution and formation of a leading and trailing edge was also a morphology not previously observed (see CD, Folder 1: N1E-115 VH2 domain for video). This data suggests that the VH2 domain could be responsible for causing a change in the rate of actin and focal complex turnover in the N1E-115 cells resulting in the increased motility of the cells, as seen with PIX and GIT1 complex formation with αPak (Zhou et al., 2000). The function of this peptide is possibly via the binding of monomeric G-actin, causing a shift in the equilibrium of G-actin to F-actin in the cell leading to increased rate of actin dynamics to result.
Fig. 21: Frames of fluorescence time lapse imaging of N1E-115 cells transiently transfected with the pXJ40 actin-GFP and VH2 domain constructs. Frames were taken every 10 seconds over a 40 minute time period.
5.3: Measuring Filopodia Dynamics in N1E-115 Cells:

Actin is polymerised and depolymerised in a manner controlled by effectors and the Arp2/3 complex (Welch, 1999; Hollien, 2002) dependent on the requirements of the cell. Filopodia are believed to be the sensory devices of a growing neurite and the mechanism of growth cone guidance. It is thought that these peripheral structures sense the surrounding environment and hence determine whether the neurite continues to follow that current trajectory or change direction (Zheng et al., 1996). Therefore their turnover is an important determinant on the decision making by the cell for its direction of growth.

Comparisons of the dynamics of the filopodia produced by overexpression of the various proteins were carried out. The half-life of the filopodia were measured by observing filopodia at time zero (assuming this as the point of formation) and determining how long before they retracted. This was repeated for each protein and compared to those of cells overexpressing the GFP-actin construct alone. This gave a relative half-life of the filopodia for each of the proteins making it possible to compare the proteins used in the assay (fig. 22).
Fig. 22: Dynamics of filopodia of transfected cells in real time. For each construct the time of existence of 40 filopodia across 4 experiments were measured. Time frame zero was taken as the beginning of the filopodia existence. Error bars represent the SEM values for this experiment.

* $p = 0.04$ level of confidence for rate of filopodia turnover in VH2 domain transfected cells compared to GFP-actin control cells.

** $p < 0.001$ level of confidence for rate of filopodia turnover in N-WASP transfected cells compared to GFP-actin control and IRS-58 expressing cells.
Assessment of the filopodia like structures of N1E-115 cells transfected with N-WASP revealed that they retracted in a much shorter period of time compared to the control cells transfected solely with GFP-actin (fig. 22). The IRS-58 overexpressing cells, did not have a statistically significant increased relative half life compared to that of control cells, but were vastly more stable than those of N-WASP transfected cells. The relative half-life of control cell filopodia was more than five times that of the N-WASP transfected cells (fig. 22).

Cells transfected with the VH2 domain of N-WASP showed a filopodia turnover, which was greater than that of control cells but far lower than that of N-WASP cells (fig. 22).

In cells overexpressing N-WASP, there was a very low actin content in its filopodia like structures, which are very dynamic and unstable. In comparison, IRS-58 displayed a very high actin content in its filopodia like structures, which did not differ in their stability from control cells. The stability and apparent actin content of the filopodia of IRS-58 expressing cells and the GFP-actin (control) expressing cells, were not dissimilar from one another. Collectively this data suggests a correlation between the actin content of the filopodia produced and their relative stability.
5.4: Summary:
The effectors IRS-58 and N-WASP both generate filopodia like structures in neuroblastoma cells, as does the GTPase Cdc42V12. The morphologies of these filopodia like structures produced are very different both from each other and the control, serum deprived cells. IRS-58 overexpression in N1E-115 cells causes the production of actin rich filopodia with a relative half-life equivalent to those of control cells. However, N-WASP overexpressing cells produce filopodia like structures with a very rapid turnover rate and low actin content. Cells which have been transfected with the VH2 domain of N-WASP results in cells possessing a defined leading and trailing edge, at which there is a rich and reduced actin content respectively.
Chapter Six

Results IV
6: Results IV:

6.1: Role of N-WASP in Filopodia Formation:

Miki and co-workers have shown that N-WASP plays a crucial role in filopodia formation (Miki et al., 1998), however it has been shown by other groups that N-WASP is dispensable for this process (Snapper et al., 2001; Lommel et al., 2001). To attempt to understand whether N-WASP is required for filopodia formation, a fibroblast cell line generated from transgenic N-WASP null mice (courtesy of Ralf Kuhn) were used in fluorescent live imaging studies. These N-WASP knockout cells do not express the N-WASP protein, but have been shown to still produce filopodia under certain conditions (Lommel et al., 2001) allowing the study of whether other effectors such as IRS-58, which promotes filopodia formation, requires N-WASP or not in this process.

The knockout cells (NWASP^−/−) were microinjected with dominant negative Rac (Rac^N17), wild type Cdc42 and GFP-actin and were then observed under fluorescence in real time. The Rho GTPases display a hierarchal signalling system of regulation (Nobes and Hall, 1995). Cdc42 signals downstream to Rac, which morphologically causes the formation of filopodia over which the lamellipodia extend. Using the combination of RacN17 and wild type Cdc42 should result in the activation of the Cdc42 pathway and hence the formation of filopodia. If the formation of filopodia is dependent solely on N-WASP, and no other effectors can facilitate this process no filopodia should be observed in the knockout cells. The control cells for this cell line (NWASP^floxfloxfloxs), which do express NWASP, were compared.
Overexpression of dominant negative Rac with wild type Cdc42 have been previously shown to form filopodia/microspikes in fibroblast cells (Kozma et al., 1995; Lommel et al., 2001). However, the microinjection of both RacN17 and Cdc42 into the NWASP<sup>−/−</sup> cells resulted primarily in the formation of lamellipodia (fig. 23, red arrow, 10 mins 10 secs frame) with limited filopodia formation observed (fig. 23, yellow arrow, 10 second frame). In the NWASP<sup>flx/flx</sup> control cells, filopodia formation was observed as in fibroblastic cell lines (fig. 24). This data illustrates that the removal of N-WASP from this signalling pathway results in a reduction in filopodia formation and an increase in lamellipodia (see CD, Folder 2: NWASP<sup>−/−</sup> cells transfected with RacN17 and Cdc42/ control cells transfected with RacN17 and Cdc42 for videos). The observation of membrane ruffles in place of filopodia in the NWASP<sup>−/−</sup> cells is somewhat controversial. The conclusions made by Lommel et al, was that the NWASP defective cells are still able to produce filopodia when microinjected with Cdc42V12 (Lommel et al., 2001). However, the images of the NWASP<sup>−/−</sup> cells microinjected with Cdc42V12 appear to display a reduction in the number of filopodia compared to control cells, as well as the formation of membrane ruffles, which are not seen in the comparative control cells. Cdc42, which signals to its downstream effector N-WASP, is a known activator of the Arp2/3 complex and is believed to be the mediator by which Cdc42 effects the actin dynamics of the cell (Rohatgi et al., 1999). These results therefore suggest that N-WASP plays a key role in the formation of filopodia in fibroblastic cells as well as in N1E-115 neuroblastoma cells.
Fig. 23: NWASP^{del/del} cells microinjected with RacN17, Cdc42 and GFP-actin. Cells were imaged approximately 4 hours after injection over a period of 40 mins with a frame every 10 seconds.
Fig. 24: NWASP<sup>flox/flox</sup> cells microinjected with RacN17, Cdc42 and GFP-actin. Cells were imaged approximately 4 hours after injection over a period of 40 mins with a frame every 10 seconds.
6.2: Co-operation between IRS-58 and N-WASP in Filopodia Formation:

IRS-58 overexpression has been shown to induce the formation of robust, actin rich filopodia like structures in N1E-115 cells on a laminin matrix in the presence of serum (Govind et al., 2001). Interactions between IRS-58 and members of the WASP family proteins, WAVE and Mena have been shown to occur via its SH3 domain found at the C-terminus (Krugmann et al., 2001). IRS-58 appears to play a role in the formation of filopodia as does N-WASP, but the two proteins produce filopodia like structures of very different stabilities and morphology. To determine whether any co-operation occurs between N-WASP and IRS58 in this process, NWASP^del/del cells were microinjected with IRS-58 and GFP-actin. Once the GFP fluorescence was visible (approximately 4 hours following injection) these cells were imaged using fluorescence confocal microscopy.

Microinjection of IRS-58 into NWASP knockout cells in the presence of serum surprisingly resulted in the formation of lamellipodia (fig. 25, red arrows), no visible filopodia were observed in the 40 minute period of observation by fluorescence imaging. However, overexpression of IRS-58 under the same conditions in the corresponding control cells (of this cell line) resulted in the formation of robust, actin rich filopodia like structures (fig. 26, yellow arrows) characteristic of fibroblastic cells overexpressing IRS-58 (Govind et al., 2001). This data again is suggestive of a requirement for N-WASP by IRS-58 in the production of the filopodia structures.

(See CD, Folder 2: NWASP^+/ cells transfected with IRS-58/control cells transfected with IRS-58 for videos).
Fig. 25: NWASP<sup>del/del</sup> cells microinjected with IRS-58 and GFP-actin. Cells imaged over a period of 40 minutes and a frame taken every 10 seconds.
Fig. 26: NWASP$^{flox/flox}$ cells microinjected with IRS-58 and GFP-actin. Cells imaged over a period of 40 minutes and a frame taken every 10 seconds.
To determine if the loss of filopodia like structure formation normally observed by overexpressing IRS-58 in fibroblastic cells is indeed due to N-WASP absence or some other effect in the knock out cells, these cells were microinjected with both IRS-58 and N-WASP cDNA (and the GFP-actin construct). These cells were imaged as before using fluorescence time-lapse photography using the GFP-actin as a marker for cells which were microinjected and expressing the constructs.
Fig. 27: NWASP<sub>del/del</sub> cells microinjected with IRS-58, N-WASP and GFP-actin. Cells imaged over a period of 40 minutes and a frame taken every 10 seconds.
Replacement of the absent endogenous NWASP by microinjection of pXJ40-NWASP in the knockout cells in the presence of overexpressed IRS-58 resulted in the formation of a few very long filopodia like structures (fig. 27, yellow arrows). This is further evidence that N-WASP plays a crucial role in the formation of filopodia in fibroblast cells and suggestive of co-operation occurring between N-WASP and IRS-58 in this process.

(See CD, Folder 2: NWASP<sup>+/−</sup> cells transfected with IRS-58 and N-WASP for video)

6.3: Increase in Actin Filaments by the VH2 Domain:

The VH2 domain of N-WASP in N1E-115 cells caused increased motility, possibly due to its binding to G-actin leading to a change in the equilibrium between G-actin and F-actin and hence the rate of actin dynamics in the cell (Miki and Takenawa, 1998). To try to elucidate whether the VH2 domain is acting to deplete N-WASP function or whether this domain is having a more general effect on actin dynamics, NWASP<sup>+/−</sup> and control cells were microinjected with the VH2 domain of N-WASP.

In VH2 domain transfected cells there is a large increase in stress fibre formation (blue arrows in figs. 28 and 29), these cells also produced membrane ruffles (red arrows in figs. 28 and 29). These cells show dynamicity in their attachment/detachment from their support. These morphologies and activities suggest an upregulation in actin polymerisation/depolymerisation, leading to the subsequent turnover of focal adhesions in these cells. Due to no obvious differences in morphology being observed in either the NWASP<sup>+/−</sup> or the control cells when microinjected with the VH2 domain of N-WASP, suggests that the VH2 domain of N-WASP could be acting to interfere with N-WASP
activity. The VH2 domain therefore could potentially be having an inhibitory effect on the Cdc42 to actin signalling pathway, thereby leading to an upregulation in Rho and Rac activity and hence resulting in the formation of the actin stress fibres, lamellipodia and increased focal complex turnover in these cells.

(See CD, Folder 2: NWASP<sup>−/−</sup> cells transfected with VH2 domain/control cells transfected with VH2 domain for videos).
Fig. 28: NWASP<sup>del/del</sup> cells microinjected with VH2 domain of N-WASP and GFP-actin. Cells were imaged approximately 4 hours after injection over a period of 40 mins with a frame every 10 seconds.
Fig. 29: NWASP$^{\text{floxed/floxed}}$ cells microinjected with VH2 domain of N-WASP and GFP-actin. Cells were imaged approximately 4 hours after injection over a period of 40 mins with a frame every 10 seconds.
6.4: Summary:

Overexpression of dominant negative Rac (RacN17) together with wild type Cdc42 has been previously shown to cause filopodia formation in fibroblast cells. However, in NWASP knockout cells this did not occur, instead a large number of lamellae were produced. Similarly, microinjection of the IRS58 cDNA into NWASP<sup>−/−</sup> cells resulted not in the formation of filopodia but in the production of lamellipodia, whereas in the corresponding control cells filopodia are produced normally. Replacement of N-WASP in the knockout cells overexpressing IRS-58 inhibits lamellipodia formation and restores some of the filopodia characteristically produced by IRS-58 overexpression in fibroblasts (Govind et al., 2001). The VH2 domain of N-WASP causes an increase in stress fibre formation and attachment/detachment of both the NWASP<sup>−/−</sup> and NWASP<sup>lox/lox</sup> from their support. This suggests, this peptide domain functions possibly to inhibit Cdc42 signalling to the actin cytoskeleton via N-WASP, resulting in an upregulation of the Rho and Rac pathways leading to the formation of actin stress fibres, lamellipodia and increased focal complex turnover.
Chapter Seven

Discussion
7: Discussion:

7.1: Effectors in Neurite Outgrowth:

N-WASP is a Cdc42 GTPase effector, which binds Cdc42 directly at the CRIB motif (Miki and Takenawa, 1996). The binding of Cdc42 and PIP$_2$ results in a conformational change in N-WASP, in which the VCA region at the C-terminus is unmasked. This allows the interaction of the Arp2/3 complex (Rohatgi et al., 2000), directly involved in actin dynamics (Rohatgi et al., 1999), with the CA region and of monomeric actin units with the VH regions of N-WASP. N-WASP is a potent activator of the Arp2/3 complex and in vitro has been shown to increase the rate of actin nucleation events (Yarar et al., 1999; Zalevsky et al., 2001). During the course of this project, N-WASP was shown to induce neurite outgrowth in PC12 cells (Banzai et al., 2000) and here in NlE-115 neuroblastoma cells (see 3.1). Delivery of antibodies specific to N-WASP inhibited neurite outgrowth in NlE-115 serum deprived cells (which would normally induce neurite production) suggesting a necessity for N-WASP in neurite formation. A truncated form of N-WASP (NN-WASP(i$_2$)) in which the C-terminus and important VCA region has been removed was unable to induce neurite outgrowth above that of control cells. This suggests that N-WASP and specifically the C-terminal VCA region of N-WASP is required to induce neurite outgrowth in NlE-115 cells.

Another Cdc42 specific effector investigated, IRS-58, has also been shown to induce neurites in N1E-115 cells and to promote the formation of filopodia/micropikes (Govind et al., 2001). These filopodia-like structures, in which both IRS-58 and F-actin appear to colocalise are both actin rich and have a robust appearance (fig. 3, 3.1).

An effector of Rac and Cdc42, Pak, was used to study neurite outgrowth in the N1E-115 neuroblastoma cell line (fig. 4, 3.1). Pak binds Cdc42 with a higher affinity than Rac (Manser et al., 1994), however, via its interaction with the Rac exchange factor,
Discussion

PIX, it is believed to act upstream of Rac, thereby possibly sustaining its own activation by interaction with Rac and is a candidate for mediating cross talk between Cdc42 and Rac (Manser et al., 1998).

All three proteins (N-WASP, IRS-58 and PakCAAX) promote neurite formation, however, their overexpression results in a very different morphology. N-WASP overexpressing cells produced neurites possessing varicosities and peripheral structures of low actin content (fig. 2, 3.1). Whereas, IRS-58, displayed no such enlarged areas along the neurite shaft and the filopodia-like structures had a much higher actin content and robust appearance (fig. 3, 3.1). αPakCAAX (a membrane targeted form of αPak), induced long, thin neurites that tapered as their distance from the cell body increased (fig. 4, 3.1), much like the morphology described for dendrites (Jan and Jan, 2001). Unlike the neurites generated by N-WASP and IRS-58, very few peripheral structures were visible on the αPakCAAX induced neurites.

Signalling hierarchies have been proposed to exist between the Rho GTPases in their control of the actin cytoskeleton (Nobes and Hall, 1995). Pak interacts with both Cdc42 and Rac (Manser et al., 1994) and once in the activated state, Pak can interact with its downstream partner, PIX, which is an exchange factor for Rac. The overexpression of another Rac GEF, Tiam1, in N1E-115 cells has been shown to cause extensive branching (Collard et al., 1996). Overexpression of Pak, which is preferentially a Cdc42 effector, potentially also increases Rac signalling via PIX, hence coordinating the Cdc42 and Rac signalling, possibly leading to the production of neurites which have a highly branched, tapered morphology.

Both IRS-58 (Govind et al., 2001) and N-WASP are Cdc42 effectors (Miki et al., 1996). N-WASP has been shown to also interact with Rac, but with extremely low affinity (Miki et al., 1996). Miki et al, reported that IRSp53 is also a Rac effector and
the intermediate between Rac and WAVE (Miki et al., 2000). However, it has also been reported that IRS-58 does not bind Rac directly and that IRS-58 is a Cdc42 specific effector (Govind et al., 2001; Krugmann et al., 2001). In addition to interacting with WAVE, IRS-58 has been shown to bind mDiaphanous (Fujiwara et al., 2000). This provides evidence for IRS-58 as being a potential mediator between Cdc42, Rac and Rho signalling pathways. This mediation could explain the formation of the actin rich filopodia-like structures resulting from IRS-58 overexpression in N1E-115 cells.

None of these effectors increases the number of neurites per cell. These data are indicative that although these effectors are important for driving the neurite formation pathway, they are not sufficient to initiate neurite formation.

(See model 1).
Model 1: Effector Signalling Interactions:

This model attempts to explain the possible interactions occurring between the effectors and the cross talk between the p21's leading to the resultant effects on the actin cytoskeleton. Only effectors discussed are shown, others have been omitted for simplicity.
7.2: Co-operation of Effectors in Diverse Morphology:

Overexpression of combinations of the effectors IRS-58, N-WASP and αPakCAAX did not result in increased neurite length, suggesting these proteins do not simply function to increase neurite length. Combinations of these effectors did result in new morphologies being observed (figs. 6B, D, F, 3.2), the most elaborate resulting from the combination of αPakCAAX and N-WASP over expression. A spider web like morphology of outgrowth was observed (fig. 6F, 3.2). These two effectors dually expressed, induced an increase in neurite branching significantly above that of IRS-58, IRS-58 plus N-WASP, or GFP (control) expressing cells (fig. 7B, 3.2), resulting in morphologies not observed with any other combination of effectors. These combinations of effectors did not significantly increase branching above that of cells expressing N-WASP or αPakCAAX alone. This morphology is similar to that of N1E-115 cells expressing Tiam-1 (a Rac GEF) (Collard et al., 1996) and appears to be dominated by a PakCAAX like morphology. This suggests, N-WASP and PakCAAX act downstream of Cdc42 and Rac respectively or both act downstream of Cdc42 but Pak is also able to activate Rac via PIX in a regulated manner, leading to the two effects on the actin cytoskeleton and the complex morphology that results.

The varying morphologies resulting from the overexpression of the effectors IRS-58, N-WASP and PakCAAX, alone and in combination, are indicative of varying roles of these proteins in the cell. IRS-58 produces robust, actin rich filopodia when expressed alone and appears to reduce complexity when coexpressed with either N-WASP or PakCAAX (figs. 6B and 6D, respectively, 3.2). This is indicative of IRS-58 acting to stabilise actin structures within the cell. In comparison N-WASP, when expressed alone and in combination with PakCAAX produces a complex morphology of varicosed and branched neurites (fig. 6F, 3.2). The filopodia-like structures of these cells are poorly
defined and of low actin content, suggesting this protein is responsible for actin turnover in the cell, which is supportive of its known function of activation of the Arp2/3 complex and involvement with actin dynamics (Miki et al., 1996; Rohatgi et al., 1999). Cells overexpressing PakCAAX both alone and in combination with N-WASP produce neurites with extensive branching, and a lack of filopodia (fig. 6F, 3.2), a morphology with characteristics of PakCAAX alone.

Together these data suggest that effectors of either Rac or Cdc42 signalling pathways can be upregulated to induce the neurite outgrowth pathway by the effectors N-WASP, IRS-58 and PakCAAX. These effectors do not appear to act synergistically to increase neurite length when coexpressed, indicative of them having a more complex role in the formation of neurites. The morphologies resulting from coexpression of the effectors N-WASP, IRS-58 and αPakCAAX, suggests cross talk exists between these pathways, leading to increased complexity of the outgrowth produced. Based on the morphology observed, αPak and N-WASP may have a potential role in the formation of dendritic branches.

7.3: The PIX and GIT1 Effect:

PIX has been shown to directly interact with Pak at the third, atypical, SH3 binding domain (residues 182-203 of αPak) (Manser et al., 1998). GIT1 interacts with PIX (Premont et al., 1998), which allows the localisation of Pak with focal complexes (Manser et al., 1998); hence, a complex is formed between Pak, PIX and GIT1. GIT1 increases the turnover of focal complexes in a PIX and C-terminal paxillin binding domain dependent manner (Zhou-shen et al., 2000). Cotransfection of either PIX or GIT1 with IRS-58, N-WASP or PakCAAX in neuroblastoma cells resulted in a motile like morphology (fig 8, 3.3). The cells were flattened with a leading and trailing edge
like morphology, which is typical of motile cells. This is supported by the observations of Zhao et al., where GIT1 was shown to induce motility in Hela cells (Zhao et al., 2000). Rac has been shown to lead to a flattened morphology in N1E-115 cells and Cdc42 in the formation of peripheral microspikes (Kozma et al., 1995; Kozma et al., 1997). Both Cdc42 and Rac have been implicated in cell polarity via the formation of a complex with PAR3, PAR6 and the atypical protein kinase C isoform, PKCc (Qiu et al., 2000; Lin et al., 2000). The motile cell like morphology observed in cells overexpressing IRS-58, N-WASP or αPakCAAX together with the downstream interactors of Pak (PIX and GIT1), is suggestive of an upregulation of Rac and potentially an increase in focal complex turnover.

PIX and GIT1 inhibit neurite outgrowth normally observed by the overexpression of the effectors IRS-58 and N-WASP (fig. 9, 3.3), possibly due to their effect of upregulating the Rac pathway, subsequently reducing activity of Cdc42 and the Cdc42 specific effectors IRS-58 and N-WASP. PIX and GIT1 could also be inhibiting outgrowth via their actions on the increase of focal complex turnover. Focal complex formation act as anchorage points in the cell to its matrix, rapid turnover of these structures allows increased motility (DeMali et al., 2002). Such an increase in motility may therefore inhibit neurite outgrowth due to a lack of the stability required for neurite outgrowth. This suggests PIX and GIT1 drive the pathway downstream of Rac responsible for cell motility, thereby inhibiting neurite formation signalling.

Increased focal complex turnover via PIX's interaction with GIT1 (Zhao et al., 2000), combined with increased Rac activity due to PIX's GEF function, could explain the motile, flattened appearance of cells overexpressing PIX, rather than the extensive branched outgrowth observed for the Rac GEF Tiam-1 (Collard et al., 1996), which is not implicated in focal complex turnover. The PIX and GIT1 inhibition of neurite
Discussion

outgrowth is not as great in αPakCAAX expressing cells (fig. 9, 3.3). Pak is both a Rac and Cdc42 effector (Manser et al., 1994), hence this effector could be activated sufficiently to cause outgrowth, thereby causing the promotion of neurite outgrowth and inhibiting cell motility signalling. This could be potentially achieved by Pak forming a complex with PIX and GIT1, hence regulating/removing these proteins from the system allowing neurite outgrowth to occur.

7.4: The Verprolin Homology 2 Domain:
N-WASP consists of multiple domains, which have various functions in the protein and its role in actin dynamics of the cell (Fukuoka et al., 1997). The most intensively studied of these domains are those found at the C-terminus and are known collectively as the VCA (verprolin homology domain, cofilin homology domain, acidic domain) region. Structural analysis has shown the cofilin homology (CH) domain to interact with the CRIB motif. This interaction is responsible for the formation of the autoinhibited structure of WASP (Kim et al., 2000). The verprolin homology (VH1 and VH2) domains of N-WASP interact directly with actin. This is believed to bring monomeric actin units to the Arp2/3 complex bound to the CH and acidic (A) regions of C-terminus of N-WASP (Rohatgi et al., 2000). The Arp2/3 complex upon binding to N-WASP is activated leading to actin nucleation events (Rohatgi et al., 2000; Zalevsky et al., 2001). Hence, the C-terminus of N-WASP was thought to be the region of N-WASP active in controlling actin dynamics of the cell via its direct interaction with the Arp2/3 complex. N-WASP, unlike other WASP family members, possesses two VH domains and has been shown to be responsible for increased activation of the Arp2/3 complex (Yamaguchi et al., 2002). The individual VH domains of N-WASP, which were cloned into the mammalian expression vector, pXJ40, were shown to have some effects on the actin
cytoskeleton of the N1E-115 cell line. The second of the two VH domains (known as VH2) significantly inhibited serum deprival induced neurite outgrowth as well as neurite outgrowth induced by the effectors IRS-58, αPakCAAX and N-WASP itself (fig. 12, 4.2). The VH2 domain however did not inhibit the formation of filopodia, instead, it promoted the formation of actin rich, robust peripheral filopodia-like structures (fig. 13, 4.2). This suggests the processes of filopodia formation and neurite outgrowth are independent of one another.

The morphology of the cells expressing the VH2 domain is motile-like, a leading edge with extensive ruffling and filopodia and a trailing edge possessing none of these peripheral structures. Expression of the VH2 domain of N-WASP in N1E-115 cells visualised in real time, displayed this morphology in which the cell forms a leading and trailing edge and translocates across the field of view during the time of observation (40 minutes) (fig. 21, 5.2). GIT1 is involved in focal complex turnover and morphology of cells transfected with GIT1 or the VH2 domain of N-WASP are very similar (both have a motile cell like morphology). This data implies that the VH2 domain of N-WASP is affecting both the actin dynamics of the peripheral filopodia and focal complex turnover. This may, result in an increase in cell motility and the resultant inhibition of outgrowth as observed in PIX and GIT1 transfected cells (fig. 8, 3.3).

N-WASP is downstream of Cdc42 and is a potent activator of the Arp2/3 complex. The VH2 domain of N-WASP is required for the binding of G-actin and the subsequent actin nucleation events by the associated Arp2/3 complex (Rohatgi et al., 2000). The VH2 domain in solitude has an affinity for monomeric actin and can increase actin depolymerisation events in vitro (Miki and Takenawa, 1997). Therefore, in the cell the VH2 domain may be removing the G-actin units causing a shift in the equilibrium of
F-actin:G-actin in the cell, leading to an increased rate of actin dynamics. The VH2 domain may potentially disrupt the function of N-WASP by interfering with the binding to G-actin and/or Arp2/3 complex. N-WASP is linked directly to actin dynamics via the Arp2/3 complex (Rohatgi et al., 2000; Zalevsky et al., 2001) hence the VH2 domain may disrupt the Cdc42 signalling via N-WASP and the Arp2/3 complex to the actin cytoskeleton. This may therefore lead to an upregulation of Rac signalling and subsequently cell motility signalling cascades, via complexes such as PAR6/PAR3/PKCζ complex, which is known to be involved with cell polarity (Lin et al., 2000; Qui et al., 2001). The more flattened and motile morphology observed from the overexpression of the VH2 domain in N1E-115 cells, could result from effects on polarity and adhesion, for example.

N-WASP (both overexpressed and endogenous proteins) is translocated to the membrane in the presence of the VH2 domain (fig. 14, 4.3). This domain could be interfering with the formation of the autoinhibited structure of N-WASP by competing with binding for the associated G-actin bound to N-WASP, locking N-WASP in a constitutively active conformation. This may result in N-WASP collecting at sites of high actin content, thereby inhibiting N-WASP's normal function, causing an increase in actin dynamics, leading to cell motility. Alternatively, this translocation of N-WASP could be due to the VH2 domain unbalancing the G-actin: F-actin equilibrium by binding G-actin causing an increase in actin severing to occur to compensate. This could lead to the recruitment of proteins, such as N-WASP, to the periphery, to restore the normal monomeric to polymerised actin equilibrium in the cell. This potentially accounts for the observed increase of N-WASP localisation at the periphery of cells transfected with the VH2 domain.
Coexpression of the VH2 domain of N-WASP with IRS-58, N-WASP and αPakCAAX also inhibited neurite outgrowth (fig. 12, 4.2). IRS-58 has been shown to interact with WAVE (a WASP family member) via its SH3 domain and therefore may lie upstream of N-WASP in the signalling pathway of Cdc42 to the actin cytoskeleton. If IRS-58 signals through N-WASP to the Arp2/3 complex and the VH2 domain acts to interfere with the function of N-WASP in actin polymerisation via the Arp2/3 complex and subsequently neurite outgrowth, this could explain the inability of IRS-58 to induce outgrowth when coexpressed with the VH2 domain.

Microinjection of the VH2 domain into an NWASP+ fibroblast cell line induced the formation of stress fibres and membrane ruffles and attachment/detachment to their support (CD, Folder 2: NWASP+ cells transfected with VH2 domain). This morphology was also observed in the comparative control cells microinjected with the VH2 domain (CD, Folder 2: control cells transfected with VH2 domain). These data are indicative that full length N-WASP itself is not responsible for cell motility, but the effect of its VH2 domain (perhaps due to its interaction with monomeric actin) on the actin cytoskeleton, is responsible for an increase in cell motility. The apparent increase in motility in response to overexpression of the VH2 domain, which is not observed in response to overexpression of full length N-WASP, may simply be because the VH2 domain lacks the normal regulation of the full length protein. Overexpression of the VH2 domain in NWASP+ cells and the respective control cells resulted in identical morphologies being observed in both (figs. 28 and 29, respectively, 6.3). These data suggest the VH2 domain of N-WASP is interfering with N-WASP activity and possibly the signalling of Cdc42 to the actin cytoskeleton. This could potentially lead to an increase in Rac and Rho signalling. An increase in Rac signalling could account for the
membrane ruffling and attachment/detachment due to increase focal complex turnover as shown by the downstream effector of Pak, GIT1 via PIX (a Rac GEF) (Zhao et al., 2000). Upregulation of Rho is known to induce stress fibre formation in fibroblast cells and could account for the increase in actin cables seen in these cells overexpressing the VH2 domain of N-WASP.

(See model 2)
Model 2: VH2 Domain Mode of Action:

The VH2 domain of N-WASP may bind to and deplete the pools of monomeric G-actin. In response, N-WASP is recruited to the membrane, where it severs actin filaments and the VH2 domain competes for the G-actin units, thereby preventing N-WASP deactivation by forming a constitutively active form. This inability of N-WASP to 'recycle' leads to a subsequent downregulation in Cdc42 signalling through N-WASP and a resultant upregulation in Rac and Rho signalling. Rac and Cdc42 act to signal through other pathways such as PAR6/PAR3/PKCζ complex causing cell motility, membrane ruffling, inhibition of outgrowth and an increase in stress fibre formation.
7.5: Is N-WASP Essential For Filopodia Formation?:

The literature shows a conflict as to the importance of N-WASP in filopodia formation. In an attempt to shed a little light on this issue, an assay measuring the stability of filopodia induced by the effectors IRS-58, N-WASP and the VH2 domain of N-WASP was employed. The assay utilised fluorescence live imaging techniques using a GFP-actin construct. In real time, it was possible to visualise the differences in dynamicity of the filopodia-like structures produced by the effectors N-WASP and IRS-58 overexpressed in N1E-115 cells. The filopodia-like structures of IRS-58 transfected cells appeared actin rich and were as stable as those of GFP-actin expressing (control) cells (fig. 22, 5.3). N-WASP transfected cells however, were significantly more dynamic and appeared to contain a far lower actin content (fig. 19, 5.2), suggesting that N-WASP is required for the rapid turnover of actin in filopodia. This significant increase in actin turnover and relative instability compared to control cells, suggests a combination of proteins, such as, IRS-58 and N-WASP are required, respectively, for formation and turnover of filopodia.

To further examine the requirement of N-WASP in filopodia formation, NWASP−/− cells were employed in this live imaging assay. Microinjection of IRS-58 into the NWASP−/− cells resulted in the extensive formation of lamellipodia. Conversely, in the corresponding control cells, filopodia-like structures were observed, as previously reported for the overexpression of IRS-58 in fibroblast cells (Govind et al., 2001). These results imply that IRS-58 requires N-WASP to produce its filopodia-like structures. In the absence of N-WASP, IRS-58 may interact with another WASP family member, such as, WAVE. WAVE2, has been shown to induce lamellipodia formation and interact with IRSp53 at its SH3 domain (Miki et al., 2002). This collectively suggests IRSp53/58 can interact with WASP family members to cause indirect effects on the actin cytoskeleton.
IRS-58 has been shown to interact with the WASP family member WAVE. The use of NWASP<sup>−/−</sup> cells has indicated that IRS-58 is possibly signalling through N-WASP to produce filopodia-like structures. To determine if N-WASP was indeed the protein required by IRS-58 to produce the filopodia-like structures that are observed in the control NWASP cells, NWASP<sup>−/−</sup> cells were microinjected with cDNA of IRS-58 and N-WASP. These cells reverted from producing lamellipodia to producing filopodia-like structures (fig. 27, 6.2). The numbers of filopodia-like structures observed however are far fewer than in the control cells. One possibility is that this is simply because of overexpression of cDNA of N-WASP is not able to compensate for endogenous N-WASP protein. Another possible reason could be the deletion of N-WASP from these fibroblast cells has resulted in an increase in other proteins, such as WAVE, hence simple replacement of N-WASP does not revert to the normal system in the control cells. Alternatively, IRS-58 requires a combination of proteins including N-WASP, such as WAVE or Mena, to form these peripheral structures. There may be competition existing between N-WASP and these others proteins for binding of IRS-58 and the correct balance of interaction is required for the formation of the filopodia-like structures seen in cells overexpressing IRS-58.

(See model 3)
Model 3: IRS-58 and N-WASP Co-operativity in Filopodia Formation:

IRS-58 and N-WASP interact via the SH3 and PP domain respectively, forming a complex. N-WASP interacts with the Arp2/3 complex, resulting in the depolymerisation and polymerisation of actin at the periphery. Interaction of WAVE and Mena with IRS-58 is blocked by this N-WASP/IRS-58 complex formation. These proteins, plus others such as zyxin, work together to form actin bundles, resulting in the formation of filopodia.
87.7: **What All the Evidence Suggests:**

IRS-58 is a possible mediator for Cdc42, Rac and Rho signalling either via direct interaction, or via interaction with effectors WAVE and mDiaphanous respectively. N-WASP is located furthest downstream of the other Cdc42 effectors in the signalling pathway to the actin cytoskeleton as it directly associates with and activates the Arp2/3 complex linked to actin dynamics (Rohatgi *et al.*, 2000; Zalevsky *et al.*, 2001). IRS-58 requires N-WASP for filopodia formation and is possibly located upstream of N-WASP in the signalling cascade from Cdc42 to the actin cytoskeleton. N-WASP could potentially interact with IRS-58 as has been shown for the family member WAVE (Miki *et al.*, 2000).

The VH2 domain acts as a dominant negative on neurite outgrowth, possibly by altering the equilibrium of G-actin:F-actin in the cell, thereby increasing the rate of actin depolymerisation. Alternatively, the VH2 domain may be interfering with the function of N-WASP by inhibiting its ability to form an autoinhibited structure and so forming a constitutively active conformation. Either way, the VH2 domain leads to a change in the normal localisation of N-WASP in N1E-115 cells to a more peripheral position (fig. 14, 4.3), compromising N-WASP function. This may lead to an alteration in actin dynamics and hence block neurite outgrowth induced by serum removal or the effectors IRS-58 and αPakCAAX (fig. 12, 4.2). This disruption to N-WASP function could also interfere with Cdc42 signalling to the actin cytoskeleton, with a resultant increase in Rac and Rho activation. Rac has been shown previously to increase cell flattening and Rho has been shown to be responsible for neurite collapse (Kozma *et al.*, 1995, 1997). Both Rac and Cdc42 have been implicated in cell polarity via interaction with the PAR6/PAR3/PKCζ complex (Lin *et al.*, 2000; Qui *et al.*, 2001). An increase in Rho signalling, could account for the increased flattening and lack of outgrowth observed in the N1E-115 cells.
expressing the VH2 domain of N-WASP. This upregulation of Rac and Rho could also explain the morphology of increased stress fibres and membrane ruffling in the NWASP<sup>−/−</sup> cells when microinjected with the VH2 domain of N-WASP.

Overexpression of the VH2 domain of N-WASP in N1E-115 cells produces a morphology almost identical to that observed with the overexpression of PIX and GIT1 in this cell line. GIT1 has been shown to increase focal complex turnover and subsequently increase cell motility in Hela cells (Zhao et al., 2000). The VH2 domain, PIX and GIT1 all inhibit neurite outgrowth induced by serum deprival or the effectors N-WASP, IRS-58 and PakCAAX. This suggests that the VH2 domain possibly could be increasing focal complex turnover and enhancing the cell motility signalling pathways, leading to a downregulation in neurite outgrowth induction.

N-WASP appears to be required for rapid turnover of filopodia and IRS-58 acts as a more stabilising factor in filopodia formation. Both of these effectors produce filopodia-like structures of very different morphologies and actin dynamics. N-WASP filopodia-like structures are of low actin content and show a rapid turnover. IRS-58, however produces actin-rich filopodia-like structures of stability equivalent to control cells. Therefore, a combination of these two properties is close to that of what is seen of filopodia in control cells. Together these data provide a strong case for the requirement of N-WASP in combination with other effectors in filopodia formation and dynamicity. The VH2 domain of N-WASP inhibits neurite outgrowth and increase cell motility in the N1E-115 cell line. This emphasises the importance of the conformational change, which N-WASP undergoes upon activation, unmasking the VCA region and controlling the actin dynamics of the cell. Without this conformational change and control actin dynamics and signalling events of the cell can be altered as seen by the use of the VH2
domain of N-WASP which lacks such controlling features as conformational change seen in the full length protein.

**Model 4: Decision Making In Actin Dynamics:**

Signalling events downstream of the Rho GTPases influence the actin dynamic state of the cell. Upregulation of effectors, such as PIX and GIT1, lead to the inhibition of neurite outgrowth and increase cell motility. Effectors such as IRS-58 and N-WASP, however, act to stabilise the cell and induce neurite outgrowth. The understanding of the mechanism, by which cell motility or neurite outgrowth is stimulated is poorly defined and is an area requiring much investigation, if the puzzle of neural network development is to be solved.
References
References

8: References:


65. Chavrier, P., Goud, B. The Role of ARF and Rab GTPases in Membrane Transport. 


References


References


Toker, A., Stossel, T. P. Thrombin Receptor Ligation and Activated Rac Uncap
Actin Filament Barbed Ends Through Phosphoinositide Synthesis in Permeabilized


1999, 22, 511-539.

129. Hawkins, P. T., Eguinoa, A., Qiu, R. G., Stokoe, D., Cooke, F. T., Walters, R.,
Wennstrom, S., Claesson-Welsh, L., Evans, T., Symons, M., et al. PDGF stimulates
an increase in GTP-Rac via activation of phosphoinositide 3-kinase. Curr Biol.
1995, 5, 393-403.


66, 1-41.

Regulation of NADPH Oxidase Activity by Rac GTPase Activating Protein(s).


181. Kuroda, S., Fukata, M., Nakagawa, M., Fujii, K., Nakamura, T., Ookubo, T.,
Izawa, I., Nagase, T., Nomura, N., Tani, H., Shoji, I., Matsuura, Y., Yonehara, S.,
Kaibuchi, K. Role of IQGAP1, a Target of the Small GTPases Cdc42 and Rac1, in


183. Kwiatkowski, D. J. Functions of Gelsolin: Motility, Signaling, Apoptosis, Cancer.

184. Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D.,
McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., . A Protein Kinase
*372*, 739-746.


186. Lemmon, M. A., Bu, Z., Ladbury, J. E., Zhou, M., Pinchasi, D., Lax, I., Engelman,
D. M., Schlessinger, J. Two EGF Molecules Contribute Additively to Stabilization

187. Lemmon, M. A., Ferguson, K. M. Signal-Dependent Membrane Targeting by


References


References


291. Rudel, T., Zenke, F. T., Chuang, T. H., Bokoch, G. M. P21-Activated Kinase

292. Sadowski, I., Stone, J. C., Pawson, T. A Noncatalytic Domain Conserved Among
Cytoplasmic Protein-Tyrosine Kinases Modifies the Kinase Function and
1986, 6, 4396-4408.

293. Sanes, J. R., Lichtman, J. W. Development of the Vertebrate Neuromuscular

294. Sarner, S., Kozma, R., Ahmed, S., Lim, L. Phosphatidylinositol 3-kinase, Cdc42,
and Rac1 act downstream of Rasin integrin-dependent neurite outgrowth in N1E-

295. Sasaki, T., Takai, Y. The Rho Small G Protein Family-Rho GDI System As a

296. Satoh, S., Tominaga, T. MDia-Interacting Protein Acts Downstream of Rho-MDia
and Modifies Src Activation and Stress Fiber Formation. *J. Biol. Chem.* 2001, 276,
39290-39294.


304. Scita, G., Tenca, P., Frittoli, E., Tocchetti, A., Innocenti, M., Giardina, G., Di Fiore, P. P. Signaling From Ras to Rac and Beyond: Not Just a Matter of GEFs. 


Appendices
8.1.1: Full Length N-WASP and N-WASP Constructs:

NN-WASP

VH1

VH2

VH1-Cof

8.1.2: C-terminal Constructs DNA and Protein Sequences:

VH1

VH2

VH1-Cof

269
8.2: Vector Maps:

8.2.1: pXJ40-HA:

Appendices
8.2.2: pXJ40-FLAG:

[Diagram of pXJ40-FLAG plasmid]

pXJ40-FLAG
4281 bp

Sst I 516
Pst I/Stu I 608
Rabbit B globin intron II

SV40 poly A site

BSM13+

Sal I 1500

T7 promoter

1296 EcoRI

> FLAG <<

BamHI
HindIII
XhoI
NotI
SmaI
PstI
SstI
KpnI
BglII
1352

pXJ40- MCS

EcoRI

T7 >> GAA TTC ACC ATG
Kozak M D Y K D D D K G

BamHI  HindIII  XhoI  NotI  PstI

GGA TCC AAG CTT CTC GAG
GCG GCC GCC

CCG GCC TGC AGG AGC TCG

G S K L L E A A A P G C R S S

SmaI

KpnI  BglII

GTA CCA GAT CTT

V P D L
8.2.3: pXJ40-GST:

Appendices

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pXJ40-GST:

- Pvu II/Xba I 1
- HCMV promoter
- Sst I 516
- Pst I/Stu I 608
- Rabbit B globin intron II
- SV40 poly A site
- Sal I 1500
- T7 promoter
- 1296 EcoRI
- BamHI HindIII
- XhoI NotI
- SmaI
- PstI
- SstI
- Kpnl
- BglII
- 1352

pXJ40-MCS

- EcoRI

T7 >> GAA TTC ACC ATG
Kozak M

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KpnI, BglII

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V P D L

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272
8.2.3.1: GST Tag Sequence:

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273
8.2.4: pXJ40-GFP:

pXJ40-GFP
4281 bp

- Pvu II/Xba I
- HCMV promoter
- Sst I 516
- Pst I/Stu I 608
- Rab B globin intron II
- T7 promoter
- SV40 poly A site
- Sal I 1500
- BSM13+

pXJ40-MCS
- EcoRI

T7 >> GAA TTC ACC ATG
Kozak M

- BamHI
- HindIII
- XhoI
- NotI
- PstI

GGA TCC AAG CTT CTC GAG GCC GCC CCG GCC TGC AGG AG C TCG
G S K L L E A A A P G C R S S

- Smal

KpnI

GTA CCA GAT CTT
V P D L
8.2.4.1: GFP Tag Sequence:

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GFP Tag Sequence:

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