In vitro function and interactions of the actin associated protein Caplin: A member of Transgelin gene family

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ABSTRACT

Caplin is a member of the Transgelin (Tg) multigene family of actin binding proteins (ABPs) associated with the actin filament network that regulates the physical status and interactions of actin.

Caplin is the second member of a polypeptide doublet of 22KDa originally identified on a SDS-PAGE by a monoclonal antibody and named PC4L (Shapland, 1988). The upper isoform of this doublet, Transgelin (Tg), was purified from sheep aorta and was found to cross-link actin filaments in vitro (Shapland et al., 1993). Transgelin was found to be down regulated by oncogenic transformation and changes in cell shape (Shapland et al., 1993).

Caplin is present in all cells thus far examined, including transformed fibroblasts and lymphocytes, and in these cells, Caplin was found to be the only member of the PC4 doublet present. The cDNA sequence of Caplin was obtained from human T cell lymphoma (HTCL) and was found to localize, by light microscopy, along actin filaments (Martin Smith PhD thesis).

The in vitro function of this isoform was unknown and the objectives of my thesis was to purify and investigate the in vitro function of protein Caplin in normal cells.

To allow this objective, I generated a pGEX fusion protein. Caplin cDNA sequence was obtained by RTPCR of mRNA obtained from normal mouse thymocytes using oligonucleotides derived from HTCL cDNA sequence (Martin Smith, PhD Thesis). Fusion protein was obtained by in frame ligation of Caplin cDNA to an EcoRI site in the bacterial expression vector pGEX-4T-3. Purification of fusion protein was achieved by chromatography followed by thrombin cleavage of fusion protein Caplin.

The translated product of normal mouse thymus Caplin open reading frame consist of 199 amino acids with a calculated molecular weight of 22.391Da, an estimated PI of 8.41 and
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an extinction coefficient of 1.302. Caplin cDNA sequence analysis indicated the presence of a putative nuclear localisation signal, two potential EF-hand sites (23-34 and 108-118) and protein kinase C phosphorylation (180-182) site.

Database searches indicates that Caplin amino acid sequence is highly homologous to protein NP25 (83%), Transgelin (82%) and Calponin (58%) and significantly homologous to sm20 (56%), VAV (54%) and UNC87 (48%). Further analysis indicates that Caplin has the same modular structure as found in the calponin family, consisting of an amino terminal CH domain (1-134), followed by an ABS domain (152-173) and a carboxyl terminal R domain (174-198).

Cell permeabilization and immunofluorescence showed that Caplin binds directly to actin filaments and also indicated staining at nuclear level.

*In vitro* functional studies including low shear viscometry, fluorimetry, sucrose density and sedimentation assays indicates that Caplin is a monomeric protein that binds to actin filaments with an estimated binding constant of \( Ka=4\times10^5 \text{M}^{-1} \), and acts *in vitro* as a calcium independent actin barbed end capping protein.

Because this protein caps the barbed ends of actin filaments and is a member of the Transgelin family of actin binding proteins, I named it Caplin.
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The cell cytoskeleton

The cytoskeleton plays a central role in the majority of cellular functions (Drubin and Hirokawa, 1998). It is a complex cohesive meshwork of afferent filaments that not only provide a dynamic skeleton for cells but also maintains cell integrity by mediating processes such as cell shape, which is fundamental in the processes of morphogenesis and development, organelle and cell-fate-determinant positioning, cytokinetics including cell division, movement of cell surface receptors, cell motility and the formation of cell extensions (Drubin and Hirokawa, 1998; Luby-Phelps, 1994).

Of central importance for the dynamic behaviour and the mechanical stability of the cytoarchitecture are three distinct, yet interconnected filament systems: microtubules, intermediate filaments and microfilaments. The diversity of cytoskeletal functions is the result of the association between these three major cytoskeleton components and a growing number of associated-proteins which are responsible for mediating cellular events (Pollard and Goldman, 1991; Cleveland and Mooseker, 1994; Fowler and Vale, 1996).

A growing area of research concerns the interactions between the cytoskeleton and signal transduction pathways (Fowler and Vale, 1996). The cytoskeleton responds very rapidly and effectively to extracellular signals. Upon extracellular stimuli the cytoskeleton components interact and thus control regulatory pathways integrating information from signal responding by changing cell morphology, dynamics or structure.
The roles, mechanisms and regulation of the cytoskeleton are rapidly being elucidated. In this introduction I will briefly review microtubules (MT) and intermediate filaments (IF) and will focus on microfilaments and its growing number of associated proteins.

1.2 - Microtubules

Microtubules (MT) are ubiquitous structural elements of the eukaryotic cytoskeleton that play an important role in organising the spatial distribution of organelles within the cell (Mandelkow and Mandelkow, 1992). MT are formed by the self-assembly of alpha and beta tubulin heterodimer into polar fibers of around thirteen parallel protofilaments aligned lengthwise (Wade and Hyman, 1997). These protofilaments make lateral contacts with each other to form a hollow tube (25nm in diameter) with an external wall along which a variety of microtubule associated proteins (MAPS) and motor proteins bind (Bloom, 1992; Downing and Nogales, 1998). The cylindrical form of MT sustains compression and bending forces. In the cytoplasm of cells they exist as single filaments that radiate outward throughout the cytoplasm from a position close to the nucleus providing a system of fibers along which organelles and vesicles can move regulating cell shape, movement, and also along which chromosomes segregate during mitosis. In addition, microtubules provide elongated cylindrical cellular structures such as axons (allowing communication between neurons and non-neuronal cells), flagells and cilia (mediating their own movement eg. sperm and epithelia of trachea and oviducts).

The development of cryoelectron microscopy and rapid freezing techniques have contributed to a reliable image of the microtubule structure (Downing and Nogales, 1998). Studies of microtubule polarity by kinesin decoration has shown that microtubules are polar
structures with an arrowhead moire pattern which point to the plus end when the protofilament skew is right-handed and towards the minus end when left-handed.

Essential to microtubule function are both polarity and their dynamic nature (Caplow, 1992; Downing and Nogales, 1998; Tran et al., 1997). MT assembly is highly dynamic switching between phases of growth and shrinkage and the most striking example is the assembly and disassembly of the mitotic spindle during cell division. They can also be static structures providing cells with structural support and movement such as found in ciliary axoneme and flagellum, neuronal processes and the marginal band of erythrocytes (Caplow, 1992).

The presence of a tubulin gene family accounts for the existence of many isotopes and reflex the different functions of microtubules. Both alpha and beta subunits exist as several isotope forms and are often found with different distributions in different cell types. Different sets of microtubules can be found in a single cell where they can also co-polymerise. Microtubule assembly in vivo is initiated by δ-tubulin complexes that are targeted to microtubule organising centers (Schiebel, 2000). Another factor contributing to this diversity is four post-translational modifications that have been reported to take place on the tubulin molecule (Luduena et al., 1992).

1.2.1 - STRUCTURE

Tubulin is the structural subunit protein of microtubules (Luduena et al., 1992). It is a 100KDa heterodimer (proteins of ~450 amino acids each) consisting of two 50KDa tightly linked globular subunits designated α and β (Mandelkow and Mandelkow, 1992). Both have been isolated from a variety of sources including plant, animal, fungal and protozoan.
Chapter 1

The atomic model obtained by electron crystallography shows that α and β subunits are basically identical structures. The monomer structure is very compact consisting of two interacting β-sheets surrounded by α-helices. The structure can be divided into three functional domains: an amino-terminal domain (the site for nucleotide binding) an intermediate domain (taxol-binding site) and the carboxyl-terminal (site for microtubule-associated proteins and motor proteins binding).

The peptide sequence of α and β subunits have between 36 to 42% homology. Each monomer binds a guanine nucleotide and the binding site varies between the two. In the α subunit the binding is nonexchangeable and occurs at the N-site, whereas in the β subunit the binding is exchangeable and occurs at the E-site (Downing and Nogales, 1998). Rapid freezing has allowed the capturing of different structural states of MT conformations during both assembly and disassembly. There are different nucleotide-bound states, or two tubulin conformations: a ‘straight’ conformation of tubulin with bound GTP at the exchangeable E-site and a ‘curved’ conformation containing tubulin bound to GDP.

Both α and β tubulins exist as families of isotypes differing in their amino acid sequences (Luduena et al., 1992). Still the differences are mainly clustered in the carboxyl-terminal region. In Drosophila there are four isotopes of β tubulin. β3 has 72% homology with other isotypes but when expressed in the postmitotic germ lines of the testis, where only β2 type is normally expressed, these cells do not form axonemal MTs (Luduena et al., 1992). Also, δ tubulin, a new member of the tubulin family, has been isolated from the microtubule-organising centre from a variety of organisms and has shown 66% homology between isoforms and 35% homology to both α and β. Amino acid sequence analysis has shown highly conserved GTP binding regions within each subunit and a less acidic carboxyl termini. The region that varies most in sequence, both in different species and isotypes, is the
carboxyl-terminal region. This region is rich in glutamate which confer this termini with an overall negative charge and is involved in binding of MAPs and discrimination among isotopes. This region is exposed on the outside of the microtubule and enhances the idea that there are isotype-selective binding providing the functional basis for isotope variety and their particular function (Downing and Nogales, 1998). Removal of this region enhances polymerization in the absence of MAPS, suggesting it to be an endogenous inhibitor of tubulin polymerization (Mejillano and Himes, 1991a). Proteolysis studies on tubulin dimers have shown five major proteolytic regions in each monomer; of which two regions in the $\alpha$ and one in the $\beta$ subunits become protected upon polymerization (Downing and Nogales, 1998).

**1.2.11 - MICROTUBULE ASSEMBLY**

Microtubule dynamic instability, the ability of a MT end to abruptly and stochastically switch between phases of elongation and rapid shortening, is based on the binding of tubulin-GTP or GDP using a proposed mechanism called the cap model (Downing and Nogales, 1998; Joshi, 1998; Tran et al., 1997).

There are three distinct steps in the life cycle of a microtubule: nucleation, assembly and disassembly (Joshi, 1998; Wade and Hyman, 1997). The tubulin dimer is organised with $\beta$ tubulin pointing towards the microtubule fast-growing plus end and with the $\alpha$ tubulin towards the minus end (Wade and Hyman, 1997). A microtubule nucleates and assembles by addition of subunits to the growing ends and disassembles by endwise loss of grouped subunits.
Nucleation is very different in vitro compared to in vivo. In vitro, microtubules self-nucleate and grow from both ends, with the plus end growing faster (Wade and Hyman, 1997) at a rate that is proportional to the concentration of free tubulin. Therefore the plus end grows approximately three times faster than the minus end (Zheng et al., 1995). In vivo, microtubules nucleate within the microtubule-organising centre (MTOC) to which the minus end is anchored, so that the plus end grows into the cytoplasm. It is now clear that nucleation of microtubule assembly is facilitated by δ tubulin which is arranged in discrete rings tethered at the centrosomes (Joshi, 1998). The mechanisms for assembly and attachment remain to be solved. It is known, however, that cells which contain centrosomes depend on them for microtubule nucleation as kinetochores are responsible for the generation of bipolar spindle during cell division (Waters and Salmon, 1997). It's been shown, in vitro, that δ tubulin can bind to the minus end of microtubules serving as anchorage at the centrosome and also that when δ tubulin is overexpressed it accumulates throughout the cytoplasm disrupting the radial microtubule arrays (Joshi, 1997). Studies in Xenopus egg extracts showed that the MTOC is responsible for microtubule nucleation. The MTOC consist of a pair of centrioles surrounded by a cloud of electron-dense pericentriolar material (PCM) and when MTs from this region are nucleated in vivo or in vitro they have 13 protofilaments whereas in vitro they mostly have 14 protofilaments, indicating that MTOCs specify the exact structure of the MTs they nucleate (Zheng et al., 1995) centrosomal microtubules are released from their sites of nucleation gliding over nuclear surfaces due to microtubular translocation and treadmilling.

Assembly and disassembly is related to GTP hydrolysis and it’s interaction with other proteins. The current accepted mechanism for dynamic instability at the plus and minus ends is the cap model. During elongation assembly predominates and dimers are added to the growing MT before a 'catastrophe' occurs at the end switching abruptly to rapid loss of
subunits and consequent shortening. This dissociation of dimers from the end is resumed to elongation by a ‘rescue’ event. In this, GTP hydrolysis leads to conformational changes in the tubulin -GDP dimer. Both α and β bind GTP but only GTP-bound β tubulin undergoes hydrolysis and exchange. Upon hydrolysis the microtubule protofilament losses the GTP-bound β tubulin cap straight conformation resulting in an uncapped protofilament (possessing a GDP-bound beta tubulin) with a ‘curved’ conformation (Downing and Nogales, 1998). The GTP cap model postulates that the hydrolysis of GTP-tubulin produces a labile core of GDP-tubulin subunits which are capped by the newly added GTP-tubulin at the growing end. A catastrophe occurs when this GTP cap is lost allowing the labile GDP-tubulin to rapidly dissociate. Rescue is proposed to occur when a shortening end is recapped with GTP-tubulin (Tran et al., 1997). This model predicts that severing an elongating end will produce plus and minus ends with exposed GDP-tubulin and that these should convert immediately to shortening. However when this model was directly tested by using a UV microbeam to sever axoneme microtubules it was shown that severed plus ends shortened as predicted by the model but severed minus end did not. Instead the minus end resumed elongation revealing the existence of a metastable intermediate state between elongation and shortening states with different kinetics at plus and minus ends (Tran et al., 1997).

This observation has also been used to elaborate the mechanism by which they preferentially attach via the plus end. The nucleotide position in the amino-terminal domain interacts with the next monomer both at the interdimer and at the intradimer interfaces. The N-site of α tubulin is always occluded in the dimer regardless of the state of assembly. On the other hand, the nucleotide at the E-site is exposed in the dimer but becomes occluded upon polymerization becoming unexchangeable. These two observations combined seem to favour the hypothesis of the plus growing end of the β tubulin.
The Mandelkow laboratory has developed a technique in which microtubules are decorated with kinesin such as each tubulin dimer interacts with just one motor domain (Song & Mandelkow, 1993). This technique combined with cryoelectron microscopy has been used to investigate the microtubule growth of protofilaments via sheet-like extensions. The protofilaments run lengthwise with lateral contacts so that the neighbouring subunits follow the shallow-pitch three-start helix form, i.e., during each complete turn of the helix the pitch rises by three subunits. Structural analysis of flagella tubules showed that the α-β heterodimer can be packed in two distinct ways in the microtubule surface lattice, the A-lattice (complete) and the B-lattice (incomplete). For the A-lattice the three-start helix is made of α and β subunits whereas for the B-lattice the helix is made up of one subunit type only. The 13 protofilaments arrangement of microtubules found in most cells have a B-lattice organisation, implying that the helices of microtubules in cells have discontinuities or seams (Wade and Hyman, 1997). This observation is incompatible with a helical growth process, suggesting that filaments are more likely to grow via sheet-like extensions which close into tubes (Wade & Hyman, 1998).

The most dramatic example of microtubule assembly and disassembly occurs in dividing cells during the morphogenesis of the mitotic apparatus (Joshi, 1998). During formation of the spindle, different classes of microtubules can be observed. The two centrosomal poles are connected via kinetochore microtubules, microtubules connect with each other (pole-to-pole microtubules) or with the cellular cortex (astral microtubules). Experiments using non-centromeric Xenopus eggs demonstrated that multimeric proteins in the vicinity of chromatin such as kinesin and cytoplasmic dynein interact with microtubules orientating them via plus and minus ends (Song & Mandelkow, 1993; Joshi, 1998).
The microtubule assembly during growth of flagella has been well described in an algal biflagellate cell type, *Chlamydomonas*. It consisted of a fusion of wild type cells that contained untagged tubulin in their half-length growing flagella with cells containing tagged tubulin with full length flagella. The resulting quadruflagellate cell showed that the new tubulin assembled at the tip of the flagella suggesting that transport and assembly of tubulin are distinct highly regulated processes (Joshi, 1998). There is however a great deal of controversy over the issue as to how microtubule assembly occurs in neuronal axons (Baas, 1997; Joshi, 1998). The controversy is based on whether or not tubulin is actively transported down the axons as assembled MT or as free subunits. Bass and Brown (1997) support the view that microtubules are nucleated at the centrosome within the neuronal cell body and then are released and actively transported in the form of microtubules and not as free units down the axon (Bass, 1997). They even suggest that it may be possible that during neuronal differentiation molecular motors shift from tethering MT to the centrosome to actively transport them down the axon. On the other hand, Joshi and Miller injected fluorescent tubulin into growing neurons of rat superior cervical ganglion and detected fluorescence primarily in the cell body and at the newly grown tip of the axon. No detectable fluorescence was seen within the proximal axon region despite the presence of a large reservoir in the cell body. They interpreted this result as the function of free tubulin assembling and disassembling from the free tips of MT distributed along the axon (Joshi, 1998). It seems that this controversy will remain until a model for tubulin transport within the axon is formulated. Ideally, more definitive evidence could be obtained by expressing a mutant form of tubulin that cannot assemble but this has not been achieved (Joshi, 1998).
1.2. III - MAPS

The microtubule associated proteins (MAPS) exert a profound influence on cells. They influence cell shape, the movement of cells, cell division and intracellular transport of vesicles and membrane bound organelles. Their function of affecting the MT dynamics is of more significance than of the isotopes themselves.

MAPs co-purify with tubulin through cycles of assembly and disassembly, although kinesin and dynein are associated with MT *in vivo* but do not recycle with them *in vitro* (Luduena and Roach, 1991). The common feature among MAPS is the presence of three to four imperfectly repeated sequences which bind and stimulate tubulin polymerization. Each of these repeats bind to one tubulin molecule suggesting that a single molecule of MAP can interact with several tubulins subunits (Luduena and Roach, 1991).

MAPs can be categorised into two major classes, structural MAPs and motor MAPs (Bloom, 1992; Olmsted, 1991).

1.2. III.a - Structural MAPs

Structural MAPs have been identified by their ability to bind to the surface of microtubules and to stimulate microtubule assembly *in vitro* (Olmsted, 1991). The mechanisms by which they promote assembly varies and can be further regulated by post-translational events. MAP-2 and Tau are the most abundant neuronal brain MAPs which stimulate MT assembly by promoting nucleation by decreasing the rate of microtubule depolymerization causing a reduction in the rate of MT shortening (Hamill et al., 1998). They carry a common carboxyl-terminal microtubule binding region comprised of imperfect repeats (Olmsted, 1991). The tau protein family is generated from alternatively spliced transcripts originating from one gene (Lee, 1990). The primary structure of tau protein consists of a
stretch of 31 or 32 residues that is imperfectly repeated 3 or 4 times in the carboxyl terminal half of the molecule. MT polymerization assays using one-repeat peptides has shown that such peptides are capable of promoting MT assembly and that the MTs formed are normal polymers (Lee, 1990). Also it has been shown that when tau cDNA is expressed in fibroblast cells in vivo it causes bundling of MTs (Kanai et al., 1989). The carboxyl terminal of tau protein has a hydrophobic ‘zipper’ sequence that mediates the cross-linking between tau molecules and MTs. This ‘zipper’ region has also been identified in MAP2 and indeed it’s been shown that MAP2 also bundles MTs (Lee, 1990). Tau is regulated by differential phosphorylation of specific Ser and Thr residues (Davis and Johnson, 1999). Phosphorylation is accompanied by decreased ability of Tau protein to bind and promote microtubule assembly. The extent to which microtubule binding capacity of tau is reduced by phosphorylation is highly dependent on which sites are phosphorylated; phosphorylation of tau just a few sites within the MT binding region virtually abolishes the association of tau with MTs whereas phosphorylation outside this region reduces but does not abolishes binding of tau to MTs (Davies and Johnson, 1999). Tau may also have a role in facilitating the cytoskeletal reorganisation that occurs during apoptotic cell death (DiTella et al., 1994). During apoptosis of neuronal PC12 cells tau protein was found to be hyperphosphorylated significantly reducing MT binding capacity. Furthermore, the MT binding function of tau from apoptotic cells was restored following dephosphorylation (Davies and Johnson, 1999; DiTella et al., 1994).

MAP-4 comprises a class of MAPs that have not been identified in brain. They have a molecular mass around 200KD, are thermostable and exists as complexes of related polypeptides (Olmsted, 1991). MAP-4 stimulates assembly and stability of microtubules by binding to the plus end. This function is abolished when this protein is phosphorylated (Hamill et al., 1998). EMAP is the major MAP found in sea urchin which promotes
microtubule dynamics by increasing elongation and shortening velocities at the plus end (Hamill et al., 1998). Furthermore MAP60 has been purified from plant cells showing *in vitro* an analogue function with animal systems.

1.2. III. b - Motor MAPS

Microtubule motor proteins are mechanochemical enzymes that generates the forces necessary for the movement of organelles, chromosomes and nuclei along MTs (Bloom, 1992). They can both stabilise and destabilise microtubules arrays, therefore regulating microtubule polymerization and depolymerization. Motor proteins have a property of moving quickly to the ends of MTs (such as centrosome, kinetochore, cell cortex or near the overlap zone of pole-to-pole MT) (Joshi, 1998). Members of the kinesin and dynein superfamilies are microtubule associated motor proteins (Hirokawa, 1998; Hirokawa et al., 1998). Motor proteins bind to microtubules transducing chemical energy, provided by ATP hydrolysis, into kinetic energy in the form of the movement of these motors along the microtubules (Hirokawa, 1998; Hirokawa et al., 1998).

A significant progress in identifying new members of motor proteins has been achieved with the development of novel techniques including the reconstruction of mitosis in *Xenopus* egg extract cell-free systems, immunodepletion, microinjection of antibodies, antisense oligonucleotides or recombinant protein and the development of model systems. This led to the identification of an increasing number of novel MAPs and the diversity and redundancy nature of them make their classification still unclear.

Several members of the kinesin superfamily of proteins (KIFs) have now been identified and characterised. They are involved in numerous cell biological processes including organelle transport, maintenance of endoplasmatic reticulum and intermediate filament
distribution, organisation of spindle MT, chromosome segregation, flagellar growth and positioning of developmental morphogens. All KIFs have motor domains (a region of ~350 amino acids, force generating element) with an ATP binding domain and a microtubule binding domain (Woehlke et al., 1997). The crystal structure studies indicate that kinesis consist of four domains: the catalytic core, the neck, the stalk and the tail. The catalytic core is a highly conserved globular domain, and the members of this family can be further classified according to the position of the catalytic domain as amino-terminal-type, middle type and carboxyl-terminal-type (Sablin, 2000). Kin N proteins are plus-end-directed MT motors whereas Kin C proteins move in opposite direction. Adjacent to the catalytic core is a stretch of amino acids that consist of the neck region. This region displays distinct three-dimensional structures and interacts with the catalytic domain to confer unique properties to different families of kinesis. The term motor domain is used to refer to the co-ordinated functional unit consisting of both the catalytic and neck regions (Sablin, 2000). The motor domain is followed by an α-helical stalk and a globular tail domain which mediate oligomerization.

In vitro motility assays have implicated two MT ATPases, kinesin and dynein, responsible for the MT-based transport (Hirokawa, 1996; Hirokawa et al., 1998). Identification and characterization of Kinesin superfamily proteins (KIFs) have been studied using neurons and polarised epithelial cells as model systems to understand the mechanisms underlying the transport of organelles and protein complexes (Hirokawa, 1996; Hirokawa et al., 1998). Kinesin is a tetramer composed of two heavy chains and two light chains forming a rod-like molecule. The heavy chain form heads that bind to MTs whereas the light chains form fan-like ends which are probably associated with membranous organelles(Hirokawa, 1996; Hirokawa et al., 1996). Well-characterised members of this superfamily include KIF1A, unc104, KIF1B, KIF2, KIF3, Kinesin II (KRP85/95), Fla10, Osm3, KIF4 and kinesin
Three major types have been identified and they are classified according to the position of their motor domain. Amino-terminal types KIFs include KIF1A, KIF1B, KIF3, KAP3 and Kinesin II. KIF1A and KIF1B are monomeric KIF which move along MT towards the plus end of these filaments, KIF1A being a neuron-specific motor and KIF1B a ubiquitously expressed motor involved in the transport of mitochondrial machinery (Goodson et al., 1997; Hirokawa, 1998; Hirokawa et al., 1998). KIF3 is composed of a KIF3A/KIF3B heterodimer and KAP3, forming a complex at 1:1:1 ratio involved in the anterograde transport of membranous organelles. KAP3 has two isoforms and binds to the tail of KIF3A/KIF3B domain regulating the membrane binding of KIF3 heterodimer, and their interaction is regulated by G protein GDP dissociation factor phosphorylation. KRP85 and KRP95 heterodimer are homologues of KIF3A and KIF3B respectively, proposed to be involved in the axonal anterograde transport they form a heterotrimeric complex (Kinesin II) with a KAP3 homologue (KAP115) likely to be required for the elongation of cilia. KIF4 forms a homodimer and moves towards the plus-end of MT abundant in juvenile tissues (Hirokawa, 1998; Hirokawa et al., 1998).

Middle type KIFs have the motor domain positioned in the middle of the molecule. KIF2 form a homodimer and is responsible for the anterograde transport of vesicles important for axonal extension in developing neurons (Goodson et al., 1997).

The carboxyl-terminal-type members exhibit MT minus-end directed motility. KIFC2 is the first KIF shown to be involved in dendritic transport in cells. It forms a homodimer with an ATPase activity localised in the cell body and dendrites (Hirokawa, 1998; Hirokawa et al., 1998).

Novel motors have been identified during cell division. Ncd and XTCK are minus-end-directed KIFs engaged in spindle assembly; Ncd is a chromatin-associated minus-end KIF (Amos and Hirose, 1997).
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On the other hand, members of the dynein superfamily have a very different mode of action. These cytoplasmic motor proteins work as complexes performing a broad range of cellular functions such as chromosome segregation, spindle formation, nuclear migration, Golgi positioning, retrograde membrane transport and functioning in the endocytic pathway (Hirokawa, 1998; Hirokawa et al., 1998). Cytoplasmic dynein is a minus-end-directed motor composed of two identical 500KDa heavy chains, three 70KDa intermediate chain, and four 53-59KDa light chains. Studies in culture cells derived from mice lacking dynein heavy chain showed fragmentation of the Golgi and dispersion of lysosomes and endosomes implying that cytoplasmic dynein is essential for the formation and positioning of Golgi complex and for the transport of endosomes and lysosomes to the cell center (Hirokawa, 1998; Hirokawa et al., 1998). Deletion of heavy chain dynein gene in fungi is not lethal suggesting the existence of a backup system for nuclear migration and division possibly involving Kinesin as the predominant motor (Yamashita and May, 1998a; Yamashita and May, 1998b).

Dynein vesicle transport is mediated by dynactins. Dynactin is a complex of nine polypeptides of which p150Glue is the largest and binds both to the intermediate chain of cytoplasmic dynein and microtubules. Phosphorylation of both reduces the rate of retrograde transport by decreasing the levels of membrane association.

Despite the discovery of a large number of members of these superfamilies many are still likely to be unknown. It is however clear that various kind of kinases, phosphatases and small G proteins contribute to the wide range of functions that these motor proteins carry out.

1.2.IV - MICROTUBULE AND DISEASE
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Alzheimer's disease is one of the commonest neurological diseases and the fourth leading cause of death in the developed world (Duff and Hardy, 1995). The pathology of the disease is complex but there are two hallmarks: the presence of a neuritic plaque—an extracellular lesion consisting of β-amyloid with other components and the neurofibrillary tangle, consisting largely of abnormally phosphorylated tau protein. The neurofibrillary tangles are composed of intraneuronal 10nm filaments forming paired helical filaments (PHF). These filaments are a pathological indication of Alzheimer's disease and tau protein is a component of PHF (Lee, 1990).

Unfortunately, much of research has been focused on the hypothesis that the amyloid deposition is central to the pathological process of dementia. Mouse models have been made by mutation of the β-amiloid precursor protein gene in order to understand the relationship of the various aspects of this pathology. Unfortunately, very little is known as to the role of Tau protein in Alzheimer's disease.

1.3 – Intermediate filaments

Intermediate filaments (IFs) are responsible for maintaining cellular integrity and the mechanical properties of the cytoplasm (Chou et al., 1997). They are dynamic components of the cytoplasmic and nuclear cytoskeleton reversibly linking the plasma membrane to other cytoskeletal components modulating cell shape and conferring resistance to mechanical stress (Herrman and Aebi, 2000; Houseweart and Cleveland, 1998). IFs are a superfamily of predominantly elongated linear fibrous proteins which upon polymerization form filaments of 10nm in diameter related by the homology of a central core, the α helical domain, which interwine in a coil-coil fashion to form the subunit structure of these filaments (Fuchs and
IFs can assemble *in vitro* in the absence of auxiliary proteins or factors indicating that all necessary information to form filaments are present within the primary sequence of the IF polypeptide (Fuchs and Weber, 1994).

Members of the IFs superfamily exhibit cell-type specific and often complex patterns of expression. In contrast to actins and tubulins which are highly evolutionary conserved, IF proteins sometimes share as little as 20% sequence homology. This observation, together with the association of IFs with a range of IF associated proteins (IFAPs), accounts for their diversity and the performance of specialised cellular functions (Fuchs and Weber, 1994; Herrmann and Aebi, 2000).

The development of transgenic and gene deletion mice systems and specific peptide inhibitors capable of disrupting IF network have contributed greatly not only to the identification of new IFAPs but also to understanding the novel mechanisms underlying cellular functions (Houseweart and Cleveland, 1998). Mimetic peptides derived from the amino acid sequence of the helix 1A domain of IF protein chains have been microinjected into cultured fibroblasts and shown to cause a rapid disassembly of the IF network. BHK-21 and 3T3 cells were injected with vimentin 1A peptide at 1:1 ratio and the result was analysed by indirect immunofluorescence with vimentin antibody. This experiment showed that upon microinjection, vimentin IF disassembled into small oligomeric complexes and monomers resulting in a dramatic alteration in cell shape from the typical spread to a rounded configuration (Goldman et al., 1996).

The dynamic organisation of IFs *in vivo* is mediated by protein phosphorylation and by IFAPs which mediate the connection between IFs and other cytoskeletal elements (Chou *et al.*, 1997). Protein phosphorylation has emerged as a major regulator of IF assembly and has been associated with increased pools of IF subunits and enhanced rates of exchange between soluble and polymerised forms of IFs. Although these ideas are still speculative, protein
kinase p34\textsuperscript{cdk2} has been shown to phosphorylate plectin and cause its dissociation from vimentin during mitosis (Houseweart and Cleveland, 1998).

1.3.1 – IF STRUCTURE AND CLASSIFICATION

IFs are composed of proteins that share a common sequence and structural features (Parry and Steinert, 1992). All IF proteins have a central $\alpha$-helical domain, the rod, which is flanked by non-helical head (amino-end) and tail (carboxyl-end) domains (Fuchs and Weber, 1994). The $\alpha$ helical domain of two polypeptide chains interwine in a coil-coil fashion. This domain contains a heptad structure with hydrophobic amino acids such that the first and the fourth of every seven residues are apolar providing a hydrophobic seal on the helical surface allowing for the coiling configuration (Fuchs and Weber, 1994; Parry and Steinert, 1992). The IF $\alpha$-helical rod is used to classify IF polypeptides and they are referred as helices 1A, 1B, 2A and 2B; the identity among all IF proteins being particularly high at start of helix 1A and near the end of helix 2B. The rods of types I-IV are \~{}350 aa long whereas the rods in lamins are 356aa long due to an insertion of 6 heptads in the helical domain 1B (Fuchs and Weber, 1994). A non-random distribution of acidic and basic residues are also found within the rod domain that stabilises the helice formation and electrostatic interactions with other structures (Fuchs and Weber, 1994; Herrmann and Aebi, 2000).

The non-helical tail domains, N-terminal and C-terminal tails are not conserved and vary in length (Heins and Aebi, 1994). The greatest variation occurs in the tail ranging from 9 residues in K19 to 1491 in nestin (Fuchs and Weber, 1994). The removal of desmin tail segment, deletion of vimentin tail and mild proteolysis of keratins I and II type tails has a
minor effect on filament assembly in vitro suggesting that tails may play a role to control lateral associations and filament diameter (Parry and Steinert, 1992).

IFs of various cell types are chemically heterogeneous and consist of different proteins. There are about 60 IF proteins grouped into four sequence homology classes (Types I to VI): keratins, desmin, vimentin, neurofilament protein (NF), nuclear lamins, glial acidic protein (GFAP) and beaded filaments (Herrmann and Aebi, 2000; Houseweart and Cleveland, 1998).

1.3.1.a - Keratins: Type I and type II IF proteins

Keratins are the largest and most complex group of obligatory heteroforming IF proteins and are essential for the maintenance of mechanical properties of epithelial tissues (Chou et al., 1997). There are at least 30 keratins ranging from 40 to 67KDa and they are divided into type I and II classes according to their sequences (Fuchs and Weber, 1994; Herrmann and Aebi; 2000). Type I are acidic (pKi=4-6) and is composed of eleven epithelial keratins K9-K20 and four hair keratins Ha1-Ha4. Type II are basic (pKi=6-8) including eight epithelial proteins K1-K8 and four hair keratins Hb1-Hb4. Both types are expressed differentially in different tissues at various stages of development. Epithelial cells synthesize keratins in a pairwise fashion, the most broadly expressed example is K5 and K14 in stratified squamous epithelia (Fuchs and Weber, 1994) and K8 and K18 found in simple epithelia (Chou et al., 1997). Keratins assembly in vitro in a 1:1 ratio of any combination of type I and type II generating filaments with distinctive properties that suit tissue-specific structural requirements of flexibility, tensile strength and cellular dynamics. Also, the mechanical strength of epidermal tissues is the result between the association of keratin IFs across cell boundaries via desmosome and hemidesmosome cell specialised junctions (Green & Jones, 1996). These cell surface attachment sites mediate the anchorage of IF at sites of cell-cell and
cell-substrate contact creating a transcellular network necessary to resist mechanical stress (Green & Jones, 1996).

**1.3.1.b - Desmin and Vimentin: Type III IF protein**

Vimentin is the most widely expressed IF of this class produced by mesenchymal cell types and a variety of transformed cell lines and tumors. Desmin is a muscle specific homopolymer forming IF protein expressed in cardiac, skeletal and smooth muscle (Houseweart and Cleveland, 1998; Milner et al., 1996). In developing mammalian muscle desmin is initially co-expressed with vimentin but upon terminal differentiation vimentin is down regulated and desmin accumulates around the Z discs of maturing cells. Recent studies using gene disruption to engineer mice lacking desmin have shown that although desmin-deficient mice developed weaker skeletal muscles at the early stages of muscle differentiation, cell fusion occurred normally indicating that desmin is not crucial during myogenesis but play crucial role in maintaining the structural integrity of muscle cells (Houseweart and Cleveland, 1998; Chou et al., 1997; Milner et al., 1996).

**1.3.1.c - Neurofilaments and alpha-internexin: Type IV IF proteins**

Neurofilaments (NFs) are the predominant type of IF in most adult neurons of both central and peripheral nervous system (Lee and Cleveland, 1994). Three types of neurofilaments are coexpressed in neurons: Human NF-L (light), NF-M (medium) and NF-H (heavy) of ~ 62,102 and 110KDa respectively. These NFs control axonal diameter during synapse formation, an observation that has been validated in studies using transgenic mice lacking NFs (Marszalek et al., 1996; Xu et al., 1996; Lee and Cleveland, 1994). This transgenic mice was designed so that each subunit composition was altered. The increase of
each NF subunit inhibited radial axonal growth whereas the increase of both NF-M and NF-H reduces growth more severely (Xu et al., 1996). The mice displayed decreased axonal growth, delayed regeneration after nerve injury and 15-20% loss of motor and sensory axons at 2 months of age (Cote et al., 1993).

Furthermore, it seems that increased axonal radial growth requires a balanced ratio of NF-L subunits and of the cross-bridge-forming NF-M and NF-H subunits which make NFs obligate heteropolymers. The mechanism by which this is achieved remains to be elucidated. It has been demonstrated, however, that when NF transgenic mice express NF-H four times the endogenous levels, axonal transport is slowed resulting in the accumulation of NFs in the cell bodies and swollen axons of these mice. It seems overexpression leads to assembly of more NFs that can be effectively transported. These NFs accumulations are now known to be a common hallmark of several human neurodegenerative diseases (Houseweart and Cleveland, 1998).

Alpha-internexin is also a member of type IV IF protein of 66-70KDa. It is expressed in neurons although it’s role seem to be at embryonic development. On contrary to NFs, alpha-internexin protein is able to form homologous polymers of 10nm filaments (Fuchs and Weber, 1994).

1.3.I.d - Nuclear lamins: Type V IF proteins

Although highly conserved and closely related to cytoplasmic IFs, nuclear lamins are found exclusively in the nucleus. Lamins form a fibrous structure, the nuclear lamina, that lie at the interface between the nuclear envelope and the nucleoplasm providing a framework and mechanical support for the nucleus (Fuchs and Weber, 1994, Spann et al., 1997). Apart
from being the major constituent of the nuclear lamina, nuclear lamins are also found in foci in the nucleoplasm, distinct from the peripheral lamina (Spann et al., 1997).

In vertebrates, five lamins have been reported and are divided into types A, B and C according to expression pattern and exon positions (Spann et al., 1997). Early vertebrates embryos express only B-type lamins, whereas somatic cells synthesize B-type, A-type and C-type lamins. A-type lamins play a specialised role in cellular differentiation and chromosome organisation. All lamins have sequences that signal their transport to the nucleus. A recent method to study the nuclear assembly system has been developed using *Xenopus laevis* egg extracts into interphase stage of mitotic division. The addition of truncated A-type lamin disrupts the formation of nuclear lamina and decreased the ability to replicate DNA (Spann et al., 1997).

### 1.3.5. *Glial fibrillary acidic protein and Nestin: Type VI IF proteins*

This group of IFs comprises members of the IF superfamily based on sequence and structure comparisons that do not classify in previous major types (Herrmann and Aebi, 2000).

Glial fibrillary acidic protein (GFAP) is expressed in astrocytes of the central nervous system, enteric glia and myelin-forming Schwann cells. Most astrocytes initially express vimentin but switch to GFAP type once they mature. Results from studies using GFPA-deficient mice are controversial indicating that possibly expression of vimentin may compensate for the absence of GFPA filament assembly in these mice, therefore buffering the effect of GFPA filaments absence alone (Houseweart and Cleveland, 1998).

Nestin show a 33% homology to type II IF proteins but it has a nonhelical tail that resembles more of a type IV protein (Fuchs and Weber, 1994).
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1.3.1f - Filensin and Phakinin

Filensin and plakinin are two lens specific members of a novel class of IFs composed of beaded filaments (BFs) (Gounari et al., 1997). This class of IF proteins have a distinctive nodular appearance and coassemble to form a beaded filamentous network underneath the plasma membrane of the lens fiber cells, the BFs (Georgatos et al., 1997). This beaded fibers were first observed in crude fractions of chicken lens by Maisel and Perry in 1972 (Maisel and Perry, 1972). They identified two polypeptides which now is known to be the building blocks of BFs: CP95 and CP49 now renamed filensin and phakinin respectively. Both polypeptides share structural homology and are able to co-polymerise into filaments in vitro. By scanning transmission electron microscopy it was concluded that BFs comprise a core filament composed of four phakinin protofilaments surrounded by a shell of four heterotypic filensin/phakinin protofilaments responsible for the 'beaded appearance of these filaments (Georgatos et al., 1997). Furthermore these filaments assemble both in vivo and in vitro at a 1:3 filensin/phakinin ratio. It is also known that these two proteins evolved from two different subfamilies of IF proteins, typeIII (filensin) and type I (phakinin) thus yielding a unique structure (Gounari et al., 1997).

1.3.II - IF ASSEMBLY

IF form filaments spontaneously, therefore, assembly does not require additional auxiliary proteins or an energy source (Stewart, 1993). The generation of intact IF is the result of a series of separate assembly steps involving different levels of structure. Single polypeptides are produced on ribosomes which upon molecular recognition form homo or heterodimers. The first step in filament assembly is the formation of a protofilament in which two polypeptide chains coil around a common axis in a left-handed parallel fashion. Most IF
proteins form homodimers eg. Lamins in a head-to-tail fashion. Keratins have, however, a unique assembly in which heteropolymers are formed with the assembly of type I and type II polypeptide, accounting for the heteropolymeric nature of these filaments (Fuchs and Weber, 1994). It seems that residues within the heptad region are responsible for the specificity of polypeptides pairing (Stewart, 1993). Although types I and II are obligatory heteropolymers, they do not co-assemble with other types of IFs (Herrmann and Aebi, 2000). Another heteropolymer-forming IF protein pair is found in the beaded filaments of lens fiber cells where phakinin and filesin co-polymerize at a 3:1 molar ration (Gergoratos et al., 1997).

Dimers (protofibril) of cytoplasmic IF proteins associate to form tetramers (protofilaments), and this association is based on the rod domain. Three distinct antiparallel arrangements of dimers are described: near half-staggered coil 1B alignment (Desmin, keratin), near half-staggered coil 2B alignment (Keratins, desmin) and dimer without stagger (prevalent form in Keratins) (Fuchs and Weber, 1994). Four protofibrils compose the 10nm diameter IF. The mechanisms underlying the packing and association between protofilaments remain to be solved. It is now clear that the head and tail ends of IFs have no contribution to IF assembly, in fact it has been shown that the removal of the tail of both type III and IV filaments may actually accelerate assembly (Fuchs and Weber, 1994; Herrmann and Aebi, 2000).

The observation of the dynamic status of IF reorganisation in response to cell cycle or cell differentiation inferred phosphorylation as a possible mechanism for regulation of IFs assembly and disassembly. In vivo, mitosis mediated reorganisation of vimentin filaments and nuclear lamina disassembly coincides with a transient increase of IF phosphorylation. Both mechanisms are controlled by a cdc2 kinase-mediated phosphorylation cascade (Peter et al., 1990). Lamin A is specifically phosphorylated at serines 22 and 392 (Heald and McKeon, 1990). When these serines are removed by mutagenesis it leads to inhibition of lamina
breakdown during mitosis. *In vitro* phosphorylation of vimentin serine 55 residue causes disassembly of vimentin filaments (Fuchs and Weber, 1994). Furthermore, IF proteins have been shown to be excellent substrate for several transduction effectors such as protein kinase C, cAMP-dependent protein kinase and CaM kinase II (Chou *et al*., 1997).

**1.3.III - INTERMEDIATE FILAMENTS ASSOCIATED PROTEINS (IFAP)**

Perhaps the most exciting new development in the IF field has been the recognition that IFs bind to specific proteins, the IFAP, which work as cross-bridges binding a variety of cytoskeletal components of the cytoskeleton of a variety of cell types generating a three-dimensional cytoplasm and suggesting a potential role as dynamic regulators of IF network integrity. IFAPs are also gaining more attention as more diseases in human and in mice are shown to arise from mutations in these proteins (Houseweart and Cleveland, 1998).

**1.3.III.a – The plakin family**

Desmoplakin, plectin, bullous pemphigoid antigen 1 (BPAG1) and envoplakin are large proteins, members of the plakin IFAP family. They are all sequence related proteins that can link IF not only to the plasma membrane but also to different cytoskeletal networks (Ruhrberg *et al*., 1996). Although they all been discovered by different ways at different times, they have been found localised to intermediate filaments and their plasma membrane anchorage site. Plakins' structure have been predicted based on the primary structure of desmoplakin: it is likely to consist of an amino termini composed of alpha helices and a carboxyl termini consisting of alpha-helices and beta sheets both separated by a central coiled-coil rod domain.
The structure of human plectin, BPAG1 and envoplakin genes have been determined and found to contain a large number of small exons increasing the potential for alternative splicing therefore increasing functional versatility (Foisner et al., 1991a; Foisner et al., 1991b; Foisner and Wiche, 1991; Ruhrberg et al., 1997; Ruhrberg and Watt, 1997).

Desmoplakin was identified as a constituent of desmosomes isolated from stratified epithelia which migrates in a SDS-PAGE as a protein doublet of 240-285KDa (desmoplakin I) and 210-225KDa (desmoplakin II). DPI and DPII are splice variants; DPI mRNA is expressed in all epithelia and cardiac muscle whereas DPII mRNA is not detectect in cardiac muscle (Ruhrberg et al., 1997; Ruhrberg and Watt, 1997; Yang et al., 1996). Molecular mapping studies using transient transfection of constructs containing specific domains indicated that the carboxyl terminus interacts directly with IFs (Green & Jones, 1996).

Plectin is the most abundant cytoskeletal cross-linker interacting with other proteins such as vimentin, glial fibrillary acidic protein, keratins, lamin B, MAPs and alpha-spectrin (Wiche, 1989a; Wiche, 1989b). Furthermore, immunoelectron microscopy revealed that plectin forms cross-bridges between IFs and microtubules, links IFs and actin filament bundles, associates with myosin filaments in cultured cells and decorate vimentin filaments (Houseweart and Cleveland, 1998). Three alternatively spliced plectin mRNAs have been identified so far. It remains to be known whether any of these variants have a tissue specific expression (Ruhrberg et al., 1997; Ruhrberg and Watt, 1997). Recently it has been found that mutations in the gene for plectin cause the human disease muscular dystrophy with epidermolysis bullosa (Smith et al., 1996). This inherited disease is characterised by muscle degeneration and skin blistering due to a failure to anchor the cellular IF network to the plasma membrane via hemidesmosomes (Green & Jones, 1996). Analysis of human and rat plectin sequences showed the existence of a putative amino-terminal actin binding domain (Houseweart and Cleveland, 1998). A region that is essential for the interaction between
plectin and vimentin IFs have been sequenced and found to be at the carboxyl-terminal. Interestingly, this domain also contain a nuclear localisation sequence (NLS) suggesting that vimentin IFs may serve as docking sites for proteins containing the NLS signal and that plectin may also function as a regulator of the nuclear transport apparatus (Nikolic et al., 1996; Chou et al., 1996). There are several proteins which are suspected to be plectin variants as the monoclonal antibody for plectin recognises the same antigen, however, determination of their primary sequence is vital to stablish a relationship of these variants and plectin.

BPAG1 is a 230KDa hemidesmosomal protein that was first identified in patients with the autoimmune disorder bullous pemphigoid (Yang et al., 1996). There are two splice variants of BPAG1, the neuronal isoforms (BPAG1n1/dystonin and BPAG1n2) expressed in parts of peripheral and central nervous system and an epidermal form (BPAG1e) expressed in the epidermis. The neuronal isoforms differ from BPGA1e by the presence of a functional actin-binding domain and a neurofilament binding sites (Yang et al., 1996; Ruhrberg and Watt, 1997). The ability of BPAG1n to connect the actin cytoskeleton and neurofilaments may be essential for many sensory neurons, mice with a mutation for BPAG1n showed signs of neuronal degeneration (Ruhrberg & Watt, 1997). Recent knockout studies revealed that basal epidermal cells lacking BPAG1e have hemidesmosomes with seemingly normal structure but severed connections of keratin IF cytoskeleton (Chou et al., 1997).

Envoplakin is the most recently identified member of the plakin family. Envoplakin is structurally homologous to desmoplakin and found to be present at desmosomes suggesting that together with desmoplakin it can be involved in the process of anchoring keratin filaments to desmosomes. Functional studies with individual envoplakin domains still remain to be carried out to verify it’s function (Ruhrberg et al., 1996). It is known, however, that envoplakin is not a constitutive component of desmosomes and because it is upregulated
during keratinocyte differentiation it may be that envoplakin is involved in the maturation of desmosomes (Ruhrberg et al., 1996).

1.3.III.b - Epinemin

Epinemin is a 44.5Kda protein associated with vimentin filaments (Lawson, 1983). This IFAP was identified by a monoclonal antibody and found to be intermittent along vimentin filaments by electron microscopy.

1.3.IV – IF AND DISEASE

IFs display a tissue specific and developmentally regulated pattern of expression (Fuchs and Weber, 1994; Houseweart and Cleveland, 1998; Milner et al., 1996). Mutations on IF genes can lead to dominant expression giving rise to genetic diseases.

Most of the genetic disorders involving IF have been found to lead to blistering skin diseases involving keratin genes.

Epidermolysis Bullosa Simplex (EBS) is a rare autosomal dominant skin disease often present at birth. The disease is characterised by appearance of blisters in the skin. There are three forms of EBS: a mild form (Weber-Cockayne) in which blistering occurs at hands and feet, an intermediate form (Koebner) and a severe form (Dowling-Meara) characterised by the occurrence of clumps of keratin within the basal cell cytoplasm (Coulombe et al., 1991a; Coulombe et al., 1991b). EBS result from point mutations on K14 and K5 genes and the severity of EBS phenotype is related to the degree to which each gene is mutated (Coulombe et al., 1991a; Coulombe et al., 1991b; Fuchs and Weber, 1994; Houseweart and Cleveland, 1998). D-M EBS patients have a single substitution (Arg- Cys/His) in the conserved amino
end of the K14 alpha-helical rod or a single substitution (Glu-Gly) in the conserved carboxyl end of the K5 rod. When these point mutations are expressed in transfected human epidermal cells it causes shortening of IF. The degree of severity is possibly because mutations affect domains which are involved in multiple contacts within IFs. Epidermolytic Hyperkeratosis (EH) is an autosomal dominant disease, with point mutations in the rod domains of K1 and K10 (Arg-His/Cys). Epidermic Palmoplantar Keratoderma (EPK) is the result of a point mutation in the gene encoding K9 (Arg-Trp/Gln), a keratin specifically expressed in the suprabasal layer of this type of skin. Remarkably all the three skin related conditions are result of mutations of an argenine amino acid.

The mechanism by which keratin mutation cause cytolysis remains to be elucidated, however, it's been hypothesized that cells without a proper IF network become fragile and prone to breakage upon mechanical stress suggesting that keratin filaments impart a mechanical framework to an epidermal cell (Fuchs and Weber, 1994).

In addition to numerous mutations of epithelial keratins, mutations in cornea specific keratins have also been described. Meesmann corneal dystrophy is a disorder characterised by fragility of the corneal epithelium due to missense mutations at K3 and K12 (Houseweart and Cleveland, 1998).

At the last count 14 of the 20 epithelial keratin genes and one of the 10 α-keratin genes had been shown to harbour mutations causing human genetic disorders. With the aid of novel genetic techniques it will not be long for the discovery of additional mutations which will hopefully elucidate the mechanisms underlying the function of IF in the cells. Recently, in order to investigate the function of desmin (encoded by single gene) in muscle tissues, a desmin null mice was generated through homologous recombination which showed a multisystem disorder involving cardiac, skeletal and smooth muscle. Histological and
electron microscopy analysis of both heart and skeletal tissues revealed severe disruption of muscle architecture and severe cell degeneration (Milner et al., 1996).

1.4 - Microfilaments

Microfilaments (MFs) provide the physical basis for the structure and dynamic properties of the cell cytoskeleton and the muscle sarcomere and serve as scaffolding of various cellular events. MFs are formed by the assembly of actin monomers into filaments of 6nm in diameter. Assembly and disassembly of MFs is a highly organised event that is regulated and modulated by its association with a growing number of actin binding proteins (ABPs), metal ions and ligands such as cytochalasins (Pollard and Cooper, 1986; Carlier, 1998; Puius et al., 1998).

The vast variety of cellular functions carried out by MFs such as cell motility, cytokinesis, secretion, protein sorting, mRNA localization, spatial ordering of glycolysis and signal transduction and generation of contractile force in both muscle and nonmuscle cells, is the result of direct link between actin isoforms and actin binding proteins (ABPs) (Rubenstein, 1990, Carlier, 1998; Weber, 1999).

1.4.I - ACTIN STRUCTURE

Actin is the major protein of MFs. It is a ubiquitous protein found throughout the eukaryotic world regarded as one of the most versatile and abundant protein in cells, found as high as 15% of the total cell protein content, essential for the maintenance of complex structural and dynamic functions of the cell (Reisler, 1993; Carlier, 1998).
Actin was originally isolated by Straub in 1942, from a crude rabbit muscle preparation followed by protein purification. In the absence of salt, actin exists as a globular protein, G actin. G actin is a single polypeptide chain of 375 amino acids residues with a molecular weight of 43KDa.

In the presence of salt and ATP, the globular proteins polymerizes into a highly asymmetrical fibrous protein, F actin (filamentous actin). Filamentous actin structures posses unique biophysical and biochemical properties and are required for cell locomotion, cell division, compartmentalization and morphological processes (Puius et al., 1998). In animal cells there are at least three characteristic levels of organisation of actin filaments: stress fibers and bundles (antiparallel arrays that are homologous to the myofibrillar organisation), filopodia and microvilli (parallel arrays that form protrusive structures at the cell surface) and arrays of filaments underlying the plasma membrane (Jockusch et al., 1995).

1.4.1. a-Actin atomic structure.

The atomic structure of G-actin molecule was elucidated by Kabsch et al (Kabsch et al., 1990) by X-ray analysis. In order to prevent actin polymerization which prevents the formation of crystals—a necessary requirement for the three dimensional structure by X-ray analysis—rabbit skeletal muscle actin was coupled to bovine pancreatic deoxyribonuclease I both at ATP and ADP forms at a 1:1 complex. The results showed that the actin molecule consists of two domains with a nucleotide (ATP or ADP) and an associated calcium ion bound in the cleft between the two domains. Furthermore, they observed that each domain consisted of two subdomains. Subdomain I contains residues 1-32, 70-144, 338-372 and both the carboxyl and amino terminals. It consists of five-stranded β-sheets surrounded by five α-helices. A hydrophilic residue Ser 344 forms a hydrogen bond with the main chain carbonyl
group of Asp 24. The authors speculate that this helix may be responsible for the binding of ABPs.

Subdomain II contains residues 33 to 69 and consist of a three-stranded anti-parallel β-sheets with an α-helix connecting the two domains.

Subdomain III contains residues 145-180 and 270-337 which consists of a five-stranded β-sheet surrounded by three helices. The β-sheet composition is identical to that in subdomain I. This observation strongly suggest that actin has evolved by gene duplication and that although at primary level they do not share identity it may be possible that soon after the duplication event each domain evolved to carry out different functions (Kabsch et al., 1990).

Subdomain IV contains 181 to 269 residues and consist of a two-stranded anti-parallel β-sheet and four α-helices. The helix is followed by a 10 residue loop and another helix from subdomain III (residues 274-282) believed to be involved in a contact across the helix axis with another pair of actin molecules in the F-actin form.

The binding of DNase I bridges subdomains II and IV and reduces the ATP-ADP exchange rate. However, they showed that ATP and ADP are involved in a large number of interactions. In the ATP structure, the β and δ phosphates form hydrogen bonds with residues 14-16 and 157-159 respectively, whereas in the ADP form, the β phosphate bonds with 15-16 residues (Kabsch et al., 1990).

They also found a calcium ion localised in a deep hydrophilic pocket formed by the phosphate groups of ATP or ADP and actin residues. The exact position can only be hypothesised as actin is known of have a single high affinity site (HAS) for a divalent cations and several low affinity sites of mono- di- or trivalent cations. For rabbit skeletal muscle
actin there are four low affinity calcium binding sites, one of which is near an acidic residue at the N-terminus.

1.3.1. **b - Actin filament structure**

Following this discovery the authors also engaged in producing an atomic model for the filament structure of actin. They generated a filament model by comparing three-dimensional maps of vertebrate muscle thin filaments decorated with myosin heads (SI) obtained by cryoelectron microscopy coupled with image analysis in different ways with the appropriate helical symmetry (Milligan et al., 1990). The resulting filament model consist of monomer binding along the filament helix with a maximum diameter of 90-95 Å with subdomain I and II lying further from the filament axis then II and IV. There are both longitudinal and diagonal contacts between monomers and each actin subunit interacts with four of its neighbours. They also proposed that the only position where the ADP-riboylated actin could be accommodated is at the barbed end whereas the point end is involved with DNase I binding (Milligan et al., 1990).

1.4.11-**ACTIN ISOFORMS**

The dynamic nature of functions that actin carry out in muscle and non-muscle cells is due to the existence of many actin isoforms (Herman, 1993). The actin gene family encodes a number of structurally related but functionally distinct protein isoforms. Multiple forms of actin, or isoactins, have been described in a number of organisms and are the product of different genes (Rubenstein, 1990). Isoactins are designated as α, β and δ according to their mobilities on isoelectric focusing gels; the α being the most acidic with PIs of 5.40, 5.42 and
5.44 respectively (Garrels and Gibson, 1976). Isoforms are expressed in muscle and non-muscle cells in temporally and spatially regulated patterns and their expression is tissue and cell type specific and conserved across species (Herman, 1993). Mammals and birds synthesize eight different isoforms of actin in a tissue specific fashion. The muscle isoactins are highly conserved, smooth muscle δ isoactin differs from skeletal muscle α isoactin at only 6 of 375 amino acid residues. To study the specific function of each isoform in a tissue, isoforms were quantitated based on differences of their N-terminal peptides (Vandekerckhove and Weber, 1978). The N-terminal region of actin has a cluster of acidic residues irrespective of actin species which is rich in negative charges (Sutoh et al., 1991). In order to investigate the functional importance of the N-terminus, a series of experiments were carried out by Vandekerckhove and Weber in which they compared the amino terminal sequences of actin isoforms in different cell types and tissues (Vandekerckhove and Weber, 1978). From these experiments they suggested that among higher vertebrates, actin divergence involves tissue rather than species diversity. All α isoforms and one δ isoform are restricted to muscle, whereas nonmuscle cells contain the β and δ isoforms (Herman, 1993; Hofer et al., 1997).

The structural and functional role of the two cytoplasmic actin isoforms β and δ remain to be elucidated. These two isoforms were analysed by immunostaining in chicken auditory hair cells (Hofer et al., 1997). These are highly specialised cells with a distinct architectural features with three major actin assemblies: the stereocilia, cuticular plate and zonula adherens ring. The results indicated that not only the β and δ genes encode functionally distinct cytoarchitectural information, the δ isoform was the ubiquitous actin isoform whereas the β isoform displayed a very restricted distribution to the stereocilia (Hofer et al., 1997).

These isoforms have now been divided into two classes according to post-translation modifications acetylation-dependent which occur at the N-terminus of each isoform: class I
isoactins comprises the non-muscle isoforms, whereas class II isoactins comprises muscle isoforms. In class I isoactins, the N-terminus is acetylated early in translation and upon completion of translation N-Ac-Met is removed and either Asp or Glu is acetylated to yield the mature form of actin. In class II, the genes encoding muscle isoactins specify a Met-Cys-Asp (Glu) at the N-terminus. Met is removed early in translation and Cys is acetylated. Upon translation completion, Ac-Cys is removed and the new N-terminus is acetylated (Rubenstein, 1990). Many experiments have been designed to obtain information about the functional importance of the negatively charged N-terminal. By site-directed mutagenesis the aspartic acid residues in the N-terminal of Dictyostelium actin were replaced with histidine residues, producing mutants with a reduced ATP dependent actin-myosin interaction, indicating that disruption of the negative cluster at the N-terminal resulted in loss of activation of the myosin ATPase reaction and also loss of the myosin driven sliding movement of actin filaments (Sutoh et al., 1991). Following these observations, Cook et al. studied the structure-function relationship of the actin N-terminal region in vivo and in vitro by site directed mutagenesis of yeast Saccharomyces cerevisiae actin (Cook et al., 1992). They developed two mutant actins: the first one, DNEQ mutant, in which Asp and Glu have been changed to Asn and Gln and in vivo this actin retained its initiator Met and the N-terminal was acetylated, and a second one, ADSE actin mutant, in which the Asp-Ser-Glu tripeptide was completely deleted and in vivo this mutant protein is not acetylated yielding a net positive charge. Both mutants were unable to activate the myosin S1 ATPase activity as well as wild type actin. In vitro analysis also showed that the removal of the N-terminal negative cluster promoted the bundling of actin filaments. Furthermore, the in vivo function of these mutants was assayed by the ability of promoting movement of secretory vesicles. The results indicated that the mechanism for vesicle transport requires actin N-terminal negative charges for maximal efficiency (Cook et al., 1992). Since then yeast and Dictyostelium have been used as model systems for the
expression of actin mutants as these systems provide means of obtaining functional actin in milligram quantities (Reisler, 1993). Yeast has only one single gene for actin and although the actin from yeast does not activate the myosin ATPase as well as rabbit α-skeletal actin, in vitro polymerization and DNase I binding properties are reasonably similar (Nefsky and Bretscher, 1992). Actin from the yeast *Saccharomyces cerevisiae*, encoded essentially by ACT1 gene, is 89% identical to mouse cytoplasmic actin (Lees-Miller et al., 1992).

Taking into account that both N and C-terminus are located in subdomain I and that both were thought to be involved in the binding of actin with other proteins, amino acids from the C-terminal end of actin were deleted in order to investigate the effect of these residues on actin. Mutants lacking C-2, C-3,C-10, C-20, C-30 or C-40 amino acids were constructed by inserting termination codons into full length cDNA of α skeletal muscle actin. Full length and truncated mutants were transcribed and translated in the presence of $^{35}$S methionine. The $^{35}$S-labeled actin were tested for the ability of wild type and mutants to coassemble with carrier actin, bind DNAse I, bind myosin S-1, bind a 27KDa proteolytic fragment of α-actinin and the ability to incorporate into myofibrils in vitro (Xia and Peng, 1995). The results showed the deletion of C-10 significantly decreased coassembly, binding of DNAse I and incorporation into myofibrils but did not reduced binding to myosin S-1 or 27KDa proteolytic fragment of α-actinin. Deletion of 20 or more amino acid residues, however, virtually abolished all normal actin functions tested (Xia and Peng, 1995). The authors suggest that removal of 356-365 residue affects formation of hydrogen bonds between actin segments and also that removal of 366-372 residues affect the structure or orientation of DNAse I binding loop therefore affecting the binding of DNAse I, coassembly with wild type and incorporation into myofibrils (Xia and Peng, 1995).
The discovery of ACT2 gene in yeast *Schizosaccharomyces pombe* has revealed a new class (class III) of isoactins (Lees-Miller et al., 1992). ACT2 codes for a larger (427 amino acids) and more basic protein with only 47% sequence identity to ACT1 yeast actin (Schwob and Martin, 1992). The difference in size is due to 3 and 11 amino acids insertions at residues 42 and 43 respectively, affecting the formation of the looping domains (found in class I and II) involved in the nucleotide binding cleft of the molecule. As result, class III isoactins contain only 14 out of 17 amino acids strictly conserved for the binding of nucleotide, producing an isoform that fold into proteins that posses ATP and metal binding properties but does not necessarily form 6nm diameter filaments (Schwob and Martin, 1992).

**1.4. III - ACTIN KINETICS**

The kinetics of actin polymerization still remain a focus of much interest (Weber, 1999). Cytoplasmic actin filaments may polymerize and depolymerize more frequently than skeletal muscle thin filament since about 50% of the total actin content in a non-muscle cell is of unpolymerized actin whereas most of the actin in muscle is polymerized (Cooper & Pollard, 1982). This large pool of actin monomers enables the cell to rapidly and efficiently respond to extracellular stimuli.

The polymerization of actin monomers into filaments can be divided into four steps: fast monomer activation, rate-limiting nucleation, elongation and annealing (Pollard, 1986a; Pollard, 1986b). This process implies a pseudo-first order assembly kinetics leading to a steady state resulting in a sigmoidal curve. The exponential growth of filaments is preceded by a lag phase which comprises the first two steps of activation and nucleation, followed by an exponential phase reflecting elongation of filaments leading to a plateau when the filaments' growth reaches a steady state. Each step depends on the solution condition
including ionic strength, presence of divalent cations, PH and temperature (Cooper & Pollard, 1982). Actin assembly is accompanied by stoichiometric hydrolysis of ATP (Weber, 1999). (See actin polymerization curve - appendix - B).

1.4.III.a -Monomer activation

This step is based on a change of monomeric actin conformation (G-actin) upon binding of a divalent cation at the high affinity divalent cation binding site (HAS) present in the actin molecule. The change in actin conformation has been reported (Cooper et al., 1983a; Cooper et al., 1983b) and recently has been confirmed by experiments using Ca\(^{2+}\) and Mg\(^{2+}\) bound to the HAS of actin monomers (Steinmetz et al., 1997a; Steinmetz et al., 1997b). In order to investigate the effect of the divalent cation bound to the single HAS of the G-actin molecule on the course of actin polymerization and also to establish a direct correlation between biochemical, structural and mechanical data, three well defined metal ion compositions consisting of ATP-G-actin-Ca\(^{2+}\), ATP-G-actin-Mg\(^{2+}\) and ATP-G-actin were polymerized by the addition of 100mM KCl for 1 hour. The results showed that the lag phase was relatively short with Mg\(^{2+}\)-G-actin and longer in the presence of Ca\(^{2+}\)-G-actin. The length of lag phase observed in divalent cation-free-G actin was more similar to Ca\(^{2+}\)-G-actin than to Mg\(^{2+}\)-G-actin (Steinmetz et al., 1997a; Steinmetz et al., 1997b). These findings are in agreement with earlier studies, confirming that Mg\(^{2+}\)-G-actin exhibits faster nucleation rate than Ca\(^{2+}\)-G-actin when polymerized in the presence of KCl. Also, the removal of the divalent cation preserves the ability of actin to polymerize into normal filaments.
1.4. III. b - Nucleation

Nucleation is the rate-limiting step for actin polymerization. It is based on the spontaneous association of actin monomers into oligomers from which a polymer can grow (Gaertner et al., 1989; Pollard and Cooper, 1986). The nucleus is defined as the smallest actin oligomer from which spontaneous monomer association is faster than dissociation (Pollard and Cooper, 1986). Nucleation is the limiting step because the reaction is so unfavourable due to high dissociation constants, resulting in the lag at the onset of spontaneous polymerization of actin. In this step only one contact between the protomers is formed. In all other steps each molecule binds with two binding sites due to the double helical nature of the filament (Wegner and Engel, 1975). The rate limiting step is the monomer critical concentration i.e., the actin monomer concentration where the rate of polymerization is zero (Gaertner et al., 1989).

Kinetic modelling using a computer to select a set of constants has been used to elucidate the steps during formation of dimers and trimers. It uses a set of experimental kinetic curves for the full time course of polymerization with a wide range of actin concentration (Pollard and Cooper, 1986). However, none of the approaches have revealed the rate constants for each individual steps. During spontaneous polymerization of 10-20 μM actin, the concentration of dimers and trimers is low (<<10⁻⁹M) and their life times very short, due to the high rate of dissociation which causes them to disintegrate rapidly (Pollard and Cooper, 1986). As predicted by kinetic modelling trimers are more effective nuclei than dimers (Wegner and Engel, 1975). Because kinetic models uses different assumptions, Ueli Aebi et al. tried to establish a correlation between structural, biochemical and mechanical data during this early stage of polymerization (Steinmetz et al, 1997a). To probe for the formation of distinct oligomers during early stages polymerization they used an intermolecular sulfhydryl cross linker, 1,4-PBM, and monitored actin polymerization by pyrene fluorescence.
measurements. It was found that immediately after the start of G-actin polymerization by
addition of 100mM KCl a single major "lower dimer" (LD) band of 86KDa was observed by
SDS-PAGE analysis. After 10mins of polymerization increasing amounts of a second major
band, the "upper dimer" (UD) of ~130KDa was found. During the course of polymerization
reaction there was a concomitant increase in the appearance of UD and disappearance of LD
and after 15mins when polymerization reached the steady state the cross-linked LD and UD
bands were completely absent. Furthermore, it was shown that besides the obligatory
nucleation-condensation based filament formation, a second facultative actin assembly
pathway exists involving the formation of a dimer LD which is unproductive by itself, but
which does get incorporated into growing F-actin filaments (Steinmetz et al., 1997a)

1.4.III.c - Elongation

The actin nuclei is elongated into filaments by addition of actin monomers. This step
refers to the association and dissociation of actin monomers at the ends of the filaments
(Pollard and Cooper, 1986). Association is a second order reaction that depends on the
concentration of both monomer and filament ends whereas dissociation is a first order
reaction that depends only on the concentration of filament ends (Cooper and Pollard, 1982).
The critical concentration for actin elongation is the concentration of monomeric actin in
which the rate of association equals to the rate of dissociation. Below the critical
concentration, dissociation is favoured and filaments shorten, whereas above the critical
concentration association of monomers exceeds dissociation resulting in filament growth.
Filament growth is exponential till the monomer concentration falls to the level of critical
concentration (Cooper et al., 1986; Pollard and Mooseker, 1981).
Actin filaments are polar structures. The polarity of filament was determined in the actomere of *Thyone* and *Pisaster* sperm using myosin subfragment 1 (S1) decoration (Tilney and Kallenbach, 1979). When polymerization was induced at low temperature with actin concentrations near the critical concentration, so that randomly polymerized filaments due to spontaneous nucleation was discarded, the S1 decorated filaments had an arrowhead pattern and the polarity of these filaments was unidirectional (Tilney and Kallenbach, 1979). Based on the arrowhead pattern created when actin binds to myosin heads, one end was called pointed end and the other the barbed end (Pollard and Cooper, 1986). General equations have been derived to describe this process (Wegner and Engel, 1975) and the relationship between the kinetic properties and the mechanism of full time course polymerization was tested by computer stimulation indicating that there is no simple measure to characterise the rate of actin polymerization (Frieden and Goddette, 1983). Actin polymerizes and depolymerizes at different rates at both ends (Cooper and Pollard, 1982; Weber 1999). All subunits in a filament have the same polarity, but the subunits at the ends of the filaments differ in conformation, resulting in unique reaction events at each end (Pollard and Cooper, 1986; Pollard and Mooseker, 1981). This observation suggests the possibility that the equilibrium at the two ends are different. The first attempt to directly measure the rate constants for polymerization of actin at the two ends were carried out by Pollard and Mooseker in 1981 (Pollard and Mooseker, 1981). Actin filaments isolated from intestinal brush-border microvilli were used to nucleate the polymerization of purified muscle actin. The net rate of growth in the two directions was assayed by measuring the change in the length of the filaments as a function of time by electron microscopy. The results indicated that the filament grow at constant rates from both ends; the rate at the barbed end being six times faster then the rate at the pointed end (Pollard and Mooseker, 1981). The different exchange rates at opposite ends, creates a different overall equilibrium constants at opposite ends and therefore,
Chapter 1

the critical concentration at the two ends are different. Thus both ends of the same filament must necessarily share the same monomer pool and attempt to attain equilibrium at different concentrations of monomer, there will be a net loss of monomers from the pointed end and a net gain at the barbed. Under physiological conditions (100mM KCl, 1mM MgCl₂, pH=7.5, 37°C), the critical concentration of the pointed end is almost six-fold higher (0.7μM) than of the barbed end (0.12μM). The difference in critical concentration at opposite sides leads to a steady-state monomer concentration where dissociation exceeds association at the pointed end and association exceeds dissociation at the barbed end resulting in a steady-state flux of actin molecules through the filament, a process referred as treadmilling (Cooper and Pollard, 1982; Gaertner et al., 1988; Pollard and Mooseker, 1981).

During polymerization the ATP bound to actin monomer is hydrolyzed to ADP and becomes non-exchangeable. This process lags behind monomer addition, as there is a delay between the incorporation of actin molecules into filaments and the hydrolysis of the bound ATP (Cooper and Pollard, 1982). One particularly interesting model is that hydrolysis occurs exclusively at the boundary between the central part of the filament consisting of ADP-subunits and the ends consisting of a variable number of ATP-subunits (Pollard and Cooper, 1982). This model implies a sequential mechanism in which a wave of hydrolysis would move from the centre towards the ends of the filaments. ATP bound to assembling monomers is hydrolysed not during the assembly step, but subsequent to incorporation within the filament to yield an ADP-F-actin filament via an ADP.Pi-F-actin intermediate (Carlier, 1998; Weber, 1999). Because the rate of monomer association is higher at the barbed end than the pointed end, it creates a growing filament in which the pointed end will have a terminal ADP-monomer and the barbed end an ATP or possibly an ADP.Pi-monomer, resulting on a chemical difference at opposite ends (Weber, 1999). Therefore, in the physiological ATP-
Mg-rich cell medium, actin filaments and monomeric ATP-actin are maintained in a dynamic steady-state (Carlier, 1998). The bound adenine nucleotide stabilises the protein against denaturation and the ATPase activity, although not required for actin polymerization, it affects the kinetics of assembly. The binding of ADP has an active mechanochemical role in cell function. Dynamic light scattering provides additional evidence for a difference between the diffusive motions of ADP and ATP-actin filaments. When equal concentrations of ATP or ADP-actin filaments are analysed by light scattering there is a rapid decay in scattering of ADP-actin suggesting that the intramolecular motions of ADP-actin filaments are more rapid than those of ATP-actin filaments (Janmey et al., 1990a; Janmey et al., 1990b). From this study the authors have also found that there is a difference in filament stiffness when actin filaments are bound to ATP or ADP. The width of negatively stained ATP filaments is 7-8nm and they are long and straight rods whereas ADP filaments have irregular diameters often as large as 12nm and appear entangled along their length. They suggested that ADP-bound filaments conformation traps elastic energy that could be available for release by actin binding proteins that transduce force or sever actin filaments (Janmey et al., 1990a; Janmey et al., 1990b).

### 1.4. III.d - Annealing

Annealing is the process in which filaments are joined together. It has no effect on the lag phase but accelerates the late stages of polymerization (Cooper and Pollard, 1982). The rate of annealing is very high and dependent on the concentration of filament ends. When filaments are fragmented by sonication or mechanical shearing, the numbers of ends available for elongation increases, increasing the rate of annealing. Two different methods, both using electron microscopy, have been used to directly study the annealing of actin filaments. Fixed
labelled filaments and unlabelled filaments were sheared and let to combine over time intervals. EM analysis showed the formation of heteropolymers with alternating labelled and unlabelled domains in an endwise joining of barbed and pointed end polarity pattern (Pollard & Cooper, 1986).

1.4.1V - ACTIN BINDING PROTEINS

*In vivo* actin assembly is highly controlled, filaments in cells are relatively short; some have defined length maintaining various structural and contractile elements of cell skeleton in an organised fashion (Schafer and Cooper, 1995). The diversity of actin filament forms is only made possible by the association of actin with actin binding proteins (ABPs). They can bind to G or F-actin and are involved in the dynamic assembly and disassembly of actin filaments resulting in a variety of unique cellular structures and functions (Weber, 1999).

There are a large number of different ABPs in eukaryotic cells; the majority fall into a smaller number of structural and functional families. The ABPs represent a good example of a diverse group of functionally related proteins whose diversity of function is in part due to different combinations of functional domains assembled in individual proteins to provide various combination of properties. ABPs utilizes modular design with a twist: instead of shuffling pre-existing domains with well defined functions, variants have evolved which can interact with G or F-actin and can be regulated by calcium and/or phospholipids and even interact with similar modules within the same protein (Puius et al., 1998).

This diversity generates great difficult to classify these proteins into specific groups. In addition, there are a large number of ABPs whose function is poorly categorized and structures are unknown.
Therefore, in this introduction, I’ve chosen to describe the best characterized ABPs and classified them based on functional and structural homology. Furthermore, myosins are not included as they are now regarded and classified as motor proteins.

1.4. IV. a - Gelsolin family

The gelsolin family of ABPs is characterised by the ability of the proteins to sever and cap the barbed end of actin filaments (Kwiatkowski, 1999). Members of this family include Villin, Severin, Fragmin and Adseverin/Scinderin and are all related to gelsolin (Ayscough, 1998; Weeds and Maciver, 1993). The gelsolin family has been found in organisms from Dictyostelium to Drosophila and humans although no members have been found in yeast or plants (Ayscough, 1998). Gelsolin is composed of six domains thought to have arisen from a common ancestral gene of 15Kda (Andre et al., 1988; Kwiatkowski et al., 1986). This pre-existing domain is repeated in a modular fashion within the members of the family, but the single 15Kda module have evolved giving rise to module variants which interact with G or F-actin (Puius et al., 1998; Way et al., 1992). The Fragmin and Severin members contain three copies of this gene and gelsolin seemed to be the result of a duplication event of this triplicated form. Recently, the structure of full length horse plasma gelsolin in a calcium free, inactive state has been elucidated and supports the triplication/duplication model of the family’s evolution (Burtnick et al., 1997). Villin is very similar to gelsolin only it has an additional domain at the amino terminus resulting in an extra actin bundling activity.

The severing and capping functions are mediated in vitro by calcium. Actin severing disrupts the interaction between adjacent monomers in the filament and tightly caps the barbed end of broken filaments. The capped end becomes stable to depolymerization and unable to nucleate assembly (de Arruda et al., 1992). Polyphosphoinositides (PPIs) causes dissociation of gelsolin from actin filament ends, providing sites for actin assembly.
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(Ayscough, 1998; Kwiatkowski, 1999; Weeds and Maciver, 1993). Member of this family are regulated upon extracellular stimuli is further discussed on cell signalling section.

I.4.IV.a.1 - Gelsolin

Gelsolin is a monomeric 83KDa ABP originally isolated from macrophages and now known to be present in most tissues, blood plasma and intestinal microvilli (Bryan and Coluccio, 1985; Schafer and Cooper, 1995; Weeds and Maciver, 1993). Gelsolin is a well known regulator of the cortical actin network in mammalian cells, and is best known for it’s involvement in dynamic changes in the actin cytoskeleton during a variety of forms of cell motility (Kwiatkowski, 1999; Witke et al., 1995). It interacts with actin in several ways by carrying out nucleation of filament assembly, severing and capping the barbed ends of actin filaments (Isenberg, 1991; Schafer and Cooper, 1995; Vandekerckhove and Vancompernolle, 1992). The severing activity is a specialized feature of gelsolin; it binds to the sides of actin filament cutting it into two short new filaments (Schafer and Cooper, 1995). After severing, gelsolin remains bound to the newly created end of one of the filaments and the whole reaction generates one fragment with a capped barbed end and another fragment with a free pointed end (Schafer and Cooper, 1995). These interactions are regulated by calcium and PH (<6.5), which synergistically activate gelsolin’s binding to actin (Kwiatkowski, 1999).

Gelsolin remodels the cytoskeleton in response to phosphatidylinositol 4,5-biphosphate (PIP2) and calcium during agonist stimulation. Gelsolin is activated by calcium and inhibited by PIP2. A rise on calcium physiological concentration increases gelsolin affinity for PIP2 releasing gelsolin from filament ends, providing sites for actin assembly, leading to remodelling of the cytoskeleton (Schafer and Cooper, 1995; Sun, et al., 1997; Kwiatkowski, 1999).
The primary amino acid sequence of gelsolin consists of six tandem repeats of a core domain. Gelsolin has three distinct actin binding sites, identified by fragment proteolysis, unevenly distributed throughout the six fold segment repeats (S1-S6) (Bryan, 1988; Way and Weeds, 1990; Weeds and Maciver, 1993). Segment 1 (S1) is the smallest domain of gelsolin containing 150 residues which interacts with actin and forms a 1:1 complex with G-actin stimulating the formation of actin nuclei in a calcium independent manner (Bryan, 1988; Mannherz et al, 1992; Way and Weeds, 1990). The amino acid residues crucial for actin binding have been located by deletion mutagenesis at regions 38-49 and 120-131 (Way and Weeds, 1990). Human gelsolin S1 was co-crystallized with rabbit α-actin and its structure was solved by X-ray crystallography (Mannherz et al, 1992; McLaughlin et al., 1993). The crystals of gelsolin segment 1 show that gelsolin contacts actin mainly in the bottom edge and front face of actin subdomain 1; the binding involving actin residues which lie in the cleft that divides subdomains 1 and 3. A calcium ion within actin-gelsolin complex was also located trapped between S1-actin complex (Mannherz et al., 1992; McLaughlin et al., 1993). Segments 2 and 3 (S2-S3) mediates a calcium independent F-actin binding and is responsible for targeting gelsolin to F-actin (Way et al., 1992). This F-binding site of gelsolin recognises the same binding site in actin as α-actinin, although there is no homologous relationship between gelsolin and α-actinin actin binding domains suggesting that they might adopt a similar structure (due to folding events) that recognises the same binding site on actin (Way et al., 1992). Gelsolin contains two PIP2-binding site spanning between residues 1-406 (Sun et al., 1997). Inhibition of gelsolin-actin binding by phosphoinositides is probably due to competition between PIPs and actin for a common binding region in the S2 domain (Kwiatkowski, 1999). The severing activity requires both the G and F-actin binding domains in the N-terminal half of the molecule (S1-S3) whereas, S4-S6 mediates a calcium dependent
actin monomer binding site responsible for nucleation activity and for the calcium regulation of gelsolin (Puius et al., 1998; Weeds and Maciver, 1993). This region contains the carboxyl-terminal half and deletion renders a fragment which severs and is PIP₂ regulated.

The in vitro actin filament severing activity of gelsolin has been investigated in vivo with the development of transgenic gelsolin-null mice (Barkalow et al., 1996; Witke et al., 1995). The transgenic gelsolin-null mice express no cytoplasmic or plasma gelsolin and showed normal embryonic development and longevity. However, there was a decrease in platelet shape changes, resulting in prolonged bleeding. Actin filaments in stimulated platelets from these mice elongate their actin without severing. The capping activity remained and was found to be due to the presence of heterodimeric capping protein presence in these cells. By treatment with phosphatidylinositol 4,5-biphosphate the capping activity is released suggesting that in platelets many other proteins with partial similarity to gelsolin could provide functional compensation for a complete lack of gelsolin expression (Witke et al., 1995). A role in cell motility has been described by analysis of gelsolin-null dermal fibroblast cells (Kwiatkowski, 1999). Upon stimuli, these cells display reduced ruffling activity and translocational motility. The reduction in motility is accompanied by a fivefold increase in the expression of Rac GTPase. Rac overexpression and motility defect is repaired by gelsolin transfection (Kwiatkowski, 1999).

An important role for gelsolin during apoptosis have been observed in gelsolin-null neutrophils. It was found that these cells progress to apoptosis cell death more slowly than wild type neutrophils. Further analysis showed that gelsolin is substrate of caspase-3, a protease that is activated during apoptosis, cleaving gelsolin. Expression of the cleaved amino-terminal fragment in cells has a calcium unregulated actin filament severing activity that leads to apoptosis (Geng et al., 1999).
A possible role in cell signalling has been investigated by overexpression of gelsolin in cells \textit{in vivo}. \textit{In vitro}, gelsolin modulates the activity of several important signalling enzymes through PIP$_2$ binding (Sun et al., 1997; Kwiatkowski, 1999). Overexpression of gelsolin in NIH3T3 cells demonstrated that gelsolin bind PIP$_2$ \textit{in vivo} to inhibit phospholipase activity (Sun et al., 1997). This result suggest that gelsolin may participate in signalling by controlling the availability of PIP$_2$ substrates.

1.4 IV a.2 - Villin

Villin is a 92KDa monomeric protein expressed specifically in polarized cells of the intestine and kidney brush border (de Arruda et al., 1992; Vandekerckhove and Vancompernolle, 1992; Weeds and Maciver, 1993). Villin is a multidomain protein that severs, caps and bundles actin filaments in a calcium regulated manner (de Arruda et al., 1992). \textit{In vitro}, villin cross-links actin filaments in the absence of calcium organising actin filaments into bundles which support the microvilli that cover the absorptive surface of intestinal and kidney epithelia, amphibian oocytes and visceral yolk sac endoderm. In the presence of calcium, however, villin disrupts the organisation of the cytoskeleton by capping and severing actin filaments into short fragments (Doering and Matsudaira, 1996; Rana et al., 1993). It’s structure consists of six-repeat sequence of 125-130 residues with an extra non-repeated 8KDa ‘headpiece’ at the carboxyl terminus which confers filament cross-linking activity (Hartwig and Kwiatkowski, 1991; Rozycki et al., 1994). The amino-terminal segment 1 bind to monomeric actin in a calcium independent manner equivalent to S1 in gelsolin suggesting that each repeat folds independently retaining intrinsic actin binding properties. The severing activity of villin requires much higher calcium concentrations than that of gelsolin. The structure of villin amino-terminal 14T has been solved in solution using.
heteronuclear multidimensional NMR (Markus et al., 1994) and found to be very similar to
gelsolin S1. Two calcium binding sites have been found: one at residues 85-86 which binds
Ca\(^{2+}\) weakly and another near the carboxyl terminus of the fragment. The NMR structure of
the stable headpiece has also been solved. This module is responsible for a calcium
independent F-actin cross linking activity and contain one of the two F-actin binding sites.
By scanning mutagenesis the F-actin binding site was found to be at position \(~40\) to 76 of
headpiece (Doering and Matsudaira, 1996). The headpiece does not compete for F-actin
binding with villin first two domains, gelsolin second or with \(\alpha\)-actinin suggesting that the
headpiece binds at a different site on F-actin (Way et al., 1992). Although villin headpiece
displays no homology to the repeated modules of the gelsolin family, it has an F-actin binding
site homologous to erythrocyte protein dematin (Rana et al., 1993). A region between
domains S1 and S2 of villin has been studied by point mutations and shown to play important
role in both F-actin severing and binding activities. Mutant proteins in which Arg137 residue
was substituted by alanine showed reduced severing activity by 83\% but no inhibition of
capping activity, a finding which is consistent with the proposition that capping activity is
retained in segment 1 (de Arruda et al., 1992). This experiment also demonstrated that the
second actin binding domain of villin lies at the junction of the first and second domains (de
Arruda et al., 1992).

The various actin binding activities of villin may enable the protein to play an important
role in forming and maintaining the absorptive surface area of the epithelium at different
times. In mature absorptive cells, villin cross-links actin filaments into a bundle which
stabilises the microvilli whereas the severing and capping activities might destabilize the
absorptive surface by causing the bundle to desintegrate and the microvillus membrane to
vesiculate (de Arruda et al., 1992). Microinjection of villin to cells that do not normally
express it results in the formation of structures resembling microvilli, suggesting that villin is involved in the morphogenesis of microvilli (Friederich et al., 1992). Recently, however, the analysis of villin-null mice showed no impair in the morphogenesis of microvilli an observation that lends to the existence of functional redundancy (Pinson et al., 1998).

1.4.IV.a.3 - Severin

Severin is a 40KDa protein first identified in the tips of advancing pseudops of Dictyostelium discoideum. It is an ABP that nucleates actin assembly and severs and caps the barbed end of actin filaments in a calcium dependent manner (Eichinger and Schleicher, 1992). It was found that in the cortex of Dictyostelium cells the amount of F-actin decreased with increasing calcium concentration, suggesting a possible role in the control of cell motility (Andre et al., 1988). This observation led to the development of Dictyostelium mutants which were deficient in severin. However, the severin deficient cells grew in a normal rate and showed to be neither essential for growth or normal motility and chemotaxis (Andre et al., 1989).

Severin has three highly conserved modular domains S1, S2 and S3 and each contains one actin binding site. S1 contains one F-capping and a calcium binding site; S2 and S3 have both an F-actin binding site. The binding of S2 to the side of actin filaments is calcium dependent; at low calcium concentrations severin S2 domain binds to F-actin severing the filaments. The presence of the additional binding site in S3 accounts for the nucleating activity of severin (Eichinger and Schleicher, 1992). Furthermore two PIP2-binding sites are present one in S1 and other in S2+S3. In the presence of PIP2, the severin activities are abolished (Eichinger and Schleicher, 1992).

Another mechanism for severin regulation has been described by Eichinger et al., in which a severin kinase protein was isolated causing phosphorylation of severin suggesting a
direct signal transduction pathway for the regulation of severin protein (Eichinger et al., 1996; Eichinger et al., 1998).

1.4.4 - Adseverin/Scinderin

Adseverin is a 80KDa calcium dependent severing protein originally isolated from bovine adrenal medula, found mainly in neural and endocrine tissues (the same protein was later isolated from chromaffin cells and called Scinderin) (Nakamura et al., 1994; Tchakarov et al., 1990; Vitale et al., 1991). Adseverin is the closest homologue to gelsolin, it has six homologous segments, with calcium dependent F-actin severing and capping activities. Adseverin has also two PIP₂ binding sites. The effects of calcium on actin polymerization was verified in vitro by viscometry. In the presence of calcium purified bovine adseverin expressed in E.coli promoted the nucleation of actin polymers and reduced the final viscosity of polymerized actin solution (Nakamura et al., 1994).

At first, adseverin distribution in cells was thought to be restricted to tissues with secretory functions. In situ hybridisation demonstrated that adseverin was expressed in the adrenal medulla but not in the adrenal cortex indicating a possible regulatory function in exocytosis by affecting the organisation of actin filaments present underneath the plasma membrane (Nakamura et al., 1994; Vitale et al., 1991). Recently, it’s been found high levels of adseverin expression on specific cells of the kidney and collon implying a novel role for this protein in epithelial cell function (Lueck et al., 1998). A recombinant murine adseverin antibody was raised and used for detection of adseverin in kidney tissues. Adseverin was expressed in mouse kidney and intestine at all stages of development and in human fetal and adult kidney (Lueck et al., 1998). Expression was seen in most of the kidney cells apart from
the proximal tubule where endocytosis occurs indicating that adseverin is not required for this function.

A novel severin isoform missing domain 5 has been reported in hematopoietic lineage cells; although function and distribution is yet to be known (Kwiatkowski, 1999).

1.4.IV.a.5 - Fragmin

Fragmin is a 42KDa protein isolated from slime mold Physarum polycephalum. It consists of three repeated segments of which two bind actin (Gettemans et al., 1995). Fragmin severs actin filaments and nucleates actin filament formation in a calcium dependent manner. Under these conditions fragmin binds two actin molecules and addition of EGTA to the actin-fragmin trimer releases one actin subunit reversibly resulting in an actin-fragmin heterodimer which efficiently caps actin filaments at the barbed end and nucleates actin polymerization weakly but loses it’s severing activity (de Corte et al., 1996a; de Corte et al., 1996b). This EGTA-heterodimer is also a target for an 80KDa physarum actin-fragmin kinase (AFK). However, phosphorylation has no effect on the actin binding properties of fragmin indicating that the phosphorylation site is not part of the actin binding contacts with fragmin (de Corte et al., 1996a; de Corte et al., 1996b). AFK phosphorylates the actin-fragmin heterodimer at Thr203 and Thr202 in the actin subunit. Upon in vitro phosphorylation the nucleating activity is abolished and the capping activity becomes calcium dependent (Eichinger et al., 1996; Gettemans et al., 1995). This has been confirmed by microinjection of the actin-fragmin heterodimer in living CV1 and NIH3T3 cells which caused the disintegration of actin cytoskeleton and alteration of cell morphology (Constantin, 1998). When these cells were phosphorylated by AFK in low calcium conditions this in vivo effect was blocked and capping activity was restored at high calcium concentration suggesting that actin polymerization in Physarum microplasmodia can be controlled in a calcium dependent manner through
phosphorylation. Fragmin also binds PIP2 and once the phospholipid is bound, actin no longer complexes with fragmin.

1.4.IV.a.6 - Advillin

Advillin is a 92KDa member of the gelsolin family of actin binding proteins. It was identified first by screening an adult murine brain cDNA library with a probe for bovine severin (Marks et al., 1998). Advillin cDNA is 75% homologous to villin and 65% to gelsolin and adsevrin. Its structure consists of six domains with an extra carboxyl-terminal headpiece similar to villin. The critical 7 amino acids residues for actin binding at the headpiece domain was defined by *in vitro* mutagenesis (Doering and Matsudaira, 1996). Advillin is the only member of the family to contain the 7 amino acids. *In situ* mRNA analysis of adult and embryonic murine tissues suggest a possible function in the morphogenesis of neuronal cells which are responsible for the formation of ganglia (Marks et al., 1998). The strong homology to villin and its expression in intestinal microvilli may also explain why villin-null mice have minimal defects on microvilli morphogenesis (Pinson et al., 1998).

1.4.IV.a.7 - Supervillin

Supervillin is a 205KDa F-actin binding protein isolated from bovine neutrophil plasma membranes (Pestonjamasp et al., 1997). Supervillin is a new actin-binding membrane protein that binds to the sides of actin filaments that cosediments with β-actin and δ-actin in sucrose gradients. The intracellular localisation of supervillin is a function of adherence state: in
adherent cells supervillin concentrates with E-cadherin at sites of lateral cell-cell contact whereas in subconfluent cells it is found throughout the cytoplasm and within the nucleus (Pestonjamasp et al., 1997). The amino acid sequence includes a carboxyl terminal highly similar to villin/gelsolin family and a very large amino terminal which contain several potential nuclear localisation signals (Kwiatkowski, 1999). The F-actin binding site are localized within S2-S5 region (Pestonjamasp et al., 1997).

This protein was also found in suspension of grown cervical carcinoma (HeLa) cells which lack adherens junctions and together with primary sequence motifs such as nuclear localisation site and villin/gelsolin homologous carboxyl terminal it indicates that supervillin may play additional roles in other cellular compartments other than cell-cell adhesion and/or information transfer to other cell compartments (Pestonjamasp et al., 1997).

1.4. IV. b - Capping proteins

As the name suggest, this group of ABPs binds to the ends of actin filaments with a direct effect on \textit{de novo} polymerization, modulating filament number and length (Weeds and Maciver, 1993). Free barbed ends are pre-requisite for all actin based motile processes as they allow for free access and the regulated assembly and disassembly of actin filaments (Puius et al., 1998). This type of regulatory proteins are able to increase the steady state concentration of ATP-G-actin through capping the ends of filaments, therefore, protecting against elongation and depolymerization at the capped filament ends (Carlier, 1998; Weber, 1999). Capping proteins are broadly classified as barbed end cappers or pointed end capper proteins and within each group they can be further classified according to specific roles they carry out on the cytoplasm of cells.
Cap G is the new name for a protein previously known as macrophage-capping protein (MCP), gCap39 or Mbh1 (Predergast and Ziff, 1991). The G reflects sequence similarity to gelsolin and Cap indicates that the protein caps barbed ends and nucleates actin assembly but unlike gelsolin, it does not sever actin filaments (Schafer and Cooper, 1995). It is 41KDa, Ca2+ sensitive protein which reversibly blocks the barbed ends of actin filaments but, unlike gelsolin, CapG dissociates from the ends of filaments by lowering the calcium concentration, enabling it to create new free barbed ends by uncapping. Cap G has one calcium binding site localized at the carboxyl termini that upon higher calcium concentrations leads to actin monomer binding. In lower calcium concentrations, such as found in the cell, Cap G function primarily as a capping rather than a monomer binding protein (Young et al., 1994; Schafer et al., 1995). The amino acid sequence of Cap G is composed of only the first three of the six repeats found in gelsolin (Schafer and Cooper, 1995).

Cap G was originally isolated from rabbit macrophages and the cDNA for Cap G from human monocyte cell line was cloned and expressed in *Escherichia coli*. The fusion protein was functionally identical to the native protein purified from rabbit macrophages with respect to calcium sensitivity and the ability to block monomer exchange at the barbed end of actin filaments (Dabiri et al., 1992; Young et al., 1994). Western blot analysis demonstrated that Cap G has a limited distribution and is primarily found in macrophages representing ~1% of total cytoplasmic protein (Dabiri et al., 1992).

Cap G was also isolated from human plasma by calcium dependent affinity chromatography and found to be secreted by macrophages (Johnston et al., 1990; Yu et al., 1991). Using truncated Cap G (gCAP39) generated by limited proteolysis it was found that actin capping activity requires almost the entire molecule. Cap G S1 domain is very similar
to gelsolin, however, S2 and S3 are different in that they do not bind to the sides of actin filaments. To test for domain activity function a Cap G chimera was generated and found that S1 behaves like gelsolin in binding monomeric actin but dependent on calcium for binding. It was also found that the lack of severing activity was a function of S2 and S3 inability to bind to the sides of the filaments (Yu et al., 1991).

The mbh1 (Cap G) cDNA was found during search for factors that could interact with c-Myc oncogene. The cDNA sequence showed ~50% homology to the amino terminal half of gelsolin and ~33% to severin. In this study it was also found that this protein contain a putative DNA binding site and a nuclear localisation site (Prendergast and Ziff, 1991). The significance of these observations is not known but it is indicative that Cap G may play a role in regulating cytoplasmic and/or nuclear architecture through potential interactions with actin (Prendergast and Ziff, 1991; Schafer and Cooper, 1995).

Evidence for a possible role for Cap G in cell signalling, via PIP2 modulation, was obtained in overexpression experiments in vivo (Sun et al., 1997). Cap G has one PIP2 binding site at the amino terminal which when overexpressed increases the affinity and binding to PIP2, leading to release from filament ends. This result led to the hypothesis that upon extracellular stimuli, the membrane PIP2 content changes, Cap G competes with other PIP2 binding proteins providing a pathway modulated selectively by PIP2 (Sun et al., 1997).

1.4.1V.b.2 - Capping protein: CapZ

Capping protein binds to the barbed ends of actin filaments preventing addition and loss of actin subunits leaving the pointed end unaffected (Schafer et al., 1995). It also binds weakly to actin monomers nucleating actin polymerization, therefore capping must stabilise the dimer/trimer nuclei (Schafer et al., 1995).
Capping protein is a heterodimer of α and β subunits each of ~30KDa (Weeds and Maciver, 1993). The amino acid sequences of each subunit are not similar to each other or any other protein but are conserved throughout evolution. The two subunits bind very tightly to each other and require each other for actin binding activity in vitro and stability in vivo (Amatruda and Cooper, 1992; Amatruda et al., 1992; Hug et al., 1992). Capping protein is fully active in the absence of calcium and can be inhibited reversibly by PIP2 and other anionic phospholipids (Schafer et al., 1995). Whereas lower eukaryotes have only one gene and isoform of each of the α and β subunits, vertebrates have two α genes referred to as α1 and α2. Only a single gene for the β subunit is present in vertebrates but there are two isoforms β1 and β2 which are produced by alternative splicing (Schafer et al., 1995). CP-β1 is the predominant capping protein of muscle and is found at Z-disks of sarcomeres (hence the term capZ), whereas CP-β2 is the predominant isoform in nonmuscle tissues although small amounts are present in muscle cells, but not at Z-disks (Schafer et al., 1996). The two isoforms arise from alternative splicing of mRNA which yields polypeptides that differ in 30 amino acids at their carboxyl end (Hug et al., 1992). By generating fusion proteins containing full or only the carboxyl region of the chicken β1-subunit of chicken it was demonstrated that the carboxyl terminal inhibits the ability of capZ to bind to the barbed end of actin filaments and nucleate actin polymerization, indicating that this region contain the actin binding site (Hug et al., 1992). Since the carboxyl terminal is responsible for the binding of isoforms to F-actin, the binding properties of each isoform was investigated by purifying chicken kidney and brain CP-β2 and chicken pectoral muscle CP-β1 and compared their actin binding function using three different actin isoforms: skeletal muscle α-actin, erythrocyte β-actin and brain β8-actin (Schafer et al., 1996). The results indicated that CP-β subunit isoforms did not differ in their actin binding properties even in tests with different isoforms. However, it was
found that PIP$_2$, caused rapid and efficient dissociation of CP from capped filaments suggesting that PIP$_2$ may regulate actin polymerization *in vivo* by acting as a second messenger (Schafer et al., 1996). The uncapping activity of PIP$_2$ was found to be Mg$^{2+}$-dependent.

The capping activity has been studied *in vivo* in yeast *Saccharomyces cerevisiae* (Amatruda and Cooper, 1992; Karpova et al., 1995). Null mutants of capping protein were prepared by deletion of CAP1 and CAP2 genes, the genes encoding the $\alpha$ and $\beta$ subunit respectively of capping protein in yeast. The null mutants showed an altered actin cytoskeleton consisting of a deficit in actin cables from mother cells and the appearance of actin spots in a diffuse pattern over the cortex of the mother and bud, suggesting that capping activity is required for the stability of actin structures comprising the cables (Amatruda and Cooper, 1992; Amatruda et al., 1992; Karpova et al., 1995). The role for CP in higher eukaryotes has been investigated *in vivo* by mutagenesis of $\beta$-subunit of capping protein from *Drosophila melanogaster*. Flies carrying mutant alleles exhibit a defect in bristle morphology that is correlated to disorganised actin bundles in developing bristles and the loss of CP activity is lethal (Hopmann et al., 1996).

The main characteristic of capping protein is the ability to regulate polymer length therefore affecting elongation and annealing of filaments. In order to establish how capping protein affects filament length, purified actin was copolymerized with capZ protein and capped filaments were analysed by fluorescence microscopy (Xu et al., 1999). They showed that at physiological concentration (300$\mu$m F-actin) each capZ molecule produces one filament and that these short filaments were more fluid than low concentration of long filaments formed by spontaneous assembly *in vitro*. They concluded that without cross
linking, the short filaments cannot account for the stiffness of the cell cortex (Xu et al., 1999).

A human polymorphonuclear leukocyte (PMN) cap Z protein has been identified as the nonmuscle isoform of capZ capping protein (Maun et al., 1996). It is a 65Kda heterodimeric protein which strictly binds the barbed end of actin filaments in a calcium independent manner but has no severing, nucleating or monomer sequestering activities. The capping activity of PMN capZ is modulated by PIP2. Quantitative western blots showed that PMN capZ represents ~1% of total protein in human PMN cytoplasmic extracts and accounts for the majority of calcium insensitive barbed end capping activity in these cells. This evidence suggest a role for PMN capZ during chemotaxis, phagocytosis and degranulation events which require a coordinated rearrangement of the cell peripheral cytoskeleton without calcium regulation (Maun et al., 1996).

1.4.IV.b.3 - Tropomodulin

Tropomodulin is 40.6KDa tropomyosin binding protein originally isolated from red blood cells that has been localised by immunofluorescence staining to a site near or at the pointed ends of rat skeletal muscle thin filaments (Fowler et al., 1993). Tropomodulin has a pointed-end capping activity completely blocking elongation and depolymerization at pointed ends of tropomyosin-containing actin filaments (Weber et al., 1994). Tropomodulin can bind directly to actin filaments. In the absence of tropomyosin, tropomodulin binds weakly to pointed-ends partially inhibiting elongation and depolymerization. Short gelsolin capped filaments were used as nuclei to test for elongation activity and fluorescence analysis with pyrenyl-actin indicated that the initial rate of polymerization was decreased to 20-30% of control. Depolymerization assays were carried out using gelsolin-capped filaments in the
presence of vitamin D, a monomeric sequestring protein, in order to prevent any
reassociation with the filament, and showed a decrease in the concentration of free pointed
ends upon increasing tropomodulin concentrations (Weber et al., 1994). Tropomodulin
doubles the critical concentration at the pointed-ends of actin filaments without affecting
either the rate or extent of polymerization at the barbed end indicating that tropomodulin does
not sequester actin monomers (Weber et al., 1994; Weber et al., 1999). This is due to the low
binding affinity of tropomodulin to the point end of actin filaments which leaves time for
ATP hydrolysis and phosphate release to go to completion, changing the ADP-Pi-actin to
ADP-actin at the pointed end therefore, lowering the apparent affinity of pointed ends for
actin monomers (Weber et al., 1999).

Tropomodulin has also been reported in maintaining filament length in skeletal
embryonic cardiac myocytes, a function essential for the contractile function of these cells
(Gregorio and Fowler, 1995; Gregorio et al., 1995). Microinjection of an antibody that
inhibits tropomodulin’s capping activity in vitro, results in elongation of actin filaments from
their pointed ends.

1.4.IV.b.4 - Aginactin

Aginactin is a 70Kda agonist-regulated actin capping protein identified in Dictyostelium
amoebae (Sauterer et al., 1991). It binds to F-actin in a calcium insensitive manner inhibiting
the rate of actin polymerization and also depolymerization. It neither severs nor nucleates
actin polymerization in either the presence or absence of Ca^{2+}. It has high homology to heat
shock proteins.
1.4.IV.C - Band 4.1 superfamily

The main characteristic of members of band 4.1 superfamily is the ability to mediate plasma membrane-actin network linkage (Arpin et al., 1994). They include spectrin-band 4.1, ERM and Merlin/schwannomin. The members of this growing family have been defined on the basis of similarities in their primary structure. This superfamily is characterized by an amino terminal FERM (4.1-ezrin-radixin-moesin) domain, required for specific binding interactions (Hoover and Bryant, 2000).

1.4.IV.C.1 - Band 4.1

The band 4.1 actin complex was first isolated from red cell membrane and acts as a pointed end capping protein *in vitro* (Pinder et al., 1984). It links the spectrin-actin network to the plasma membrane, by binding to the transmembrane protein glycophorin via an amino-terminal binding site while it’s carboxyl-terminal binds to the cytoskeleton (Workman and low, 1998).

A large number of isoforms have been identified, generated from tissue specific and developmental stage specific splicing events. Apart from interacting with spectrin, these proteins use specific domains to interact with a variety of molecules (band 3, glycophorin C, PIP, PIP2, calmodulin) modulating functions at the plasma membrane (Hoover and Bryant, 2000).

Although 4.1 is localised at the membrane in nucleated and anucleated erythrocyes, it has been showed to be present in the nucleus. The functional significance of this finding is not clear but it’s been suggested that 4.1 may regulate assembly of mitotic spindle and RNA processing (Hoover and Bryant, 2000).
1.4.IV.c.2 - ERM family of proteins

The ERM constitute a subfamily of three closely related proteins ezrin, radixin and moezin involved in cytoskeleton organisation (Arpin et al., 1994). They work as plasma membrane-actin filament cross-linkers that regulate cell adhesion and cortical morphogenesis and are involved in signal transduction pathways (Mangeat et al., 1999; Tsukita and Yonemura, 1997a; Tsukita and Yonemura, 1997b). The ERM family of proteins localize to and support cell surface projections by forming oligomeric head to tail structures linking the underlying cytoskeleton with the plasma membrane (Shcherbina et al., 1999a; Shcherbina et al., 1999b). These proteins are encoded by a gene family which share 70% overall amino acid identity and their amino-terminal are about ~35% identical to the amino-terminal of band 4.1 (Arpin et al., 1994; Tsukita and Yonemura, 1997a; Tsukita and Yonemura, 1997b, Henry et al., 1995). ERM proteins consist of three domains: a globular amino-terminal membrane binding domain, followed by an extended helical domain and a positively charged carboxyl-terminal actin binding domain (Tsukita and Yonemura, 1997a; Tsukita and Yonemura, 1997b). The amino-terminal halves is highly conserved with ~80% identity and is involved in membrane binding whereas the carboxyl-terminal is involved in actin cytoskeleton interactions. The in vivo function of ERM proteins have been studied by coexpression of ERM proteins in the presence of antisense oligonucleotides which selectively suppressed the expression of each ERM protein (Takeuchi et al., 1994a; Takeuchi et al., 1994b). Immunofluorescence revealed that all ERM members are colocalized at cell-cell adhesion sites, microvilli and cleavage furrows where actin filaments are densely associated with plasma membranes (Takeuchi et al., 1994a; Takeuchi et al., 1994b).
The role of amino and carboxyl- terminals in cross-linking between plasma membrane and actin filaments was investigated by point mutations and deletion of fragments (Amieva et al., 1999; Martin et al., 1995).

The presence of both actin and membrane binding domains in ERM proteins explain their cross-linking functions. However, only few are located just beneath the plasma membranes of cultured fibroblasts where they function as membrane-actin cross-linking proteins suggesting that a regulation mechanism is involved in the expression and cross linking activities of these proteins (Shcherbina et al., 1999a; Shcherbina et al., 1999b). ERM activation involves unfolding of the molecule, allowing the protein to bind to the plasma membrane directly or indirectly via linker proteins (Mangeat, 1999). The ERM proteins are essential for the assembly of actin filaments and focal adhesion complexes mediated by the GTPases, Rho and Rac (See cell signalling 1.5).

1.4.IV.c.2.a - Ezrin

Ezrin is a 80KDa cytoplasmic protein with a potential role as a membrane-cytoskeleton linker protein. The primary structure shows 37% and 23% amino-terminal identity with human erythrocyte band 4.1 and talin respectively. Ezrin is present in a variety of simple or stratified epithelial cells as well as mesothelial cells (Algrain et al., 1993). Immunoelectron microscopy indicated that ezrin was preferentially localised in the apical microvilli of epithelial cells associated with the plasma membrane of the microvilli. In fibroblasts transfected with the human cDNA encoding full length ezrin, ezrin remains associated with cortical actin filaments after non-ionic detergent extraction (Algrain et al., 1993). Using truncated forms of ezrin under the same conditions it was found that the amino-terminal domain is solubilized while the carboxyl-terminal domain is associated with cortical actin.
filaments and with stress fibers. Recently, however, GST fusions of amino-terminal ezrin showed to be resistant to detergent extraction (Amieva et al., 1999). Expression of GST fusion of amino-ezrin induced changes in filopodial shape and appearance of microextensions depleted of actin filaments (Amieva et al., 1999). It’s been suggested that the carboxyl-terminal contains at least one actin binding site and the amino terminal is necessary for the correct localisation of ezrin at the cortex of the cells. Indeed, by generating GST fusion proteins of truncated ezrin it was found that the actin binding site was localised to the carboxyl-terminal end 34 amino acids (Turunen et al., 1994). This site showed homology to the actin binding site in the carboxyl terminal of moesin, radixin and CapZ. When the carboxyl-terminal half of ezrin was introduced into insect or CHO cells, the actin based cytoskeletal organisation was affected resulting in the formation of numerous cellular protrusions (Martin et al., 1995). Furthermore, PIP2 binds to a GST fusion protein consisting only of the amino terminal but not to the GST-carboxyl terminal, suggesting that phosphorylation of the amino terminal may activate F-actin binding by interfering with intermolecular interactions, this being an additional regulatory mechanism for the binding activity of this protein (Turunen et al., 1994; Huang et al., 1999). Ezrin is a phosphoprotein with multiple phosphorylation sites and the target of various protein kinases. The relocalization of ezrin in the microvilli is accompanied by an increase in phosphorylation of serine residues. Mouse epithelial cells cultured in the presence of ezrin antisense phosphorothioate oligonucleotides showed loss of cell-cell and cell-substrate adhesion but no effect on microvilli structures (Takeuchi et al., 1994a; Takeuchi et al., 1994b).

Recently ezrin has been detected by immunoblot with specific antibodies in white blood cells such as lymphocytes, monocytes and neutrophils and in vitro assays demonstrated that ezrin was completely and readily proteolyzed by calpain (Shcherbina et al., 1999a; Shcherbina et al., 1999b).
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1.4.IV.c.2.b - Radixin

Radixin is a 68.5Kda member of the ERM family of proteins originally isolated from rat liver cell-to-cell adherens junction (Tsukita and Yonemura, 1997; Tsukita and Hieda, 1989). Electron microscopy of negatively stained acrosomal processes revealed that radixin is a barbed end capping protein and by immunofluorescence using IgG antiradixin antibody is localised at the cell-cell junctions of rat liver, heart and intestine cells. The effect of radixin on the rate of actin polymerization was studied by falling ball viscometry (Tsukita and Hieda, 1989; Tsukita and Itoh, 1989). Radixin caused a reduction in actin viscosity which was not influenced by calcium. The presence of radixin showed an effect on the annealing of the filaments; the recovery of viscosity was inhibited to 60% of control (Tsukita and Hieda, 1989; Tsukita and Itoh, 1989). Following this study, Sato et al., investigated the distribution of radixin in diving cells using a monoclonal antibody obtained using chicken gizzard radixin as an antigen (Sato et al., 1991). Indeed, they found that radixin was found in the contractile ring which is formed just beneath the cleavage furrow during cytokinesis (Sato et al., 1991). Sequence analysis of cDNA clones encoding mouse radixin showed 75.3% homology to human ezrin and confirmed it to be a member of the band 4.1 superfamily (Funayama et al., 1991). The function of amino and carboxyl domains have been studied by exogenously expressing radixin polypeptides in NIH 3T3 cells (Henry et al., 1995). Exogenous full length radixin behaved like ERM proteins, localising at same cortical structures (ruffling edges, filopodia, lamellopodia, microvilli and cleavage furrow of dividing cells) (Henry et al., 1995). However, high level expression of the carboxyl domain in the absence of amino domain dramatically alters cell morphology and division. The amino terminal domain fused to GFP was expressed in NIH3T3 cells and found to localize to the plasma membrane (Amieva et al., 1999). Mouse epithelial cells grown in the presence of radixin antisense oligonucleotides
showed a decreased ability to attach and spread on the substratum confirming its functional role in cell-cell and cell-substrate adhesion (Takeuchi et al., 1994a; Takeuchi et al., 1994b). Recently it's been reported of role for radixin in lamellipodial stability during nerve growth cone motility (Castelo & Jay, 1999). Microscale chromophore-assisted laser inactivation of radixin in growth cones caused a 30% reduction of lamellipodial area within the irradiated region whereas all other control experiments did not show any effect on lamellipodia (Castelo & Jay, 1999).

1.4.IV.c.2.c - Moesin

Moesin is the quantitatively dominant ERM protein in human blood lymphocytes, monocytes and neutrophils and the only one in platelets (Shcherbina et al., 1999a; Shcherbina et al., 1999b).

Moesin is a 77KDa protein and lacks a polyproline stretch that is present after the α-helical segment of radixin and ezrin. Point and deletion mutants of yeast moesin were examined for F-binding activity and for intra and intermolecular interactions with terminal and carboxyl domains. It was found that substitution of Threonine 558, a critical site of moesin phosphorylation, by aspartate activates moesin F-actin binding site in the carboxyl terminal indicating that the introduced negative charge controls F-actin binding not by changing affinity but rather by exposing the carboxyl terminal binding site (Huang et al., 1999). Upon replacement the carboxyl and amino terminal no longer interacted as it occurs within native protein. Moesin is required for the formation of membrane microextensions and for GTPases mediated formation of stress fibers, focal adhesion complexes and microextensions (Huang et al., 1999). Upon treatment with antisense oligonucleotides it was found the disappearance of lymphoid microvilli (in these cells microvilli function to monitor
blood vessel wall and serve as the locus of adhesion and morphological changes leading to extravasation and activation. Microvilli structures were also affected when mouse epithelial cells were grown in the presence of moesin antisense oligonucleotides, however, no effect on cell-cell and cell-substrate adhesion was observed (Takeuchi et al., 1994a; Takeuchi et al., 1994b). Immunoblots using specific antibodies were used to determine the expression pattern and functional properties of moesin in blood cells. Moesin expression was found to be as high as ~0.5% of total cell protein in lymphocytes. Moesin was also tested for calpain sensitivity (an important effector of calcium signalling and activation-associated changes in blood cells) in vitro and shown to be insensitive to calpain proteolysis whereas ezrin was completely proteolysed, suggesting that differential sensitivity to calpain contributes to specialised and non-redundant functions of these proteins (Shcherbina et al., 1999a; Shcherbina et al., 1999b). It is known that the carboxyl domain and actin filaments are co-distributed with stress fibers, but this co-distribution is not seen with full length moesin. All this evidences suggest that activation of moesin actin binding site may not occur in the cytoplasm. Recent evidence with green fluorescent protein (GFP) fusions of moesin in live NIH3T3 cells have shown that moesin is distributed along the plasma membrane where it may be bound to a variety of receptor proteins suggesting that activation may occur in the vicinity of the plasma membrane (Amieva et al., 1999).

1.4.IV.c.3 - Merlin/Schwannomin

Merlin/Schwannomin is a 66KDa protein identified as a tumor supressor of neurofibromatosis type 2 in which the lack of merlin in schwann cells results in overgrowth (Rouleau et al., 1993). It shares 63% identity with ezrin in the first 342 residues and has a
secondary structure similar to ERM family of proteins: a large amino-terminal domain followed by a large α-helix and a small carboxyl-terminal (Rouleau et al., 1993).

The gene encoding merlin was identified as a candidate tumor supressor for neurofibromatosis 2 and may play a role in signal transduction events that regulate cell division (Rouleau et al., 1993; Trofatter et al., 1993). Overexpression suppresses cell growth and the suppression of expression using antisense oligonucleotides results in reduced cell adhesion and increased growth, but the mechanism underlying these functions remain to be elucidated (Huynh and Pulst, 1996; Lutchman and Rouleau, 1995).

The similarity of merlin primary and secondary structure to band 4.1 superfamily suggest an involvement in membrane cytoskeleton organisation by providing a potential link between membrane-cytoskeletal complex and signal transduction pathways. The high amino-terminal homology to that of ERM proteins suggest that merlin may compete with ERM for membrane binding site (Tsukita and Yonemura, 1997).

1.4.IV.d - Monomer binding proteins

The monomer pool of actin in nonmuscle cells is around 50% of total cellular actin. This concentration of unpolymerized actin pool is greatly above the critical concentration for polymerization of filament ends. This unpolymerized pool is maintained by binding of G-actin to a number of monomer binding proteins, allowing for the rapid and efficient disposal of monomers for transient assembly anywhere and at any time in the cell (Schafer and Cooper, 1995, Weber, 1999).
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1.A.IV.d.1 - ADF/Cofilin family

Cofilin, actin depolymerizing factor (ADF), destrin, actophorin and depactin are closely related members of a family of small (15-22Kda) proteins that can form a binary complex with G-actin and depending on pH can also bind to F-actin causing depolymerization (Sun et al., 1995; Theriot, 1997; Weber et al., 1999; Cooper and Shafer, 2000). ADF/cofilin members are essential proteins found in all eukaryotes playing important roles in cytokinesis, endocytosis and in the development of all embryonic tissues (Carlier et al., 1999; Theriot, 1997).

ADF/cofilin from different organisms present a high degree of sequence homology. These proteins carry a phosphoinositide binding site and share sequence homology at the carboxyl terminal end with members of the gelsolin family and with the carboxyl terminal of lower eukaryote profilin. Based on this structural homology between cofilin and gelsolin S1 domain, it was hypothesized that cofilin could interact with actin in a similar manner. The ADF/cofilin fold consists of five central β-sheets flanked by three to four α-helices. Analysis of co-crystal structures has shown, however, that the actin binding surface of gelsolin S1 is distinct from cofilin (Lappalainen and Drubin, 1997; Lappalainen et al., 1997). Mutagenesis studies of yeast cofilin defined regions of the molecule interacting with both G and F-actin at the amino terminal and region interacting with F-actin only extending from the amino terminus across the beginning of the long α-helix to the carboxyl terminus (Lappalainen et al., 1997). These results also demonstrated that the amino terminal region of yeast cofilin is critical for F-actin binding and depolymerization.

The actin binding activity of various members of the family are inhibited by phosphorylation and/or by competitive binding of phosphoinositides indicating that ADF/cofilin family members are good candidates for downstream effectors of several types of
signalling cascades that cause rearrangements of the actin cytoskeleton (Theriot, 1997; Weber et al., 1999). ADF/cofilin is regulated by phosphorylation of a single conserved residue at the amino terminal region. ADF/cofilin activity is induced by dephosphorylation which correlates with increase motility and extension of cellular processes (Carlier et al., 1999; Lappalainen et al., 1997).

The interaction of cofilin with F-actin has been analysed by electron cryomicroscopy and image reconstruction. It showed that cofilin binds cooperatively between two actin subunits along actin filament. The binding site comprises the lower half of subdomain 1 and the cleft between subdomains 1 and 3. This association generates a twist on actin filament resulting in a reduction in the length of the filament. This study also demonstrated that the binding of cofilin altered and prevented the binding of phalloidin to filaments, indicating that this structural change may represent a novel mechanism through which the actin cytoskeleton may be regulated (McGough et al., 1997).

*In vitro* biochemical studies have been carried out in order to understand mechanisms underlying the effect of cofilin on actin dynamics. Bacterially expressed ADF1 form plant *Arabidopsis thaliana* has been expressed in bacteria to examine the effect on actin binding and depolymerization effects. It was observed that ADF accelerates the association rate of actin monomers at the barbed end by 12 fold without affecting the dissociation rate, whereas at the pointed end, the association rate was modestly enhance but the dissociation rate was increased by 12 fold indicating that the different between the critical concentrations of the barbed and pointed ends is greatly enhanced by the presence of ADF leading to depolymerization of actin (Carlier et al., 1997). This depolymerizing function of cofilin has been demonstrated *in vivo*. *Listeria* in *Xenopus* egg extracts are much shorter and contain ~20 times less actin in the presence of ADF/cofilin than in *Xenopus* that have been depleted of ADF/cofilin by treatment with inactivating antibody (Rosenblatt et al., 1997).
ADF/cofilins are typically concentrated at the dynamic actin-rich lamellipodia in locomoting cells. Cofilin overexpression in Dictyostelium discoideum stimulates membrane ruffling and movement. Green fluorescent protein (GFP)-Dictyostelium cofilin fusion protein was expressed and the purified GFP-cofilin bound to actin decreasing the apparent viscosity of actin solution in a similar manner to native cofilin (Aizawa et al., 1997). During phagocytosis GFP-cofilin accumulated into actin bundles in the region underlying the phagocytic cup. Upon cAMP activation of cells cofilin coassembled with F-actin at the leading edge and when the chemical stimuli was changed there was a rapid redistribution of GFP-cofilin towards the new pseudopod demonstrating that cofilin plays a crucial role in vivo in rapid remodelling the cortical meshwork into bundles (Aizawa et al., 1997).

The in vivo function of ADF/cofilin has also been studied in vivo in yeast (Lappalainen and Drubin, 1997; Lappalainen et al., 1997). Using partial loss of function alleles in the yeast cofilin gene, alleles were generated producing a range of severity in their phenotypes demonstrating that rapid cycles of actin assembly and disassembly depend on cofilin. Two mutants were also generated whose mutations resulted in lethality. They appeared to be defective in F-binding but not G-binding activity, suggesting that F-binding and stimulation of actin depolymerization are both essential functions of cofilin in yeast and most likely in all eukaryotic cells (Lappalainen et al., 1997).

The information obtained from all these experiments generated a model for the effect of ADF/cofilin on actin polymerization. It seems that under physiological ionic conditions, all ADF/cofilin recognize ADP-bound form of both G and F-actin with high specificity, with a slight preference for G actin. This causes cofilin to sequester actin monomers enhancing nucleation and the addition of ADF-ADP-actin to barbed end of actin filaments. This accounts for the rapid filament turnover seen in lamellipodia. The mechanism by which cofilin increases the number of filaments differs from the sequestering effect. Because of the
slow nucleotide dissociation from ADF-actin complex, the major monomeric actin state when
F-actin is assembled at steady state in the presence of ADF is not ATP-actin but ADF-ADP-
actin. The ADF decorated filaments rapidly lose subunits from the pointed end, compared to
the ADF-ADP-actin barbed end. This will be in agreement with the finding that binding of
ADF generates a twist in the filament which enables ADF to control filament length (Carlier
et al., 1999; Cooper and Shafer, 1999).

1.4.IV.d.2 - Thymosin- β4

Thymosin β4 is a major actin monomer binding protein present at high concentrations in
many vertebrates cells and cell lines (Nachmias, 1993; Nachmias et al., 1993). Nine
thymosins have been identified and six have been shown to bind to actin. Thymosin β4 was
found to form a 1:1 complex with G-actin under physiological conditions, and the binding
was not affected by calcium (Weber et al., 1992). In contrast to all monomeric binding
proteins, thymosin β4 does not bind to F-actin, does not elongate actin filaments at either end
nor suppress the nucleation of polymerization (Weber et al., 1992).

A role in sequestering monomeric actin was elucidated in studies in resting neutrophils in
which thymosin β4 accounts for much of the pool of G-actin (Cassimeris et al., 1992).
Platelets and leukocytes require a pool of monomeric actin in order to respond rapidly to
changes in their environment with an increase in polymerized for. In resting platelet about
half of thymosin β4 is complexed to G-actin. In response to stimulation leukocytes increase
their F-content by four fold. Uncapped filament ends take up monomers, decreasing G-actin
concentration by dissociation thymosin β4-actin complex (Weber, 1999). Kinetic studies
to bind to poly(L-proline), PIPs and actin and shown to have similar effects, although profilin I binds with higher affinity to monomeric actin than profilin II (Gieselmann et al., 1995).

The crystal structure of the complex between profilin and β-actin has directly identified the profilin binding site on domains 1 and 3 of G-actin and its binding constant is about 5 times higher than thymosin-β4 (Puius et al., 1998; Weber, 1999). Its secondary structure is composed of an antiparallel central β-sheet flanked on one side by amino and carboxyl-terminal α-helices and on the other side by two β-strands and a third helix. The face with two helices participates in actin binding (Rozycki et al., 1994; Schutt et al., 1993). The crystals data also revealed that β-actin has substantial conformational flexibility when bound to profilin, suggesting that the binding of profilin may lead actin to adopt a conformation which facilitates monomer addition to the growing barbed end enhancing actin polymerization (Schutt et al., 1993).

In the cell the primary function of profilin is the promotion of rapid actin assembly at low G-actin concentrations. Profilin-actin complexes cannot bind to the pointed ends of actin filaments because the interacting actin surface is blocked by profilin. The complex binds to the barbed filament ends and elongate actin filaments form the barbed ends after incorporation of the actin molecule into the filament. Profilin only caps when free profilin concentration is very high (Weber, 1999). Elongation stops when actin monomer concentration falls below the critical concentration. This profilin induced lowering of the critical concentration requires energy which is provided by the hydrolysis of actin-bound ATP In Thyone acrosomes, the amount of profilin is equimolar to the actin resulting in a high rate of acrosome elongation (Tilney and Inoue, 1982). Cells lacking profilin display a greater drop in F-actin levels upon temperature shifts and are slower to recover to initial F-actin levels that wild type (Yeh and Haarer, 1996).
By immunofluorescence localisation, profilin is a cytoplasmic protein. Electron microscopy revealed that some of the profilin is associated with the inner surface of the plasma membrane of platelets and leukocytes (Hartwig et al., 1989).

In addition to binding actin, all cellular profilins bind to a number of proline rich proteins in vitro and co-localize in vivo. These include the focal adhesion proteins vasodilator-stimulated phosphoprotein (VASP) and the mammalian homologue of Drosophila enabled (MENA) (Gertler et al., 1996; Reinhard et al., 1995b). VASP is a microfilament and focal adhesion associated protein which is also concentrated in highly dynamic regions of the cell cortex. Human platelet VASP binds directly to purified profilins from human platelets, calf thymus and birch pollen, suggesting that they may be involved in the organisation of microfilament organisation via external signals (Reinhard et al., 1995). MENA is a murine protein highly related to VASP and was found to be concentrated at the pole of motile Listeria monocyes associated with actin polymerization while on non-motile bacteria. MENA is uniformly distributed. When expressed in fibroblasts, MENA induces the formation of F-actin rich protrusions (Gertler et al., 1996). These proteins are involved in the formation of specific actin structures suggesting that the interaction with proline rich sequences is a targeting mechanism for the localisation of profilin and it’s actin associated activities.

Recently it’s been reported that Schizosaccharomyces pombe Cdc12, an essential gene necessary for actin ring formation, binds directly in vitro to profilin, suggesting that this connection is necessary for ring formation in cell division (Chang et al., 1997). The Rho family of small G proteins are known regulators of rapid actin reorganisation leading to the formation of filopodia, lamellipodia and stress fibers. A possible role of profilin-small G proteins mediated actin filament reorganisation was investigated by generation of recombinant profilin mutants expressed in swiss 3T3 cells. The mutant protein was generated so that only the actin binding site was removed allowing the mutant proteins to retain it’s
ability to bind to all other detectable profilin binding proteins (Suetsugu et al., 1999). Expression of Cdc2, Rac, Rho with wild type and mutant protein showed that mutant protein suppressed the formation of Cdc2 induced actin microspikes and Rac induced membrane ruffles whereas Rho-induced stress fiber formation occurred independently of mutant expression, thus indicating that profilin is indispensable for the de novo actin polymerization that occurs during microspike and membrane ruffling formation (Suetsugu et al., 1999).

1.4. IV. e - Spectrin family

This family of ABPs binds and cross-links actin filaments into higher order structures that give shape and substance to the peripheral cytoplasm of cells. Although the primary function of cross-linking proteins is to join filaments together, these proteins have the ability to bind to additional proteins such as membrane glycoproteins and cytoplasmatic proteins regulating their interaction with F-actin (Hartwig, 1995). In order to cross-link filaments each protein possesses two actin binding sites through dimerization. Members of this family share a conserved 27KDa F-actin binding domain usually located at the amino terminal of each protein (Hartwig, 1995; Puius et al., 1998).

The high similarity of the modular features among all members of this family suggest they arised from a common ancestor gene. One current hypothesis is that a gene duplication gave rise to a stable lineage leading to α-actinin genes while other duplicated gene acquired additional repeats by a series of unequal cross-over events giving rise to an spectrin ancestor gene. This large gene was split into two functional genes, each encoding a different spectrin subunit (Viel, 1999).
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1.4.1.1 - Spectrin

Spectrin is the major structural protein of the erythrocyte membrane skeleton that binds and cross-links actin filaments (Hartwig, 1995). The basic unit of spectrin is an antiparallel heterodimer composed of two homologous chains, α (280KDa) and β (246 KDa) (Pascual et al., 1997; Fujita et al., 1998). Each homologous chains associate laterally into tetramers through a head to head interaction. The initial antiparallel association of α and β spectrin to form dimers occurs and the amino terminus of the β subunit which associates with the carboxyl terminus α subunit. This site is referred as the dimer nucleation site (Cherry et al., 1999). The rest of the molecule is thought to associate in a zipper-like fashion. Two zipped dimmers associate at the tetramerization site, which is located at the opposite end of the dimer nucleation site resulting in a tetramer molecule in which the actin binding sites are exposed at it’s ends enabling the molecule to cross-link membrane-associated actin filaments (Cherry et al., 1999; Pascual et al., 1997). Additional features include an actin binding domain made of two calponin homology domain (CH), a plekstrin homology domain, a SH3 domain and a calmodulin like domain with four EF-hands (Pascual et al, 1997).

Spectrin is present in most animal tissues, although it has been most studied in red cells where binding of spectrin to the sides of short actin filaments is essential for the maintenance of the normal biconcave shape and membrane stability of red blood cells. The remarkable elasticity of these cells have been attributed to the modular structure made of sequential repeats and the association with other actin binding proteins to maintain and regulate the complex structure and physiology of these cells. The bulk of α and β subunits consist of 16 repeats of 106 amino acids predicted to fold into a left handed triple helical bundle (Pascual et al., 1997). At alkaline pH oligomers dissociate, and this event is accompanied by deprotonation of tyrosine and lysine residues within the repeat segments (Fujita et al., 1998).
Isoforms have been identified in cells and tissues ranging from plants, bacteria to humans (Hartwig, 1995). Diversity at protein level is the result of various contributing factors such as existence of multiple genes, alternatively spliced mRNA from individual genes and by the combinatorial association of different isoforms. Nonerythrocyte isoforms have been localised by immunohistochemistry to plasma membrane closely associated with plasma membrane receptors, glycoproteins and subcellular vesicles. Four genes for spectrin have been identified; two for each α and β subunit chain. The genes for erythroid spectrin are αI1 and βI1, located at chromosomes 1 and 14 respectively. The nonerythroid isoforms genes are named αII and βII on chromosomes 9 and 2 respectively. In muscle, however, the βI1 chain pairs with αII chain product (Hartwig, 1995). A novel isoform β\text{heavy} -spectrin has been identified in \textit{Drosophila melanogaster} which has a role in establishing primary epithelial polarity (Thomas et al., 1997).

The tetrameric protein form is the result of the head-to-tail antiparallel alignment of α and β polypeptide chain yielding a dimeric molecule of 100nm which associates with another dimer to produce a 200nm protein. The backbone of dimer structure consists of what is called the ‘head of dimers’ which is composed of the actin binding domain of the β polypeptide at it’s amino terminal end and the EF-hand binding site of the α polypeptide at it’s carboxyl terminal. Both α and β subunit have domains with different structure and function. Each α and β chains have 22 and 17 repetitive elements respectively. The α chain has two EF-hand motifs at repeat 22 at the carboxyl end followed by a rod domain ( repeats 21-1) containing a sequence insertion between repeat 11 and 12 responsible for calmodulin binding site. The β chain is shorter than α chain that has an actin binding site at the amino terminal end followed by 17 homologous repeats and a carboxyl end composed of a single α helix (Hartwig, 1995).
The major function of spectrin is to establish a planar network for supporting the plasma membrane. At this location, spectrin interacts with adducin, ankyrin and actin to form a two dimensional membrane skeleton to adhere it to the plasma membrane. Ankyrin connects spectrin to the integral membrane by binding via the 15th repeat of β chain. Adducin also binds to spectrin-actin complex with higher affinity than spectrin alone (Hartwig, 1995).

To study the role of spectrin in cells, mutant mice cells were generated in which cells contained β-spectrin but lacked α-spectrin. Deficiency of α-spectrin resulted in cell fragility and shape irregularity, indicating a direct effect on membrane cytoarchitecture and integrity. This idea was reinforced by studies in Drosophila lacking α-spectrin in which cells demonstrated the same irregularities (Dahl et al., 1994).

New data suggest a noncortical role for spectrin as a component of a network associated with the Golgi membrane (Holleran and Holzbaur, 1998). A novel spectrin isoform of 220KDa have been isolated that colocalizes with the Golgi. It's been suggested that spectrin might form a meshwork in the Golgi membrane similar to that found in the erythrocyte membrane, therefore mediating cargo transport and sorting of selective membrane proteins (Holleran and Holzbaur, 1998).

### 1.4.IV.e.2 - α-actinin

α-actinin is a ubiquitous actin binding and cross-linking protein of muscle and nonmuscle cells (Hartwig, 1995). In muscle cells α-actinin is localised at the Z discs of the sarcomeres where the barbed end of actin filaments insert and in nonmuscle cells α-actinin is colocalized with stress fibers, lamellipodia and filopodia. The binding of α-actinin in muscle and nonmuscle cells is regulated by calcium. Native α-actinin molecules are non-covalent
dimers composed of identical subunits which are aligned in an antiparallel fashion. Each subunit has an actin binding site at the amino terminal allowing the molecule to cross-link actin filaments to the length of the α-actinin isotype (Hartwig, 1995). The existence of many isotypes is the result of three distinct genes and alternative splicing. Three human α-actinin genes have been identified (ACTN1, ACTN2, ACTN3); ACTN1 is subjected to alternative splicing generating nonmuscle and smooth muscle isoforms (Hartwig, 1995).

Each α-actinin subunit is composed of three structural domains (Matsudaira, 1991). The amino terminal domain comprises residues 1-245 that encode an F-actin binding site which is highly conserved and related to members of spectrin family (Hartwig, 1995; Way et al., 1992). The central domain consists of four 120 amino acids repeats, which forms the rigid rod backbone of the molecule. Sequence analysis shows that α-actinin repeats R1 and R2 are homologous to β spectrin repeats β2 and β3 respectively, whereas repeats R3 and R4 are homologous to α spectrin repeats α20 and α21 (Viel, 1999). The crystallographic structure of R2 and R3 indicates that R2 from one chain interacts with R3 from the other chain generating a similar spectrin triple helix backbone structure (Viel, 1999). The carboxyl terminal contains two EF-hand calcium binding sites (Hatwig, 1995; Matsudaira, 1991).

The function of the F-actin binding domain has been investigated intensively. This domain has been highly conserved throughout species; chicken and Dictyostelium have 62% homology (Hartwig, 1995). The critical residues for actin binding extend from 120 to 134 and expression of protein constructs lacking these residues fail to bind to actin (Schleicher et al., 1995).

The carboxyl terminal contains two EF-hand domains which in muscle and nonmuscle α-actinin isoforms have different affinities (Hartwig, 1995). Muscle isoforms bind actin filaments in a calcium independent manner whereas nonmuscle α-actinin isoform binds in a
calcium dependent manner. The reason for this difference in isoform binding affinity is the presence of 9 and 12 amino acids insertion within the EF-hand domain of muscle isoforms (Schleicher et al., 1995). It's been suggested that the presence of extra amino acids in muscle isoforms may contribute to spatial targeting information to direct the isoform to the Z discs. The function of EF-hand domain has been investigated by point mutation in Dictyostelium discoideum and results showed a difference in calcium affinity for the two sites, implying a regulatory function of EF-hand domains. Disruption of the first EF-hand site abolished completely the F-actin cross-linking activity, whereas disruption of the second EF-hand structure did not alter the actin binding capacity (Schleicher et al., 1995; Witke et al., 1993).

A role for α-actinin in maintaining sarcomere integrity and in myogenesis has been identified in Drosophila mutants (Witke et al., 1992). Drosophila has a single α-actinin gene that is alternatively spliced producing one nonmuscle and two muscle isoform. Mutations affecting the muscle isoforms generate progeny which are immobile or have flight defects and muscle fragility. Electron microscopy analysis of mutants' sarcomere indicated an irregular Z line (Witke et al., 1992).

Dictyostelium double mutants lacking α-actinin and ABP 120 have been generated via homologous recombination to investigate the role of cross linking proteins on cortical cytoskeleton (Rivero et al., 1996). Cells lacking both proteins exhibit reduced growth rate, reduced cell size reflecting a reduced strength of the cortical cytoskeleton which can not resist internal forces during cell growth leading to a reduction in size. An effect on cytokinesis was observed by the presence of very large multinucleated cells, suggesting that both proteins might be involved in the division of daughter cells after DNA replication (Rivero et al., 1996).
Chapter 1

1.4.IV.e.3 - Dystrophin

Dystrophin is a 427KDa elongated protein product of Duchenne muscular dystrophy gene (Fabbrizio et al., 1993). Electron micrographs revealed its localisation in high amounts beneath the sarcolemal membrane in normal skeletal cells (Porter et al., 1992).

Dystrophin primary structure consists of four distinct regions, dominated by a large rod-shaped domain composed of 24 spectrin-like repeats with an overall length of 125nm (Koenig et al., 1988). The amino terminal domain contains two tandem calponin homology domains responsible for actin binding (Renley et al., 1998). The second region comprises 24 spectrin type repeats corresponding to the central rod repetitive sequence domain. The carboxyl end domain has a cysteine rich segment of 280 residues homologous to α-actinin carboxyl terminal with a putative calcium binding site. The last region comprises 425 amino acids specific to dystrophin only (Ervasti and Campbell, 1993; Hartwig, 1995).

Human dystrophin is a gene product of the X chromosome. The discovery of Duchenne muscular dystrophy gene revealed the presence of multiple splice sites that lead to the existence of isoforms which are also expressed in non-muscle cells (Hartwig, 1995). Isoforms lacking the amino terminal and rod shape domain have been identified in non-muscle cells in addition to alternatively spliced carboxyl terminal isoforms (Ervasti and Campbell, 1993; Hartwig, 1995).

The absence of dystrophin in muscle results cause a devastating muscular disorder called Duchenne's muscular dystrophy. The role for dystrophin in linking the actin cytoskeleton with the extracellular matrix in striated muscle have been established. Immunocytochemical studies have localised the carboxyl terminal of dystrophin to the sarcolemma intracellular plasma membrane in skeletal muscle. After limited proteolytic digestion it was found that the cysteine rich domain at the first half of the carboxyl domain remains bound to a glycoprotein.
complex therefore linking the sarcolemal membrane with the extracellular matrix (Suzuki et al., 1992a; Suzuki et al., 1992b; Suzuki et al., 1992c). However patients diagnosed with Duchenne muscular dystrophy have been shown to express a dystrophin isoform which lacks the cysteine region suggesting that the entire carboxyl region is not solely responsible for targeting of dystrophin to the plasma membrane (Ervasti and Campbell, 1993).

Nuclear magnetic resonance using synthetic peptides corresponding to the amino terminal domain, revealed that this domain contain two actin binding sites located at 17-26 and 128-156 providing evidence for the direct binding of dystrophyn to actin filaments. Recombinant amino terminal proteins, corresponding to tandem CH domain, were found to cosediment with F-actin (Fabbrizio et al., 1993).

The role of dystrophin in skeletal muscle is to link the extracellular membrane to the actin subsarcolemma cytoskeleton via a transmembrane glycoprotein complex. In order to evaluate the function of dystrophin in nonmuscle cells, and to test whether dystrophin is targeted to the muscle sarcolemma via preferential binding to muscle F-actin, dystrophin-glycoprotein complex was co-sedimented in the presence of muscle or brain F-actin. The results showed that dystrophin binds to both actin isoforms at similar affinities. It further indicates that other mechanisms might be involved in the targeting of dystrophin to the sarcolemmal membrane, other than glycoprotein complex binding, as several patients with Duchenne muscular dystrophy have been reported to express dystrophins encoding only the amino terminal and rod domains (Renley et al., 1998).

Recently it's been demonstrated that a cluster of basic spectrin like repeats near the middle of the rod domain interacts with F-actin via electrostatic interactions (Amann et al., 1999). Recombinant protein encoding repeats 11 and 14 was found to bind F-actin and binding was dramatically inhibited by increasing NaCl concentration. The functional significance of this finding still remains to be further investigated (Amann et al., 1999).
Gelation factor ABP-120 is a cross-linking protein that has been identified and purified from *D. discoideum* (Condeelis et al., 1984). It has a predicted molecular weight of 92KDa but migrates at 120KDa on SDS-PAGE gels. It is present in the cortical cytoplasm of these ameoboid cells particularly in pseudopodial projections in response to chemotactic stimuli (Condeelis et al., 1984).

ABP-120 is a homodimer rod of 40nm in length, which assembles in an antiparallel fashion (Condeelis et al., 1984). The domain structure of ABP-120 consists of the conserved amino terminal actin filament binding site of 250 residues of the spectrin family, followed by six repetitive 100 amino acid motif analogous to ABP280 (Fucini, 1999; Hartwig, 1995). The actin binding consists of two tandemly repeated CH domains whereas the rod is composed of six anti-parallel β-sheets which adopts an immunoglobulin-like fold (Fucini et al., 1999).

Dimerisation is crucial to the cross-linking function of ABP120 and is mediated by interactions involving the rod domains. cDNA constructs corresponding to all six repeats were generated and expressed in *E.coli* to examine the way in which each repeat contributes to dimerisation of ABP120 molecule (Fucini et al., 1999). It was found that fragments corresponding to repeats 1-6 and 5-6 dimerise, whereas repeats 1-5 and single repeats 3 and 4 are monomeric, indicating that stable dimerisation requires repeats 5 and 6 (Fucini et al., 1999). The authors further suggest a molecule model in which the two chains of the molecule overlap only at the C-terminus such that the actin binding domains in each molecule are widely separated which could help to ensure efficient cross-linking of actin filaments (Fucini et al., 1999).
Direct evidence for the role of ABP-120 in pseudop extension by cross-linking actin filaments has been demonstrated by mutagenesis (Cox et al., 1992). Mutant proteins have been obtained by homologous recombination and chemical mutagenesis of ABP-120 gene (Cox et al., 1992). Upon cAMP stimulation, ABP-120 null cells are rounded and have altered motility; they move extending pseudopodia which are much smaller than the wild type. Also, the amount of F-actin cross-linked in the cytoplasm of these mutants is reduced.

ABP-280, also called filamin, is a homodimeric protein that cross-link actin filaments into orthogonal networks in the cortical cytoplasm (Van der Ven et al., 2000). ABP-280/filamin is a large protein with molecular weight of 250KDa, found abundantly in smooth muscle and platelets were they represent 3-4% of total protein content. ABP280/filamin localises in actin enriched regions such as pseudopodia, membrane ruffles of moving cells and the cleavage furrow of dividing cells (Hartwig, 1995). In mammalian cells ABP-280 is concentrated near the plasma membrane and the recent identification of ligands for ABP280/filamin, such as β-integrins indicates a regulatory role in the organisation of the actin cytoskeleton (Loo et al., 1998; Van der Ven et al., 2000).

Three human isoforms have been identified. ABP280/filamin is the ubiquitously distributed isoform, encoded by a gene located at chromosome X. A second isoform, δ-filamin, was identified by PCR analysis whose gene is localised on chromosome 7. A third isoform has been identified in yeast and named β-filamin (Van der Ven, 2000).

The modular structure of ABP280/filamin consists of an amino terminal domain followed by 24 tandem 100-residue repeats that has an immunoglobulin fold and a carboxyl domain involved in dimer formation that are analogous to ABP120 (Hartwig, 1995; McCoy et
In contrast to ABP120, the two chains in the Y-shaped ABP280/filamin molecule are arranged parallel to one another. Sequence analysis indicates that domains 15-16, 20 and 22-24 are good candidates for involvement in dimerisation, although this remains to be tested (McCoy et al., 1999). Still, the two chains overlap at the carboxyl terminal exposing the actin binding site enabling ABP280/filamin to efficiently cross-links actin filaments.

The function of ABP-280/filamin have been evaluated in melanoma cell lines which do not express it. These cells display abnormal membrane stability characterized by continuous protrusions and retractions of the cell membrane (Hartwig, 1995).

Filamin has been also shown to bind to the cytoplasmic domain of β1-integrin providing a mechanism for the interaction of this cell surface receptor with cytoskeletal proteins (Loo et al., 1998). Filamin was found to bind to the cytoplasmic domain of β1-integrin via it’s carboxyl terminal (residues 2171-2647). This region comprises 4.5 tandem repeats, including the hinge domain. Recombinant protein containing the carboxyl terminal 478 amino acids was expressed in Jurkat T cell line and western blot analysis using monoclonal antibody against filamin of cell lysates showed that filamin specifically coprecipitated with β1-integrin. Additionally, fusion proteins containing the carboxyl terminal 478 amino acids of filamin was also shown to specifically bind to a synthetic peptide containing the complete cytoplasmic domain of β1-integrin in vitro. These results reinforce the role that filamin plays in regulating cell adhesion, motility and activation (Loo et al., 1998).

Recently, δ-filamin has been found to be highly expressed in mammalian striated cells. Using a monoclonal antibody that recognises both the non-muscle and muscle isoforms, immunocytochemistry and electron microscopy showed that the amino terminus of δ-filamin is localised at the periphery of the Z-disc. Western blots showed that both δ-filamin mRNA and protein are absent in proliferating cultured skeletal cells but are upregulated immediately
after induction of cell differentiation, suggesting the this isoform may play a role in Z-disk assembly (Van der Ven. Et al., 2000).

1.4.IV.e.6- Adducin

Adducin is a 200KDa heterodimeric phosphoprotein of the membrane skeleton that intermediates the association of spectrin with actin filaments (Hartwig, 1995). Although originally purified from erythrocytes, related isoforms have been identified in brain and other tissues. The α chain is the product of a gene on chromosome 4 and the β chain is the product of a gene located in chromosome 2 and is expressed differently in different tissues (Hartwig, 1995).

Adducin is a heterodimeric asymmetric molecule that consists of an α, β and δ similar subunits. Each subunit has a structurally distinct domains: an amino terminal globular head (40KDa), an 8 KDa central domain and a carboxyl terminal domain which is responsible for the association of actin and spectrin complexes. The globular head at the amino terminal contain an actin binding site with low homology to spectrin whereas the rest of the molecule is highly enriched in hydrophylic residues (Hartwig, 1995; Manunta et al., 1998).

In the absence of spectrin, adducin binds and bundles actin filaments with low affinity. However, its main role is believed to be the regulation of the assembly of spectrin and actin. To achieve such interaction, adducin modulates the exposure of transmembrane proteins in a calcium and phosphorylation dependent fashion (Manunta et al., 1998). A direct effect of such modulation role has been described in Milan hypertensive rats (MHS) in which point mutations in α and β adducin genes is associated with hypertension. Comparative analysis of normal and MHS cells indicated that the presence of mutated adducin led to an increase in F-
actin content suggesting that adducin polymorphism may affect the transport of sodium by modulating the binding of Na-K-pump to actin filaments (Manunta et al., 1998).

1.4. IV.e.7 - Fimbrin/ T & L Plastin

The smallest member of the spectrin superfamily of actin cross-linking proteins is fimbrin/ T & L plastin (68KDa). Fimbrin cross-links actin filaments into tight bundles in a calcium sensitive manner and cells lacking fimbrin have found to assemble abnormal actin cytoskeletons (Puius et al., 1998). Fimbrin differs from the other members of this family of cross-linking proteins in that it is a globular protein that has two actin binding domains tandemly arranged on the same polypeptide chain, located in the central core of the protein, which direct the formation of tight actin bundles observed in microvilli and stereocilia (Goldsmith et al., 1997; Hartwig, 1995; Puius et al., 1998).

The genes for two fimbrin/plastin isoforms have been identified and the protein products of these genes are called T and L fimbrin/plastin (Hartwig, 1995). T-fimbrin/plastin is the isoform found in all tissues except haematopoietic cells and has a molecular weight of ~70KDa whereas L is the hematopoietic isoform with a molecular weight of ~63KDa.

Fimbrin contains an amino terminal calcium binding domain (1-100aa) followed by a tandem pair of actin binding domains (101-375 and 376-624). Each of these actin binding domains is composed of two CH domains (Goldsmith et al., 1997; Hanein et al., 1998).

The amino terminal consists of two EF-hand calcium binding domains. The actin bundling activity of fimbrin is inhibited by calcium, indicating the importance of the amino terminal calcium binding domain (Hanein et al., 1998). Based on crystal structure and biochemical information, a model for the mechanism of calcium regulation has been proposed. The calcium binding domain occupies the crevice between the two CH domains
and in the presence of calcium it can disrupt actin binding by changing conformation and interaction of adjacent CH domains (Hanein et al., 1998).

The crystal structure of fimbrin actin binding domain has been determined (Goldsmith et al., 1997) and found to be composed of two tandemly arranged calponin homology (CH) domains. Each CH domain is composed of four $\alpha$-helical segments separated by a long $\alpha$-helix connecting the two CH domains (Goldsmith et al., 1997). Three helices form a loose parallel three-helix bundle with the fourth helix lying perpendicular to the bundle. Electron cryomicroscopy image analysis of first actin binding domain (1-375) bound to rabbit skeletal muscle actin shows that this segment binds to the outer face of subdomains 1 and 2 of actin and that binding causes a conformational change in subdomain 1. This result demonstrates that fimbrin do cause internal rearrangements of actin and therefore can potentially change the properties of actin dynamics (Hanein et al., 1997).

1.4.IV.f - Miscellaneous ABPs

1.4.IV.f.1 - Insertin

Insertin is an actin binding protein isolated from chicken gizzard smooth muscle which retards but does not totally inhibit actin polymerization at the barbed end (Teubner et al., 1998; Teubner and Wegner, 1998; Weigt et al., 1992). Filament assembly appears to occur by insertion of two insertin molecules at the barbed ends of actin filaments (Weigt et al., 1992). The primary structure of insertin is almost identical to amino acid residues 862 and 1212 of tensin, and this homologous domain is localised in the third quarter of tensin amino acid sequence (Weigt et al., 1992). Southern blot analysis revealed that insertin and tensin derive from the same gene; insertin likely to be a proteolytic fragment of tensin (Teubner et
al., 1998; Teubner and Wegner, 1998). As tensin occurs in focal adhesion sites playing an important role in the early events of association of microfilaments with membranes, it was suggested that the presence of the insertin domain in tensin could be part of the machinery of molecules that permit treadmilling of actin filaments in living cells by allowing the insertion of actin molecules between membrane and actin filaments (Teubner et al., 1998; Teubner and Wegner, 1998).

L4IV.f.2 - AbLIM

Actin binding LIM (abLIM) protein is a novel retinal protein (Roof et al., 1997). It has a complex primary structure with a cytoskeletal demantin like domain that is fused to a second domain composed of four double zinc finger motifs (a cysteine rich motif). Fusion protein constructs were generated of each domain (GST-Dem and GST-LIM) and GST-Dem bound with high affinity to actin filaments in vitro. Whether abLIM cross links filaments still remains to be shown. Although abLIM has high affinity to demantin it does not contain the putative cysteine residue involved in the trimer formation of demantin. It has been suggested that the LIM motif may play a fundamental role as cellular regulatory sequences through modulation of a variety of protein-protein interactions. AbLIM is a substrate for intracellular kinase and is highly phosphorylated in the retina during light adaptation. Together with demantin like motif it is possible that abLIM could play a role in establishing or altering cell morphology through actin binding and/or participate in a signalling pathway involved in cellular differentiation (Roof et al., 1997).
1.4.IV.f.3 - Demantin

Demantin was first identified in the erythrocyte membrane skeleton. It is an actin bundling protein whose sequence includes a headpiece domain homologous to that found in the villin family (Rana et al., 1993). Demantin consists of two polypeptide chains of 48 and 52KDa present in a 3:1 ratio. It can bind to and bundle actin filaments although it does not contain the six domain repeat structure of the gelsolin family. The actin bundling activity is abolished upon c-AMP-dependent protein kinase phosphorylation and is restored after dephosphorylation. In solution demantin exists as a trimer and bundles actin filaments in a phosphorylation dependent manner. Analysis of primary structure of human demantin carboxyl terminal showed ~48% homology to human villin. By limited proteolysis it was shown that loss of bundling was parallel to increasing proteolysis resulting in a fragment of 13KDa that sedimented with actin filaments. These results suggested that like villin demantin headpiece alone can bind but not bundle actin filaments. Also, demantin has one cysteine residue that is indeed phosphorylated and it may account for the fact that the actin bundling activity of demantin but not villin is regulated by phosphorylation (Rana et al., 1993).

Northern blot analysis detected demantin transcripts in heart, brain, lung, skeletal muscle and kidney. The widespread distribution of demantin transcripts in human tissues and it’s homology to villin suggest a possible role in regulating actin reorganisation via phosphorylation in tissues that do not contain villin (Rana et al., 1993).

1.4.IV.f.4 - Tensin

Tensin is a barbed-end capping protein found at cell junctions in fibroblasts and muscle (Chuang et al., 1995; Shafer and Cooper, 1995). The cDNA sequence of tensin revealed
homology to insertin and is most likely a proteolytic fragment of tensin. Recombinant tensin shows complete capping activity. Tensin has the ability to bind to actin filaments at multiple sites, enabling tensin to both cap the barbed ends of filaments and to cross link filaments.

Tensin is phosphorylated on tyrosine, threonine and serine residues. It also has a Src homology 2 (SH2) domain which makes it a candidate to bind to other proteins which are themselves tyrosine phosphorylated. The many characteristics of tensin make it to associate with the actin cytoskeleton in many different ways (Lo et al., 1997; Schafer and Cooper, 1995).

The \textit{in vivo} function of tensin has been investigated during embryogenesis and post natal development in mice (Lo et al., 1997). It was found that although tensin was expressed in many different tissues during embryogenesis, tensin null mice developed normally, suggesting that tensin’s diverse functions are redundant and may be compensated for by other focal adhesion proteins (Lo et al., 1997).

\textbf{1.4. IV.f.5 - Vinculin}

Vinculin is a 117KDa protein that localises to both cell-cell and extracellular matrix adherens junctions where it provides a structural and functional link between F-actin and cell adhesion molecules (Rudiger et al., 1998).

It’s secondary structure consists of a large 95KDa globular head amino terminus domain connected by a short proline-rich region to a 30KDa tail domain (Steimle et al., 1999). The amino terminal head contains binding sites for cytoskeletal proteins talin and \(\alpha\)-actinin whereas vinculin tail binds F-actin, paxillin and acidic phospholipids (Bakolitsa et al., 1999; Rudiger et al., 1998). The proline rich region contains distinct binding sites for vasodilator-stimulated phosphoprotein (VASP), vinexin and ponsin (Bakolitsa et al., 1999). The head
and tail domains interact folding the molecule. This intramolecular association regulates the
interactions of vinculin. The binding sites for talin, α-actinin and F-actin are blocked by
intramolecular interaction. *In vitro,* free vinculin tail domain binds F-actin and cross-links
actin filaments into bundles. Expression of vinculin tail in vinculin-deficient cells results in
decrease in actin dependent cell motility which can be reversed by coexpression of vinculin
head domain (Johnson and Craig, 2000).

The crystal structure of vinculin tail (879-1066) has been determined and consists of five
helices connected by short loops. The combination of short and long helices creates a crevice,
hendering a ‘V’ shape (Bakolitsa et al., 1999). Several studies using recombinant peptides
have attempted to localize the actin binding site (Puius et al., 1998). Two regions of the tail
corresponding to amino acids 940-1012 and 1012-1066 of chicken vinculin sequence has been
shown to constitute the actin binding sites (Johnson and Craig, 2000).

The mechanism by which vinculin regulates the assembly of the actin cytoskeleton is far
from understood. Folding of vinculin, is regulated by acidic phospholipids and possibly by
phosphorylation. It seems that in the presence of acidic phospholipids, the head and tail
interaction is released, causing a conformational change in vinculin tail allowing for
membrane insertion via electrostatic forces and exposing the vinculin head, allowing for
binding to focal adhesion proteins (Bakolitsa et al., 1999).

*1.4. IV. 6. - Metavinculin*

Metavinculin is a larger splice variant which is coexpressed with vinculin. It binds to
actin via an actin binding domain localised in the carboxyl terminal tail analogous to vinculin.
It is within this actin binding area that metavinculin has a specific insert, but cosedimentation
studies coupled to viscometry demonstrate a direct interaction between metavinculin and actin
filaments (Rudiger et al., 1998). Whereas the tail region binds well to actin, intact gizzard metavinculin binds only weakly or not at all suggesting that the biological activity of metavinculin is regulated by an intramolecular interaction between head and tail as in the case for vinculin.

1.4. IV.f.7 - Calponin

The calponin family comprise a ~30KDa actin binding proteins that regulate smooth muscle contraction. It contains three isoforms, a smooth muscle-specific variant termed CaP h1, a neutral variant CaP h2 and an acidic variant which is particularly enriched in the brain (Gimona and Mital, 1998; Stradal et al., 1998). The three variants share an overall 70% amino acid identity. All isoforms have a multidomain structure: a calponin homology (CH) domain at the amino-terminus, an actin binding site (ABS) and a series of three 29 residues tandem repeats at the carboxyl-terminus (Stradal et al., 1998). CaP h2 isoform differs in lacking a consensus ABS motif between the CH-domain and the tandem repeats, which in CaP h1 isoforms can be chemically cross-linked to actin (Gimona and Mital, 1998). However, despite the absence of CaP h2 ABS, it still co-sediments with F-actin in vitro suggesting the presence of another binding site. Indeed, it has been found that the residues responsible for the binding of CaP h2 to F actin seem to reside at the carboxyl-terminus within the tandem repeats (Gimona and Mital, 1998). The CH-domain of the calponin family has been identified in many actin binding proteins and signalling molecules where it has been proposed as the region responsible for directly linking signal transduction molecules to the actin cytoskeleton via an association with actin (Stradal et al., 1998). Data base analysis defined three groups of CH-domain containing molecules: Fimbrin family of monomeric actin cross-linking molecules; dimeric cross-linking proteins (α-actinin, spectrin, filamin) and
monomeric F actin binding proteins (dystrophin); and proteins containing only a single amino-terminus CH-domain. It was also found that proteins that contain an amino-terminus CH-domain are more similar to each other than carboxyl-terminus CH-domain containing proteins (Stradal et al., 1998). Each single CH-domain is able to bind one actin monomer in the filament which result in the low binding constant range (Stradal et al., 1998).

The crystal structure of the calponin homology domain (CH) from human spectrin has been obtained. It consists of four α-helices connected by long loops. The core of this domain is composed of two parallel α-helices forming an angle of 75° arranged in a ‘V’ shape (Carugo et al., 1997).

Although the CH domain has been identified as the conserved F-actin binding domain of many actin binding protein families, it seems that both amino and carboxyl-terminals are involved in the binding of calponin isoforms to actin filaments (Carugo et al., 1997; Stradal et al., 1998).

Transgelin is a monomeric 22KDa actin binding protein which is down regulated in transformed cells (Shapland et al., 1988). Transgelin causes gelation of actin filaments by falling ball viscometry in a calcium independent manner. EM studies showed that transgelin rapidly induces the formation of actin bundles and light scattering indicates that it does so by cross linking actin filaments (Shapland et al., 1993). Sheep aorta transgelin binds directly to actin filaments in vitro at a saturable ratio of 1:6 actin monomers with a binding constant of $7.5 \times 10^5 \text{M}^{-1}$. Transgelin is present as one copy in the mammalian genome and has been conserved from yeast to humans (Prinjha et al., 1994). Transgelin message is not present in lymphocytes and transformed SV40 3T3 cells and since transgelin is known to cross-link
actin filaments it was suggested that it may play a crucial role in cytoskeletal activation and remodelling that occur during metastasis (Shapland et al., 1993; Prinjha et al., 1994).

1.4.IV.f.9 - Cortexillins

Cortexillins are actin bundling proteins. Cortexillins I and II constitute a novel subfamily of proteins with actin binding sites of actinin/spectrin type (Faix et al., 1996). The carboxyl-terminal of these dimeric proteins contain a heptad repeat domain by which the two subunits are joined to form a two stranded parallel coil. The amino-terminal has an actin binding domain folded into globular heads (Faix et al., 1996). Cortexillin link actin filaments preferentially by binding them into anti-parallel fashion. Elimination of the two isoforms by gene disruption gives rise to flatten and large cells indicating that cortexillins play a role in determining cell shape.

1.4.IV.f.10 - Ponticulin

Ponticulin is a ~56KDa F-actin binding transmembrane glycoprotein found in Dictyostelium plasma membrane (Chia et al., 1993). Ponticulin nucleates actin polymerization in a lipid dependent manner. Disruption of the gene encoding ponticulin by homologous recombination, indicate that ponticulin is not required for cellular translocation but may play a role in cell patterning during development (Hitt and Luna, 1994).
Fascin is a 58KDa actin bundling protein found in membrane ruffles, microspikes and stress fibers (Yamashiro et al., 1998). It bundles actin filaments in vitro into structures which stabilise cellular processes ranging from mechanosensory bristles to the filopodia of nerve growth cones (Edwards & Bryan, 1995).

Fascin is encoded by a single gene and homologous proteins have been identified and characterized in a variety of tissues and organisms ranging from sea urchin to humans (Cant & Cooley, 1996). The significance of these multiple isoforms remains unclear, however it is possible that different variants are differently localised within the cell organising specific actin bundles (Edwards and Bryan, 1995).

Human fascin is a globular protein with one actin binding domain localised at the carboxyl terminal half of the molecule. This 26Kda fragment has been shown to cosediment with F-actin (Edwards and Bryan, 1995).

In order to map and investigate the function of actin binding domain Drosophila mutants were generated by introducing point mutations within the actin binding site (Cant and Cooley, 1996). In Drosophila, fascin is encoded by a single gene. Mutation in a small conserved domain near the carboxyl terminus resulted in partial inactivation of fascin; mutants had kinked bristles and were fertile. However, a more dramatic effect was observed as result of a mutation that changed a serine residue at 289 to asparagine. This mutation completely inactivated fascin and mutants displayed gnarled bristles and were sterile due to the absence of cell cytoplasmic actin bundles, therefore affecting rapid cytoplasm transport during oogenesis (Cant and Cooley, 1996).

The expression of fascin is greatly increased in many transformed cells as well as in specialized normal cells including neuronal cells and antigen-presenting dendritic cells. The
morphological characteristic of all these cells is the development of many membrane protusions in which fascin is predominantly present (Yamashiro et al., 1998). To examine this morphological changes, fascin was expressed in epithelial cells to levels as high as those found in transformed cells. Fasin transfected cells showed an increased number of longer and thicker microvilli on apical surfaces, extended lamellipodia and disorganisation of cell-cell contacts indicating that fascin is directly responsible for membrane protusions through reorganisation of actin cytoskeleton at the cell periphery (Yamashiro et al., 1998).

1.4.V - ACTIN AND DISEASES

The actin cytoskeleton is implicated in many cell functions and any defect of these processes leads to cytoskeletal changes affecting the normal cell biology resulting in many disease states (Janmey and Chaponnier, 1995). It participates in a wide variety of crucial cellular events including determining and altering shape, movement, cell division, cell-cell communication, cell anchorage and organisation of intracellular apparatus. A defect in actin or in a protein that connects some component of this network could affect any of these processes and have a consequent effect on growth control (Janmey and Chaponnier, 1995; Trofatter et al., 1993). Abnormalities in actin itself are probably lethal given the different roles of actin in cell biology. Also, abnormal intracellular expression of actin or actin binding proteins has been implicated in cell transformation, motility, fibrotic diseases and scar formation. Cell transformation is accompanied by cell shape changes and by decreased expression of actin associated proteins. Gelsolin, α-actinin, tropomyosin, vinculin, transgelin and merlin are down regulated in transformed cells and their re-expression reduces
tumorigenicity (Janmey and Chaponnier, 1995). Such proteins may function as regulator of cell growth and differentiation.

Gelsolin was identified as the most down regulated protein in transformed fibroblast and epithelial cells (Vandekerckhove et al., 1990). Overexpression of gelsolin causes an increase in fibroblast motility which is mediated by the severing activity of gelsolin. A role for gelsolin in rapid motile responses in cell types involved in stress responses such as hemostasis, inflammation and wound healing was demonstrated in the gelsolin null mice (Witke et al., 1995). The gelsolin-null mice have normal embryonic development indicating that actin severing activity of actin is not essential for motility during early embryogenesis. However, the adult animals display defects in hemostasis and platelet activation, inflammatory response and leukocyte motility, and dermal fibroblast function in vitro. A prolonged bleeding time was most likely due to diminished severing of actin filaments in platelets corresponding to a decrease in platelet shape changes (Witke et al., 1995). Cultured gelsolin-null fibroblasts had striking morphological abnormalities consistent with impaired actin depolymerization evidenced by a reduction in cytoplasmic actin severing activity (Witke et al., 1995). Transgelin is absent in both transformed mesenchymal cells where actin stress fibers are reduced in number and in non-adherent cells such as lymphocytes (Shapland et al., 1993; Shapland et al., 1988). In contrast to gelsolin and Transgelin, L plastin has been found to be expressed in many types of malignant human cells (Lin and Forscher, 1993). In Philadelphia chromosome positive human leukemias, the c-abl proto-oncogene on chromosome 9 becomes fused to the bcr gene on chromosome 22 generating Bcr-Abl fusion proteins (McWhirter & Wang, 1993). C-Abl has an F-actin binding motif at the carboxyl end. The Bcr sequence enhance F-actin binding activity associated with c-ABL. Philadelphia fused protein causes the dissociation of Abl to F-actin. inducing a redistribution of F-actin into punctuate aggregates, transforming cell shape (McWhirter & Wang, 1993).
One of the most direct evidence linking an actin binding protein to a specific disease was the finding of a gene responsible for neurofibromatosis type 2 (NF2) which codes for the ERM related protein merlin. NF2 is a dominantly inherited disorder characterised by the development of multiple tumors of the nervous system caused by inactivation of a tumor suppressor gene in chromosome 22q12 (Rouleau et al., 1993; Trofatter et al., 1993). Mutations in the NF2 genes leads to a truncated protein and loss of one functional copy causes disease. Merlin/schwannomin normally lead to a stable cell-cell- and cell-matrix interactions, but it’s absence may lead to cell migration, changes in cell shape or loss of contact inhibition events tightly related to cell transformation (Rouleau et al., 1993).

On a large contrast, normal actin organisation can also contribute for entry and propagation of various infectious diseases and the release of cytoskeleton elements into the extracellular may contribute to allergies, coagulation defects and cystic fibrosis. An intact cytoskeleton is essential for invasion and infection by various viruses and bacteria (Janmey and Chaponnier, 1995). *Salmonella typhimurium* are not internalised by human renal epithelial cells that have been treated with cytochalasin D to disrupt F-actin. *Salmonella* induce actin-mediated membrane ruffling similar to that activated by growth factor. *Listeria monocytogenes* invades macrophages and other mammalian cells competing effectively for host cell actin, nucleating actin assembly from it’s surface (Tilney et al., 1992a; Tilney et al., 1992b). Actin filaments are nucleated from the basal and lateral surfaces becoming cross-bridged and falling off the bacteria surface. New filaments are then nucleated generating a tail composed of short actin filaments translocating away from the pre-formed tail (Tilney et al., 1992a; Tilney et al., 1992b). Enteropathogenic *Escherichia coli*, one of the leading causes of infantile diarrhea and consequently death, attaches to intestinal epithelial cells inducing loss of brush border microvilli and polymerization of host actin into a structure known as pedestal. This structure is found just underneath the bacterium which remains on the
extracellular surface of the cell and are able to translocate laterally along the outside surface. The mechanism by which the organism translocate is not yet known, however it’s been shown to be inhibited by cytochalsin D suggesting that polymerization of host actin is essential for host-cell infectiveness (Higley and Way, 1997). These pathogens recruit elements of the host actin cytoskeleton that promote the polymerization of actin, which is used as the driving force to propel the bacterium through the cytoplasm and ultimately into neighbouring cells (Higley and Way, 1997).

Indirect evidences suggest that an intact epithelial may be the target of HIV infection. HIV uses the actin cytoskeleton of infected lymphocytes to form and actin-rich pseudopod for the transfer of virus into epithelial cells (Pearce-Pratt et al., 1994). Vaccinia virus was shown to be capable of inducing actin polymerization directly behind the virus particle in order to move intra and intercellularly (Cudmore et al., 1995). The first stage of vaccinia tail formation involves the recruitment of monomeric actin followed by filament elongation at the virion surface, as filaments ends in the bulk of the tail are blocked against further monomer addition. Interestingly, both viral and bacterial tails are very similar suggesting that a similar mechanism of actin turnover exists in both systems (Cudmore et al., 1995; Higley and Way, 1997).

1.5 - CELL SIGNALING

It is now known that the boundary between cell cytoplasm and cell membrane is long past. Extracellular factors such as matrix proteins, cell-cell contacts, or soluble agonists that interact with plasma membrane receptors are known to influence the rate of formation and spatial organisation of polymerized actin (Hall, 1994). Although many intracellular signalling
molecules have been identified to carry out such functions GTP-binding proteins (GTPases) play a key regulatory role.

GTP-binding proteins function as molecular switches, cycling between an inactive GDP-bound conformation to an active GTP-bound conformation. GTPases are activated by guanine nucleotide exchange.

Rho proteins belong to the small GTP-binding proteins superfamily which consist of the Rho, Rac and Cdc42 subfamilies that control the assembly and disassembly of actin cytoskeleton in response to extracellular signals (Tanaka and Takai, 1998; Tapon and Hall, 1997). An area of increasing interest is to identify downstream target molecules for rho, rac and Cdc42 that regulate their effect on the actin cytoskeleton.

The mammalian rho subfamily consist of proteins that share 50-55% homology (Hall, 1994). Members include RhoA, RhoB, RhoC, RhoG, RhoK and RhoE (Hall, 1994; Tapon and Hall, 1997). Rho is a small GTPase that exhibits both GDP/GTP binding and GTPase activities. Rho has GDP-bound inactive and GTP-bound active forms and upon extracellular stimulation GDP.rho is converted to GTP.rho regulating the actin cytoskeleton and cell adhesion (Kimura et al., 1998). The first indication of Rho function came from studies using C3 transferase, a bacterial enzyme known to inactivate endogenous rho proteins through ADP ribosylation. C3 was microinjected to quiescent Swiss 3T3 cells and readdition of serum was followed. These cells failed to induce the rapid reformation of actin stress fibers and the assembly of focal adhesions indicating a rho-regulated signal transduction pathway in Swiss 3T3 cells that links extracellular signal to the formation of stress fibers (Hall, 1994; Tapon and Hall, 1997). Cell-cell interactions mediated by the activated leukocyte integrin LFA-1 were also inhibited by C3 transferase in lymphocytes. These observations suggested a role in regulating the assembly of integrin complexes and that the formation of actin stress fibers seen in 3T3 cells was a secondary consequence. Indeed, now it is known that there are
downstream target molecules that modulate the effect of rho on the actin cytoskeleton. Identification of the myosin-binding subunit (MBS) of myosin light chain phosphatase (MLC) showed that MBS is a substrate for Rho-K in vitro and that phosphorylation of MBS leads to decrease in MLC phosphatase activity. A model was suggested whereby Rho activation results in increased level of MLC phosphorylation leading to the bundling of dispersed actin filaments (Tapon and Hall, 1997). A role for Rho-mediated cytokinesis has been confirmed with the identification of p140mDia and Bni1p proteins (Imamura et al., 1997; Watanabe et al., 1997). P140mDia is a downstream effector of Rho which binds selectively to the GTP-bound form of Rho and also binds to profilin. P140mDia, profilin and RhoA are co-localised in the spreading lamellae of cultured fibroblasts and are recruited around phagocytic cups induced by fibronectin coated beads. Upon C3 exoenzyme microinjection, this recruitment was abolished. Also p140mDia was overexpressed in COS cells and was observed to form homogeneous actin filaments. Together these evidences suggested that Rho regulate actin polymerization by targeting profilin via p140Dia beneath the plasma membrane (Watanabe et al., 1997). Cells of the budding yeast *Saccharomyces cerevisiae* possesses RHO1, a homologue of the mammalian RhoA gene and yeast RHO1 mutants are deficient in budding process. Rho1p is localised at the bud tip and cytokinesis site which suggest a possible regulatory role in bud formation. The identification of Bni1p protein demonstrate that this protein interacts directly with profilin via a formin-homology (FH1) domain therefore regulating actin polymerization. However, the mechanism by which Rho targets Bnip remains to be elucidated (Imamura et al., 1997). A rho-dependent downstream regulation of actin cytoskeleton have been described in which rho-GTPase activates rho kinase which in turn phosphorylates α-adducin enhancing the binding of α-adducin to actin filaments *in vitro* (Kimura et al., 1998).
Rac GTP binding proteins are implicated in actin cytoskeleton-membrane interaction in mammalian cells and has been shown to mediate growth factor-induced polymerization of actin to form membrane ruffles and lamellipodia (Van Aelst et al., 1996). Microinjection of recombinant Rac protein into serum starved Swiss 3T3 cells has a dramatic effect on actin cytoskeleton that is quite different from Rho-induced effects: it stimulates the rapid polymerization of actin at the plasma membrane to produce lamellipodia and membrane ruffles (Hall, 1994). However, microinjection of Rac protein prior to growth factor addition completely inhibits ruffling and lamellipodia formation but has no effect on stress fiber formation, thus establishing a distinct Rac-regulated signal transduction pathway in Swiss 3T3 cells linking plasma membrane receptors to the polymerization of actin at the plasma membrane (Hall, 1994). A more specialized role for Rac has been identified in phagocytic lymphocytes. In these cells Rac participates in the activation of the multicomponent NADPH oxidase to generate superoxide in response to microbial infection. It has been possible to reconstitute NADPH oxidase activity in a cell free system using purified cytochrome b, p47phox, p67phox (a structural protein from cytosol) and rac in the GTP-bound form. A signalling pathway for actin assembly involving Rac has been identified in which the final message is phosphoinositide-mediated F-actin uncapping. Detergent permeabilized platelets were stimulated with thrombin receptor activating peptide (TRAP) and shown to uncap actin filaments barbed ends and concomitantly synthesize PIP2. This observation was very interesting because PIPs are very effective in preventing gelsolin and other capping proteins from binding to actin filaments but inefficient in removing them from the ends of filaments. It was found that GTPase activity driven by Rac works upstream to stimulate phosphoinositide biosynthesis uncapping actin filaments and regulating platelet shape change (Hartwig et al., 1995). Rac also stimulate the assembly of multimolecular focal adhesion complexes at the plasma membrane which contain vinculin but are distinct from Rho-induced
focal adhesion. Microinjection of Rac along with C3 transferase to serum-starved Swiss 3T3 fibroblasts induces actin polymerization to form lamellipodia around to cell margin leading to the formation of vinculin containing complexes that are morphologically distinct from Rho-focal adhesions, in which they do not have the characteristic elongated, arrowed shaped of Rho-regulated focal adhesion (Nobes and Hall, 1995). Cdc42 was also microinjected to cells in order to gain insight into the function of this GTPase on actin filament distribution. After microinjection a mixture of Rho and Rac-like focal complexes appeared associated with weak actin stress fibers and occasional lamellipodia. These observations suggest a molecular model for the coordinated control of cell motility in which activation of Cdc42 in Swiss cells leads to the sequential activation of Rac and then Rho GTPases (Nobes and Hall, 1995).

A novel Rac1-interaction protein (POR1) has been isolated and identified which binds directly to Rac1 in a GTP dependent manner (Van Aelst et al., 1996). POR1 is a 34Kda protein that lacks a CRIB motif. A mutation in Rac1 effector binding loop abolishes membrane ruffling and interaction with POR1.

CDC42 has been described to be essential for the assembly of components at the bud site of dividing yeast and mutations in Cdc42 leads to general enlargement of the yeast cell and disruption of actin cytoskeleton (Hall, 1994). In mammalian cells Cdc42 triggers the formation of microspikes and filopodia (Nobes and Hall, 1995). An effector candidate for Cdc42 and Rac was recently identified as the serine/threonine kinase PAK65 and close inspection of Cdc42/Rac interactions led to identification of a consensus motif of 18 amino acids referred to a CRIB motif. Data base searches identified WASP, the protein product of the Wiskott-Aldrich syndrome (WAS) locus (Symons et al., 1996). The WASP gene is mutated in WAS patients. WAS is an X-linked recessive disorder characterised by the triad: thrombocytopenia, eczema and recurrent infections due to defects in T and B cell function. The cellular defects in WAS patients suggest a defect of actin cytoskeleton (Symons et al.,
It was found that WASP is an effector for Cdc42 but not for Rho or Rac and this interaction is dependent on the presence of G protein-binding domain. Cellular expression of full length tagged-WASP produces clusters of WASP that are highly enriched in polymerized actin whereas in carboxyl-terminally deleted WASP the cluster is no longer observed. This evidence provides a novel link between Cdc42 and the actin cytoskeleton. Furthermore, the WASP sequence contains two domains that are homologous to other proteins involved in actin organisation. Yeast protein Bee1 is homologous to WASP and disruption causes a striking change in the organisation of actin filaments in yeast resulting in defects in budding and cytokinesis (Li, 1997).

The ERM proteins have been directly implicated in Rho and Rac dependent cytoskeletal reorganization. A direct involvement of Rho family in regulating the actin cytoskeleton via ERM system was described with the finding that Rho GDI directly interacts with ERM proteins initiating the activation of Rho subfamily members by reducing the Rho GDI activity (Takahashi et al., 1997). Rho GDI is a general regulator that forms a complex with GDP-bound inactive form of the Rho family members and inhibits their activation. When cells were treated with agonists or calcium the GDP-bound RhoA protein complexed with GDI in the cytosol is activated to GTP-bound form and translocated to the same areas as ERM proteins. This activation seemed to be via CD44 integral membrane protein. By immunoprecipitation it was found also that Rho-GDI and CD44 were co-precipitated with moesin (Takahashi et al., 1997). To examine the CD44-ERM protein interaction in vitro, mouse ezrin, radixin and moezin and GST fusion protein containing the cytoplasmic domain of CD44, generated by means of recombinant baculovirus infection, were generated to construct an in vitro assay for the binding between ERM and the cytoplasmic domain of CD44 (Hirao et al., 1996). It was found that ERM proteins bound with high affinity to GST-CD44 and in the presence of PIPs this high affinity was maintained suggesting that Rho is a
likely regulator of PIP activation. Immunoprecipitated CD44/ERM complex contained Rho-GDP dissociation inhibitor (GDI) Results obtained from incubating living BHK cells with C3 toxin also favoured the notion that the formation of CD44/ERM complex is dependent on active Rho-GTP-bound form. C3 recruited most insoluble ERM proteins to a soluble pool leaving CD44 free from ERM proteins (Hirao et al., 1996). CD43 and ICAM-2 have also been identified to bind to ERM proteins and the binding region has been narrowed down to their juxta-membrane, positive cluster of 20-30 amino acids in the cytoplasmic domain (Yonemura et al., 1998). CD44, CD43 and ICAM-2 were expressed in mouse fibroblasts and found to be co-concentrated with ERM proteins at microvilli, whereas fusion protein constructs lacking the positive cluster were diffusely distributed on the cell surface (Yonemura et al., 1998).
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Tri-Phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propansulphonat</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribosenucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine-tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-Bis [β-amino ethyl ether] NNNN-tetraacetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>HTCL</td>
<td>Human T-Cell Lymphoma</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirits</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani Broth</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTTC</td>
<td>Mouse Thymus T Cell</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
</tbody>
</table>
BUFFERS AND SOLUTIONS

All solutions were prepared using distilled-deionised water and stored as stated.

Solutions are listed alphabetically.

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>COMPONENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (protein gels)</td>
<td>30%(w/v)acrylamide(BDH); 1%(w/v)N,N'-methylene-bis-acrylamide (BDH). Stored foil-wrapped at 4°C.</td>
</tr>
<tr>
<td>Ammonium persulphate (APS)</td>
<td>10%(w/v) APS (BioRad) freshly prepared on day of use</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>50mg/ml ampicillin(Sigma). Filtered 0.2 m(Millipore), aliquoted and stored at -20°C.</td>
</tr>
<tr>
<td>Antibody Block Buffer</td>
<td>3% BSA(Sigma) in PBS with 0.1% sodium azide (Sigma) (Immunoblotting).</td>
</tr>
<tr>
<td>Antibody Wash Buffer</td>
<td>0.3% BSA (Sigma) in PBSA</td>
</tr>
<tr>
<td>Coomassie Blue Stain</td>
<td>50%(v/v)methanol(BDH), 10%(v/v)glacial acetic acid (BDH)0.05%(w/v)coomassie Brilliant blue R (Sigma).</td>
</tr>
<tr>
<td>Destain</td>
<td>30%(v/v) methanol (BDH), 10%(v/v)glacial acetic acid (BDH)</td>
</tr>
<tr>
<td>DEPC</td>
<td>Used at 0.1% to inactivate RNAases in solutions, removed by autoclaving (Fluka)</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium, 500ml (Sigma). Stored at 4°C.</td>
</tr>
<tr>
<td>Eletrobolt Transfer Buffer</td>
<td>25mM Tris base(Boe), 192mM glycine(Sigma), 20%(v/v)methanol (BDH); pH=8.3. Ethylene-diamine-tetraacetic acid 0.5M (Sigma) adjusted to pH=8. Sterilised by autoclaving.</td>
</tr>
<tr>
<td>Ethylene-diamine-tetraacetic acid</td>
<td>0.5 M EDTA (Sigma) adjusted to pH=8.0</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Preweighted 11mg EtBr (Bio Rad) tablets dissolved in 11mls ddH₂O, in fume hood, to 1mg/ml. Stored foil-wrapped at 4°C.</td>
</tr>
<tr>
<td>Laemmli Sample Buffer</td>
<td>80mM Tris-Cl pH=6.8, 2.3%SDS(BDH), 0.002% bromophenyl blue(BDH), 14.2M 2-mercaptoethanol(BDH), 20% glycerol (Fluka). Made fresh on day of use.</td>
</tr>
</tbody>
</table>
Luria Bertani Broth 1% bacto tryptone (Difco), 0.5% bacto-yeast extract (Difco), 1% NaCl (Sigma). pH=7.4. Sterilised by autoclaving.

Magnesium sulphate 1M stock MgSO₄ (Fluka). Sterilised by autoclaving.

2-mercaptoethanol 14.2M 2-ME (BDH). Stored at 4°C in dark bottle.

Phenol Liquefied phenol (Fluka) was saturated with DEPC'd ddH₂O.

Phenol/Chlorophorm Phenol (Fluka), chloroform (BDH) mixed at ratio 1:1 stored at 4°C.

Phosphate Buffered Saline \(1\times PBSA:137\text{mM } \text{NaCl (Sigma)}, 2.7\text{mM } \text{KCl (BDH), } 8\text{mM } \text{NaH}_2\text{PO}_4\) (Sigma), \(1.45\text{mM } \text{KH}_2\text{PO}_4\) (Sigma), pH=7.5.

RNA Extraction Buffer 6M urea(), 3M lithium chloride (Fluka). Filtered 0.2μm. Stored at 4°C.

Sequencing Sample Buffer 95% formamide, 20mM EDTA, 0.05% xylenyl cyanolff, (sequenase kit,USB). Stored at -20°C.

SOB Medium 2% (w/v) Bacto-tryptone (Difco), 0.5% Bacto-yeast extract (Difco), 1% (w/v) NaCl (Sigma), 2.5mM KCl, pH=7.0. After autoclaving 20mM glucose and 20mM MgCl₂ (Sigma) were added to the solution.

SOB Agar SOB medium, 1.5% (w/v) agar (Difco). Sterilised by autoclaving.

Solution I 50mM glucose (BDH), 10mM EDTA (Sigma), 25mM Tris (Sigma)pH=8.0. Autoclaved and stored at 4°C.

Solution II 0.2M NaOH (Sigma), 1%(w/v) SDS. Freshly made and 0.45μm filtered.

Solution III 3M potassium acetate (Fluka), 2M glacial acetic acid (BDH). Freshly made

Sodium Dodecyl Sulphate 10% SDS (BDH), filtered 0.4μm

TE 10mM Tris-HCl, 1mM EDTA. Sterilised by autoclaving.

Trypsin ICRF media supplies; 0.8% (w/v) NaCl, 0.038% (w/v) KCl, 0.01% (w/v) disodium hydrogen orthophosphate, 0.01% (w/v) dextrose, 0.3%(v/v) Tris.HCl pH7.7, 0.25% (w/v) trypsin, 10,000u sodium penicillin, 0.01%(w/v) streptomycin, phenol red. Stored at -20°C.

Versene ICRF media supplies: 0.02% (w/v) EDTA in PBSA.

### Molecular Weight Markers

1Kb ladder and 123 ladder (Gibco BRL technologies) was used as markers in agarose gels whereas Rainbow molecular weight ladder (Sigma) was used for SDS-PAGE.

### Protease Inhibitors

<table>
<thead>
<tr>
<th>protease inhibitor</th>
<th>Used at conc.</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEBSF</td>
<td>1mM</td>
<td>serine proteases</td>
</tr>
<tr>
<td>Antipain</td>
<td>50 g/ml</td>
<td>papain, trypsin</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>50 g/ml</td>
<td>Serine proteases. NOT thrombin</td>
</tr>
<tr>
<td>Bestatin</td>
<td>10 g/ml</td>
<td>amino-peptidases(B) Not carboxypeptidases</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>60 g/ml</td>
<td>Chymotrypsin</td>
</tr>
<tr>
<td>EDTA</td>
<td>5mg/ml</td>
<td>Metalloproteases</td>
</tr>
</tbody>
</table>

120
### Methanol/chloroform extraction of proteins

This is a method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids (d. Wessel, U.I.Flugge, 1984). 150\(\mu\)l of sample was mixed with 750\(\mu\)l of methanol : chloroform (ratio 4:1) in an eppendorf tube and vortexed for 2 minutes. 450\(\mu\)l of \(dH_2O\) was added to it and mixed by vortexing. The eppendorf tube is centrifuged at 10,000g in a bench microfuge for 1 minute at room temperature. The aqueous top layer was removed with a Pasteur pipette leaving the interphase undisturbed. To the bottom organic layer, 450\(\mu\)l of methanol (100\%) was added, vortexed and centrifuged at 10,000g for 5 minutes at room temperature. All supernatant is removed and the protein pellet left to air dry. The pellet is resuspended in SDS sample buffer and analysed by SDS-PAGE.

### 2.1 - Biochemistry

#### 2.1.1 - SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

This technique uses an anionic SDS detergent in combination with a reducing agent and heat. SDS binds to polypeptides and confer a net negative charge. The binding of SDS is directly proportional to the polypeptide molecular weight and independent of it's sequence.

SDS-PAGE gel system of 12% acrylamide were assembled using a Biorad Miniprotein II vertical electrophoresis system. Glass plates were cleaned and assembled onto Biorad gel mould. A solution of 12\% (v/v) acrylamide (Biorad), 0.37M Tris.HCl (PH=8.8)(Boehringer Mannheim), in distilled water was degassed under vacuum, crossed linked by addition of 0.1\% (w/v) SDS, 0.033\% (w/v)(Pierce), ammonium persulphate (APS)(Biorad), 0.00033\% (v/v)
NNN’N’-tetramethylenediamine (TEMED) (Biorad) and poured into the gel mould. The mixture was overlayed with isobutanol and allowed to polymerise. After polymerization, the isobutanol overlayer was rinsed off with distilled water and a stacking gel consisting of 5.5% acrylamide, 0.138M Tris.HCl (PH=6.8) in distilled water was degassed under vacuum and crossed linked by addition of 0.1% (w/v) SDS, 0.056% (w/v) APS, 0.00056% (v/v) TEMED. The sacking gel was carefully poured onto the surface of the polymerised gel and a Teflon 10 well comb was inserted and allowed to polymerise at room temperature for 1 hour.

Samples were heated at 100°C for 3 minutes in Laemmli sample buffer (0.8mM Tris.HCl PH=6.8, 2.3% (w/v) SDS, 0.003% (w/v) bromophenol blue, 1.42M 2-Mercaptoethanol (Biorad) and 20% (v/v) glycerol (Fluka)). Also 5µl of dye-coupled molecular weight markers (BRL Life technologies) were loaded on each gel.

After complete polymerization, the combs were removed, the gel apparatus was mounted and 1 litre of 1 x Laemmli running buffer (25mM Tris base, 192mM glycine, 0.1% (w/v) SDS, PH=8.3) was added to the top and bottom reservoirs. The wells were washed using a glass pipette and 12µl sample were loaded into each well. The apparatus was attached to an electrical power supply and run at 70 volts through the stack and 120volts through the running gel.

The electrophoresed proteins were stained with 0.05% (w/v) Coomassie brilliant blue R, 50% (v/v) methanol, 10% glacial acetic acid for several hours at room temperature with shaking. The gels were destained (30% (v/v) methanol, 10% glacial acetic acid) and stored in 7% (v/v) acetic acid.
2.1.11 - IMMUNOBLOTTING

Electrophoretically separated proteins were transferred from a SDS gel to a nitrocellulose membrane and probed with specific antibody. This technique is useful in the identification and quantitation of specific proteins in complex mixtures (Towbin et al, 1979).

The transfer procedure was carried out in a Biorad Miniprotean II transfer system. Four pieces of porous polyethylene pads, four sheet of 3mm-CHR Whatman paper and one sheet of 0.45μM pore size nitrocellulose filter (Schleicher & Schuell GmbH Dassel, FRG) were cut to the exact size of the electrophoresed SDS polyacrylamide gel. All three were prewet in 1x electrophoretic transfer buffer (25mM tris.HCl, 192mM glycine, 20% (v/v) methanol (PH=8.3) and a successive layer system was assembled onto the cathode side of the Biorad apparatus consisting of two layers of pads, two layers of 3mm paper, the electrophoresed gel, the nitrocellulose filter (overlayed avoiding air bubbles), two layers of 3mm paper and two pads. The cassette was closed firmly and assembled into the transfer apparatus. The chamber was filled with 1x transfer buffer and electrophoretic transfer was accomplished at 170vts for 1 hour with constant stirring. An ice container was also assembled into the chamber to prevent the rise of temperature.

The nitrocellulose sheet was carefully removed from the cassette and placed into a container containing antibody block buffer (3%(w/v) BSA, 0.1% (w/v) NaN3 in PBSA) for 12hours at 4°C. The filter was washed 5 times in antibody wash buffer (0.3% (w/v) BSA in PBSA) and incubated with the primary antibody (either 20μg/ml protein C4 monoclonal antibody or 2μg/ml C4 polyclonal) for 2 hours at room temperature. The filter was washed in antibody wash buffer and the secondary antibody (goat anti-mouse Ig peroxidase) for 2 hours at room temperature. The filter was developed by peroxidase or ECL methods. The peroxidase was carried out by incubating the filter in a solution of 16.6% (v/v) cold methanol, 0.015% (v/v) H2O2 and 0.05% (w/v) 4-Chloronapthol in PBSA for 15 minutes at room
temperature. The developing reaction was brought to a halt by washing the filter 2 times in PBSA.

The filter was air dried and stored in the dark.

ECL developing of filter was followed according to manufacturer’s protocol.

### 2.1.III - ECL WESTERN BLOTTING

This is a light emitting non-radioactive method for detection of immobilised specific antigens conjugated directly or indirectly with horseradish peroxidase-labelled antibodies (Amersham, Life Science)

### 2.1.IV - AGAROSE GEL ELECTROPHORESIS

1% agarose gel electrophoresis was performed to resolve small DNA fragments. 40mls gels were assembled in which 0.4g of agarose were added to 40mls of 1 X TAE buffer (0.04M Tris-acetate, 0.001M EDTA) melted in a microwave, let it cool to 60°C followed by addition of 5μg/ml of ethidium bromide. The mixture was cast into a Pharmacia GNA-100 gel electrophoresis apparatus with placing of comb to generate wells for sample loading. The gel was covered by 1 X TAE solution and run at 40v/cm. Samples were loaded after addition of sample loading buffer (0.25% (W/V) bromophenol blue, 0.25% (W/V) xylene cyanol FF, 40% (W/V)sucrose in distilled water. A 1Kb ladder molecular weight marker was always loaded in all cases.

Visualisation was carried out on a U.V. light box and photographed using a Polaroid DS34 camera.
2.2 - Antibodies

2.2.1 - ANTI-C4 MONOCLONAL ANTIBODY

This mouse anti-protein C4 secreting hybridoma was generated by D. Lawson (1983).

2.2.1.a - Immunogen preparation:

Fresh chicken gizzard was homogenised in a Waring blender at top speed in 10 volumes of 0.5 mM phenylmethylsulfonylfluoride (PMSF). The homogenised mixture was centrifuged and the pellet obtained was resuspended and blended at low speed. A series of centrifugation and pellet resuspensions took place and the resultant supernatant was adjusted to a final pH = 7.2 with 10 mM MgCl₂. This supernatant was used as the immunogen.

2.2.1.b - Immunisation:

BALB/c mice were immunised subcutaneously at dosage of 100 μg of immunogen per injection. Day 1: subcutaneous in complete Freund’s adjuvant; days 14, 63 and 77 interperitoneal in complete Freund’s adjuvant; day 81 tail bleed and test for antibody response; days 84, 85 and 86 intravenous injection and day 87 fusion.

2.2.1.c - Fusion and Hybridoma production:

This was also performed by Dr. D. Lawson using the non-secretor SP2 myeloma cell line cultured in DMEM medium.

2.2.1.d - Screening and Cloning Hybridomas:

The hybridomas were tested by immunofluorescence on formaldehyde fixed methanol when wells were two thirds confluent. The antibody producing cells were cloned by limiting dilution into 96 well limbro round bottom microtitre plates with a feeder layer of adherent BALB/c macrophages at a concentration of 5 x 10³ cells/ml. This procedure was repeated.
twice and cells were frozen in 5% DMSO, 95% FCS at -27°C for 10 minutes and stored in liquid nitrogen. Ascites tumours were raised by injecting nu/nu mice with 10 x 10^7 cells and the asctic fluid was harvested 10 days later. The anti-C4 monoclonal was used at 20µg/ml in biochemical analysis and immunofluorescence.

2.2.11 - ANTI-C4 POLYCLONAL ANTIBODY

This antibody was generated by Shapland et al (Shapland et al., 1993). Rabbit anti-Transgelin polyclonal antibody was raised from sheep aorta tissue samples electrophoresed on 3mm thick SDS-PAGE gels. The samples were electroeluted into 1 x Laemmli running buffer, collected in dialysis tubing (Spectra/Por) followed by acetone precipitation. The precipitated protein was resuspended and dialysed against 0.1M MOPS (3-[N-Morpholino]propane sulfonic acid, Sigma) pH=7.5 and injected into rabbits over a three month period. A DEAE 52 (Whatman) was used to obtain an IgG fraction. The fraction was further passed over an affinity column of purified Transgelin (2mg) coupled to Affigel 10 (Biorad), eluted with 50mM diethylamine (Sigma), pH=11.5, neutralised with 1M Tris.HCl pH=7.5, dialysed into PBSA and concentrated by millipore filtration. The anti-C4 polyclonal was used at concentration of 2-10µg/ml in biochemical analysis.

2.3 - Tissue culture

2.3.1 - MOUSE THYMUS LYMPHOCYTES

Mouse thymus were obtained by sterile dissection of 8 days old BALB/c mouse. The thymus were teased in a Petry dish containing 1 x PBSA with a sterile needle. Cell suspension was centrifuged in an MSE Benchtop at 150g for 10 minutes at room temperature. The cell pellet was carefully resuspended in 1ml of PBSA. These cells were used for RNA extraction.
The remaining lymphocytes were placed on a 250ml UV sterilised flask (Falcon) incubated at 37°C in a humidified incubator (LEEC) in an atmosphere of 5% CO₂ overnight. The supernatant was removed from the flask by aspiration, cells were briefly rinsed with 10mls trypsin/versene (4:1) which was discarded and a further 5mls of fresh trypsin/versene was added to the flask allowing to incubate at 37°C for 5 minutes till all cells detached from surface. The cell suspension was transferred to a universal tube containing 15mls of DMEM medium with 10% FCS and centrifuged in a MSE Benchtop at 200g for 5 minutes at room temperature. The cell pellet was resuspended in 1ml of fresh DMEM medium and counted in a Neubauer haemocytometer and either grown in a flask at a concentration of 5 x 10³ to 5 x 10⁴ and maintained at 37°C in 100% humidity and 5% CO₂, or cells were plated in coverslips at concentration of 2.5 x 10³ to 2 x 10⁴ per ml.

2.4 - Immunofluorescence

All experiments were carried out by Dr D. Lawson

2.4.1 - FIXATION

Aliquots onto 13mm diameter sterile coverslips in a 24-well Linbro plates (Flow laboratories) for 3 rat embryo fibroblast cells were adjusted to 1 x 10³ cells/ml in DMEM, plated in 1ml days. Cells were rinsed briefly in PBSA and then permebilised by formaldehyde fixation 3.5% (v/v) in PBSA for 12 minutes at room temperature followed by cold methanol (10 minutes at -20°C). After brief rinsing in PBSA cells were incubated for 10 minutes in antibody block buffer (1% (v/v) BSA, 100mM lysine in PBSA), probed with 20μg/ml anti-C4 monoclonal antibody for 30 minutes at room temperature. Cells were rinsed in PBSA and incubated with an IgG-specific goat anti-mouse rhodamine at a dilution of 1 in 100 in antibody block buffer for 30 minutes. Coverslips were rinsed in PBSA and mounted
onto glass slides using Gelvitol mount (Monsanto Polymers Ltd, USA) and allowed to air dry. The coverslips were visualised on a Nikon Optiphot fluorescence microscope with a 60 x objective and a 10 x eyepiece. Fluorescent images were observed by using an epifluorescence attachment.

### 2.4.11 - DETERGENT EXTRACTION

Secondary cultures of rat embryo fibroblasts were plated on glass coverslips at a concentration of $2.5 \times 10^3$ cells/ml in DME, 10% FCS rinsed briefly in cold buffer then were detergent extracted for 15 minutes at 4°C in 2mls of buffer + 0.5% CHAPS, rinsed in buffer, plunged into -20°C methanol and blocked for 5 minutes at room temperature in 3% BSA + 100mM lysine in PBS. Control coverslips were briefly rinsed in buffer, plunged into methanol, rehydrated and blocked as above. Antibody labelling and immunofluorescence were carried out as previously described.

### 2.4.11.1 - REBINDING OF CAPLIN TO PERMEABILIZED CELL SYSTEM

This protocol was used to allow for the rebinding of protein Caplin to permeabilized cells. Rat embryo fibroblasts were detergent extracted for 15 minutes at 4°C in 2mls of buffer + 0.5% CHAPS, rinsed in buffer and blocked for 5 minutes at room temperature in 3% BSA, 100mM lysine buffer and then incubated at 4°C for 30 minutes with 3μl FPCaplin in a total volume of 60μl. The coverslips were fixed in 7mls of 1% glutaraldehyde (Fluka) for 30 seconds at 4°C, immediately transferred to 2% formaldehyde (BDH) in PBSA for 5 minutes at room temperature. They were rinsed briefly and incubated in 1mg/ml sodium borohydride in PBSA at 37°C for 10 minutes to block the binding of free aldehyde groups. Antibody labelling and immunofluorescence were carried out as before.
Chapter 2

2.5 - RNA preparations

2.5.1 - TOTAL RNA EXTRACTION

All reagents and tubes were made RNAase free by autoclaving or baking at 180°C for 24 hs. DEPCdH2O was used in all solutions. Freshly dissected mouse thymus tissue was placed in a 50ml polypropylene tube (Falcon) and teared with a sterile seringe needle in a Petri dish with 10mls of ice cold 6M Urea/3M LiCl solution. Extraction was carried out overnight at 4°C. The mixture was transfered to a 15ml corex tube (Du Pont Instruments) and centrifuged a 16700g at 0°C for 30 minutes in a JA17 rotor (beckman J2-21 centrifuge). The supernatant was discarded and pellet was resuspended in 8mls of 6M Urea/3MLiCl solution.

Centrifugation was repeated as before. Supernatant was discarded and pellet obtained was resuspended in 6mls of 10mM Tris.Cl PH 7.5 and 0.5% SDS at room temperature for 2hs. The mixture was then transfered to a 14ml polypropylene tube (Falcon) and phenol extracted twice with equal volume. The aqueous layer containg total RNA was transfered to a fresh tube and extracted once with equal volume of phenol:chloroform solution. The remaining top aqueous layer was finally extracted with equal volume of chloroform. The aqueous layer was transfered to a 30ml corex tube and 0.1 volume of 3M Na acetate PH 6.5 was added and mixed thouroughly. Two and a half volumes of EtOH were added and allowed to react overnight a -70°C in a 30mls corex tube. The mixture was centrifuged at 16700g in a JA17 rotor (Beckman J2-21 centrifuge) for 30 minutes. Supernatant was discarded and pellet was washed and vortex in 10mls of 80% EtOH. Centrifugation was repeated for 30 minutes. The pellet obtained was air dried and dissolved in 0.5mls of DEPCdH2O in a eppendorf tube. Total RNA concentration was obtained by reading optical density at 260nm.
2.5.11 - SELECTION OF POLY A+ RNA

Approximately 5% of total RNA is messenger RNA (mRNA). mRNA carry a poly(A+) tail at the 3' end and is isolated by affinity chromatography on a oligo d(T) cellulose column (Edmonds et al, Aviv PNAS). Total RNA in 0.5mls of dH2O was heated at 65°C for 5 minutes and quickly cooled down to room temperature by briefly placing it on ice. An equal volume of 2 x column loading buffer (20mM Tris.cl PH 7.6, 0.5M NaCl, 1mM EDTA PH 8.0, 0.1% sodium lauryl sarcosinate) was added and the mixture was transferred to a 14ml polypropylene tube containing oligo d(T) beads. Incubation was carried out for 40 minutes at room temperature with constant shaking. The mixture was centrifuged at 3000rpm for 6 minutes. The supernatant was carefully removed, transferred to a eppendorf tube, heated at 65°C for 5 minutes and allowed to cool at room temperature. The denatured supernatant was added to a 14ml tube containing 40µg of oligo d(T) cellulose beads and incubated at room temperature with constant shaking for 15 minutes. Centrifugation was carried out at 3000rpm for 6 minutes. The supernatant was removed and kept at -20°C. The pellet obtained was washed 5 times in 5mls of 1 x column loading buffer (high salt buffer to prevent nonspecific binding), centrifuged at 3000rpm for 6 minutes. The aqueous layer was discarded and pellet containing normal mouse thymus Caplin poly A+ RNA bound to oligo d(T) cellulose beads was eluted four times with 1ml of elution buffer (10mM Tris.Cl PH 7.6, 1mM EDTA PH 8.0, 0.05% SDS). Washes were interspersed by centrifugations at 3000rpm for 6 minutes. Fractions obtained by each elution step were pooled, heated at 65°C for 3 minutes and allowed to cool at room temperature. To the pooled fractions, 450µl of 5M NaCl solution were added, mixed by gentle pipetting, and reapplied to fresh oligo d(T) cellulose beads. Incubation was carried out at room temperature for 45 minutes with constant shaking and contents were centrifuged at 3000rpm for 6 minutes. Supernatant was discarded and pellet containing Caplin Poly A+ RNA bound to oligo d(T) cellulose beads was washed 3
times with 5mls of 1 x column loading buffer. Supernatant from each wash was pooled and elution was carried out as before. mRNA eluted and pooled fractions was precipitated by adding 0.1 volumes of 3M Na acetate PH 5.2 and 2.5 volumes of 100% EtOH in a 30mls corex tube. Precipitation was carried out at -80°C overnight. mRNA was collected by centrifugation at 11000rpm for 45 minutes. Pellet was washed in 80% EtOH and recentrifuged at 11000rpm for 30 minutes. The pellet was air dried and resuspended in 100μl of DEPCdH2O. The concentration of mRNA was obtained by reading optical density at 260nm.

2.5.III - DETERMINATION OF NUCLEIC ACID CONCENTRATION

The concentration of mRNA was determined by measuring the optical density at 260nm/280nm using a SP8-400 uv/vis spectrophotometer (Pye/Unicam) and quartz cuvettes by the equation:

\[
\text{Conc}=\text{OD}_{260} \times \text{cuvette length} \times \text{dilution factor} \times \text{Nucleic acid coefficient (40μg/ml for RNA)}
\]

2.6 - Cloning of protein Caplin cDNA

2.6.1 - PRIMERS AND OLIGONUCLEOTIDES

Two oligonucleotides were manufactured by Genosys (Genosys Biotechnologies,Inc., Cambridge) and used as primers for PCR reactions and the sequencing of Caplin cDNA. They were each at 10μM concentration and used at 1μg/μl.
1) Sense oligonucleotide: a 34mer sense primer

MANR

5' GGAATTCTGGAATGGCAACAGGGGACCTGCATA 3'

2) Antisense oligonucleotide: a 33mer antisense primer

QUIL rev

5' GGAATTCGGGATCAGAGGATCTGGCGTGGCATC 3'

These oligonucleotides were allowed to anneal to RT Caplin cDNA and were further amplified by PCR as described below.

2.6.11 - CDNA PREPARATIONS USING REVERSE TRANSCRIPTASE

Reverse transcriptase were carried out using Stratagene First Strand Synthesis Kit. This kit was designed to convert 1μg of mRNA into cDNA strands. The kit also uses a control RNA (3' RACE SYSTEM) as template for the first strand synthesis.

Mouse thymus mRNA was used to obtain cDNA transcripts. 1μg of mRNA was used and adjust to a final volume of 13μl. 1μl of 10μM adaptor oligo-dT primer solution was added and mixed gently. The mixture was heated to 65°C for 10 minutes and chilled on ice for further 2 minutes. The contents of the mixture were obtained by brief centrifugation in a microcentrifuge. To it 2μl of 10X synthesis buffer (200mMTris-HCl (PH8.4), 500mMKCl, 25mM MgCl₂, 1mg/ml BSA), 1μl of 10mM dNTPs mix (10mM each dATP,dCTP,dGTP,dTTP) and 2μl of 0.1MDTT were added and mixed gently. The mixture was equilibrated at 42°C for 2 minutes and collected by centrifugation. 1μl of superscript reverse transcriptase (Moloney Murine Reverse Transcriptase enzyme 200U/μl- Stratagene) was added and incubated at 42°C for 30 minutes. This enzyme has been genetically engineered so that it lacks Rnase H activity therefore allowing for full length cDNA synthesis and higher yields of first strand cDNAs. After incubation the mixture was briefly centrifuged
and the tube was placed on ice. Finally 1μl of Rnase H (2U/μl) was added which degrades the original mRNA template and is specific for RNA:DNA heteroduplex and incubation was carried out at 42°C for 10 minutes. The samples containing first strand cDNA products were frozen at −85°C.

2.6.II. a - Determination of nucleic acid concentrations

The concentration of nucleic acid was determined by measuring optical density at 260nm/280nm using a SP8-400 uv/vis spectrophotometer (Pye/Unicam) and quartz cuvettes.

The sample was diluted in DEPC (diethyl pyrocarbonate) treated distilled water and the concentration was calculated using the following equation:

\[ \text{Conc} = \text{OD}_{260} \times \text{Cuvette length} \times \text{dilution factor} \times \text{Coefficient for nucleic acid} \] (50μg/ml for double stranded DNA).

2.6.III. POLYMERASE CHAIN REACTION

The samples obtained using reverse transcriptase were amplified using polymerase chain reaction (PCR) (Henry A.Erlich, Sanger,PNAs 1977)

The DNA amplification was carried out following Anglican Biotec Ltd protocol. A 50μl core mix was made containing 1X PCR buffer (5mM Tris-HCl PH=9.0, 25mM NaCl, 25mM KCl, 1.25mM MgCl₂,0.1μg/μl BSA), 0.25mM dNTPs (dATP,dTTP,dCTP,dGTP), 1μl of each primer 10μM MANR and 10μM QUILrev (see oligo section), 2.5 units of Taq polymerase overlayed with few drops of paraffin oil (BDH) to prevent condensation. Amplification was carried out in a Hybaid Thermal cycler with an initial denaturation step at 97°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, primer annealing at 50°C for 1 ½ minutes, extension at 72°C for 2 minutes. The final extension was carried out for 15 minutes and the reaction was held at 25°C. Control reactions without DNA were also carried out.
The PCR product was analysed on a 1% agarose gel and further cloned into pUC18 for sequencing.

**2.6.IV - GLASSMILK PURIFICATION OF DOUBLE STRANDED DNA**

DNA was purified from contaminants using the Geneclean II Kit Bio 101 Inc (La Jolla, CA) which uses a silica matrix called Glassmilk specifically formulated to bind to DNA.

To a previously labelled eppendorf tube containing 50μl of protein Caplin amplified DS-DNA, 150μls of 6M NaI stock solution were added, heated at 50°C for 2 minutes and placed on ice. 5μls of Glassmilk suspension of silica matrix in water was added, mix by gentle vortex and incubated for 5 minutes on ice. The mixture was centrifuged in a microfuge at 12000g for 5 seconds. The supernatant was carefully discarded and the Glassmilk-DNA pellet was washed 3 times with 700μls of New wash solution (NaCl/ ethanol solution prepared according to manufacturer’s instructions and stored at -20°C). After last centrifugation, the pellet was eluted in 10μls of TE solution and incubated at 50°C for 5 minutes. This step allowed for the detachment of DNA from the glassmilk. The glassmilk pellet was collected by centrifugation at 12000g and the supernatant containing purified DNA was carefully removed and stored at -20°C or immediately used for ligation into a pUC18 vector.

**2.6.V - LIGATION OF CAPLIN DNA INTO PUC 18 VECTOR**

The purified PCR products were cloned into an EcoRI restriction site on pUC18 DS-circular plasmid vector. Cohesive ends were generated on PCR products by digesting with EcoRI (Gibco BRL Life technologies) restriction enzyme. To 15μl of 0.18μg/μl of pUC18 vector 2.7μl of 10U/μl of EcoRI, 2μl of 10 X React 3 (50mMTris-HCl PH=8.0, 10mM MgCl2, 0.1MNaCl) and distilled water up to a total of 20μl were incubated at 37°C for 11/2
hours. The reaction was stop by placing it on ice. The whole sample was loaded on a 1% agarose gel. Electrophoresis was carried out for 3hs at 35v/cm. The bands were visualised, carefully sliced and purified by Glassmilk system. In order to prevent religation, digested pUC18 was phosphatased by adding 0.1 units of calf alkaline phosphatase (Pharmacia), 5µl of One-Phor-All Plus buffer (Pharmacia) and distilled water up to a volume of 50µl. The mixture was incubated for 30 minutes at 37°C and the reaction was stopped by bringing the temperature to 70°C for 15 minutes therefore inactivating the catalytic activity of the enzyme.

Ligation reactions were carried out by mixing 0.1µg/µl pUC18, 0.4µg/µl DNA insert, 1 X One-Phor-All buffer (Pharmacia) and 1mM ATP. The reactions were heated at 45°C for 5 minutes, placed on ice for 2 minutes following the addition of 4 units of T4 ligase enzyme (Pharmacia) and incubated at 11°C overnight. Control reactions were also carried out containing unphosphatased pUC18 with no DNA insert. Samples were stored at −20°C.

2.6.VI - TRANSFORMATION OF DH5 ALPHA E.COLI CELLS

The ligation reaction was heat inactivated at 65°C for 10 minutes and placed on ice. 3µl of each ligation sample were transferred to 1.5ml eppendorf tubes. Meanwhile, E.coli DH5a cells are thawed on wet ice. 50µls of cells were added to each eppendorf tube and further incubated on ice for 30 minutes. The cells were then heat shock at 37°C for 20 seconds and immediately placed on ice for 2 minutes. 800µl of SOC medium was added and the samples were warmed to 37°C for 45 minutes. Samples of 100µl or 200µl were plated and the remaining transformation samples was stored at −85°C with 7% DMSO. 0.5ng pUC19 was used as control to determine the transformation efficiency.
2.7 - NUCLEOTIDE SEQUENCING OF PROTEIN CAPLIN

2.7.1 - Double-stranded Sequencing

All sequencing reactions were carried out according to manufacturer’s protocol for Sequenase™ Version 2.0 DNA sequencing Kit (USB). Samples containing 5 μg of Caplin insert-containing pUC18 purified on glassmilk (as described above) and eluted into 9 μl of dH₂O plus 2 μl of 0.1 μg/μl of sequencing primer (~40, M13 reverse or oligonucleotides) were heated to 99°C for 5 minutes and immediately placed on liquid nitrogen. The individual samples were then thawed to room temperature for 5 minutes and to it 1 μl of 0.1 M DTT, 2 μl of diluted GTP labelling mix (diluted 1:3 in dH₂O), 0.5 μl of [³⁵ S]dATP (25 Ci/μl, Amersham), 2 μl of Sequenase buffer (5X concentrated: 200 mM Tris.HCl pH=7.5, 100 mM MgCl₂, 250 mM NaCl) and 9 units of Sequenase polymerase were added. The labelling reactions were mixed and incubated at room temperature for 3 minutes. Meanwhile, four 0.5 ml microcentrifuge tubes were individually labelled with G, C, A and T. To tube labelled G 2.5 μl of ddGTP termination mix (80 μM dGTP, 80 μM dCTP, 80 μM dATP, 80 μM dTTP, 8 μM ddGTP, 50 mM NaCl) was added and similarly 2.5 μl of ddCTP, ddATP and ddTTP containing termination mix were added to tubes labelled C, A and T respectively. The tubes were sealed to prevent evaporation and prewarmed at 42°C for 1 minute.

Once the labelling reaction was completed, 4.5 μl was removed and transferred to tube labelled G. The contents were mixed and incubated at 42°C for 12 minutes. In the same manner 4.5 μl of labelling reaction was individually added to tubes labelled C, A and T, incubated at 42°C for 10 minutes. The reaction was stopped by addition of 4 μl of stop solution (95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF) and samples were stored at −80°C. Samples were warmed at 90°C for 2 minutes before 2.5 μl were loaded onto sequencing gels.
2.7.11 - SEQUENCING GEL ELECTROPHORESIS

Two IMS cleaned gel plates (30cm x 40cm) were siliconised and assembled together with tape using 0.4-1.2mm wedge spacers. 140ml of 6% gel mix (Sequagel, National Diagnostics) were mixed with 1ml 10% APS and the gel mixture was carefully poured onto the assemble gels with a 50ml syringe avoiding air buble. The gel was left to polymerise overnight. The combs and tape at the bottom of the assembled plates were removed and the plated were then placed into a Gibco BRL Life Technologies Model S2 sequencing apparatus with sequencing running buffer (1 x TBE: 0.089M Tris base, 0.089M boric acid (Sigma), 2mM EDTA) added to it. The gels were pre-run for 1 hour at 60 Watts. Before loading, samples were brought to a temperature of 85°C for 2 minutes and 3μl of each termination mix loaded into the corresponding lane in a order pattern : A, G, C and T. The gel was run at 50watts for a desired time. Gels were then fixed in 5% acetic acid and 15% methanol for 60minutes and pressed onto 3mm CHR Whatman paper covered in Saran-wrap (DOW) and vacuum dried at 80°C for 1 hour in a Biorad Model 483 Slab Dryer to preserve resolution. The dried gels were put in direct contact with Hyperfilm-MP film (Amersham) for 36 hours. The nucleotide sequence was read directly from the developed film starting from the bottom, giving a 5’ to 3’ orientation from the priming site.

2.7.III - AUTOMATED SEQUENCING

Automated sequencing of Caplin cDNA was carried out at King’s College School of Medicine and Dentistry, Department of Molecular Medicine to confirm the sequence. 5μg of plasmid purified pGEX-Caplin were provided with two pGEX sequencing primers (Pharmacia Biotech) at concentration of 5pmol/μl in distilled water:

1) 5’ pGEX 23mer sequencing primer

sequence: 5’-d[GGGCTGGCAAGCCACGTTTGGTG]-3’
2) 3' pGEX 23mer sequencing primer

sequence: 5'-d[CCGGGAGCTGCATGTGTCAGAGG]-3'

The service uses an ABI 373A DNA sequencer which detects DNA molecules labelled with fluorescent dyes and automatically analyses data to determine the nucleotide sequence. Fluorescence is excited by laser and signals are analysed by computer. A chromatographic print-out was produced and the data was downloaded on to a MAC disk.

**2.7.IV - COMPUTER ANALYSIS OF SEQUENCE DATA**

All protein families and domains alignments were carried out using the National centre of biotechnology information which accesses various databases banks.

**2.8 - Cloning of Caplin cDNA into pgex fusion protein vector**

Mouse thymus Caplin cDNA sequence was expressed in *E.coli* BL 21 as a fusion protein with the Schistosoma japonicum glutathione s-transferase (GST) system. This system allows for the expression of eukaryotic genes cloned in frame with synthetic or bacterial nucleic acid sequences and can account up to 25% of total cell protein.

The vector chosen for cloning Caplin was pGEX. This vector is used in gene fusion systems for high level expression of cloned genes and purification of functional proteins. It uses a Tac promoter to ensure high levels of expression of cloned proteins in *E.Coli*. It also provide means of purifying fusion protein on a gel matrix, Glutathione Sepharose 4B, while still maintaining protein antigenicity and functional activity.

The vector of choice was pGEX-4T-3 which permits frame 3 ligation of cDNA inserts and cleavage of the recombinant fusion protein with thrombin. It is 4968 basepairs long with a multiple cloning site at position 918 to 980. Two restriction sites were chosen for cloning purposes, an EcoRI site at 938-943 and a BamHI site at 930-936. Also, there is a coding
region for thrombin cleavage extending from 918 to 935. This site specific serine protease
was used for the cleavage of the GST fragment from the fusion protein. This plasmid also
confers ampicillin resistance to the transfected cells.

2.8.1 - LIGATION OF CAPLIN DNA INTO PGEX FUSION PROTEIN VECTOR

In order to ligate protein Caplin cDNA insert into the fusion protein pGEX-4T-3 vector a
series of reactions took place. Cohesive ends were generated by EcoRI digestion on both
vector and insert and the vector was further dephosphorylated to prevent religation.

2.8.1.a - pGEX-4T-3 restriction digestion and dephosphorylation:

On an autoclaved eppendorf tube pGEX-4T-3 (500μg/ml) was incubated with EcoRI
(10U/μl) restriction enzyme in 10X react 3 buffer (50mMTris.HCl pH=8.0, 10mM MgCl₂,
0.1M NaCl) for 1 hour at 37°C. The reaction was stopped by placing it on ice. After 50
minutes of incubation a test sample was run in a 1% agarose gel at 50V to test for restriction
efficiency.

In order to prevent religation of the cohesive ends generated by restriction digestion with
EcoRI the 5’ ends of the linear plasmid were dephosphorilated by calf intestinal alkaline
phosphatase. The linear plasmid was incubated with 1U/μl of alkaline phosphatase in One
Phor-All buffer (Pharmacia) for 30 minutes at 37°C. The catalytic activity of the enzyme
was stopped by placing the tube in a waterbath at 70°C for 15 minutes. The reaction was
briefly cooled to room temperature and a phenol:chlorophorm extraction followed (section).
The sample was concentrated by ethanol extraction and store at -20°C in TE buffer.

2.8.1.b pUC18-Caplin EcoRI digestion

pUC18-Caplin (0.18μg/μl) was digested with EcoRI (10u/μl) for 90 minutes at 37°C.
The reaction was stopped by placing on ice. The sample was loaded on a 1% agarose gel run
at 35V for 3 hours. The bands were verified under U.V. light. The bands containing the Caplin insert were carefully sliced and placed in an eppendorf tube. The digested insert was purified by glassmilk purification using the GeneClean II Kit as described before.

2.8.I.c - Ligation reaction

The pGEX-4T-3 vector and the Caplin cDNA insert were ligated on a ratio of 1:3. Cohesive end ligation was carried out by adding the insert and the plasmid to an eppendorf tube and allowing the reaction to proceed at 45°C for 5 minutes. The tube was placed on ice. To the vector/insert mixture 1μl of 10X One-Phor-All Plus buffer (Pharmacia), 4 units of T4 ligase enzyme (Pharmacia) and 1mM ATP were added and the reaction was incubated for 2 hours at room temperature. The ligation reaction was stopped by bringing the temperature to 65°C for 10 minutes. Completed ligation reactions were stored at -20°C. Control ligation reactions of unphosphatased pGEX-4T-3 with and without cDNA insert, and phosphatased pGEX-4T-3 without cDNA insert were also carried out.

2.8.II - TRANSFORMATION OF E.COLI BL21 CELLS

Epicurian Coli BL21 competent cells (Stratagene) were chosen as this strain is deficient in proteases and allows high level protein expression. Transformation reactions were carried out as described by Stratagene’s protocol.

Competent cells and ligations were thwaned on ice. 100μl of the competent cells were aliquoted to eppendorf tubes and 25mM B-mercaptoethanol were added, the contents swirled gently and allowed to incubate on ice for 10 minutes. 40ng of ligated DNA was gently added to the cells and incubated on ice for further 30 minutes. The tubes were heat pulse in a 42°C water bath for 45 seconds followed by incubation on ice for 2 minutes. 900mls of SOC medium were added to the transformed cells and the tubes were incubated for 1 hour at 37°C in a Gallenkamp orbital shaker set at 225rpm. 50μl of each transformed sample were spread
onto SOB agar plates containing 50μg/ml ampicillin and incubated overnight at 37°C in a
Genlab Midi 2/55 incubator.

This kit also provided a control in which 1μl of supplied pBR322 control plasmid was
used to transform 100μl of the competent cells. Another control transformation was carried
out in which no DNA was added to the competent cells to check for sterility.

Transformation efficiency (CFU/μg) was calculated by using the equation:

\[
\text{CFU/μg} = \frac{\text{CFU pBR322 control plate} \times 1 \times 10^6 \text{ng} \times \text{dilution factor}}{\text{ng pGEX-Caplin} \times \text{μg}}
\]

2.8.II.a - Mini preparation of plasmid DNA

Single colonies generated from transformation were picked up using a sterile wire rod
and grown overnight in 2mls of LB medium containing 50μg/ml of ampicillin at 37°C with
constant shaking.

One-and-half mls of each culture sample were transferred to an autoclaved eppendorf
tube, and centrifuged at 12000g for 30 secs at 4°C. The supernatant was discarded and the
cell pellet was resuspended 100μl of ice cold solution I (solut.& buffers) and incubated for 5
minutes at room temperature. To it, 200μl of solution II (see solut.& buffers) was added and
mixed by gentle inversion and placed on ice. 150μl of ice cold solution III(see solut &
buffers) was added and briefly vortex and place on ice for further 5 minutes. The mixture was
centrifuged at 12000g for 10 minutes at 4°C in a microfuge. The supernatant was carefully
removed and transferred to a fresh eppendorf tube and extracted with an equal volume of
phenol:chloroform (1:1) with brief vortexing followed by microcentrifugation at 12000g for 5
minutes at 4°C. The aqueous layer was carefully removed and placed in a fresh tube. Two
volumes of 95% (v/v) ethanol were added and incubated at room temperature for 2 minutes.
Precipitated plasmid DNA was pelleted by microcentrifugation at 12000g for 2 minutes at
room temperature. The pellet was air-dried and washed in 1ml of 70% ethanol (v/v). The pellet was collected by centrifugation at 12000g for 2 minutes, the supernatant was carefully removed and the plasmid pellet was air-dried. The pellet was resuspended in 50μl of TE buffer containing 20μg/ml RNAase (Sigma). 5μl of dissolved plasmid was digested with EcoRI or BamHI restriction enzymes and the inserts were visualised on 1% agarose gels.

Cultures containing the correct insert were frozen in 7% DMSO in liquid nitrogen and stored at -70°C.

The amount of plasmid DNA (in μg/ml) obtained was calculated by the equation:

\[ \mu g/ml = OD_{260nm} \times \text{Cell length} \times \text{dilution} \times 50 \mu g/ml \]

2.8.II.b - *Restriction digestion to identify plasmids containing Caplin cDNA in the correct orientation*

In order to check for the correct orientation of the Caplin cDNA insert onto vector pGEX-4T-3 to each sample two restriction digestion reactions were set up using EcoRI and BamHI restriction endonucleases. 7μls of extracted plasmid DNA were mixed in an eppendorf tube with 1μl of EcoRI or BamHI (10u/μl), 1μl of 10 x React 3 buffer (50mM Tris.HCl pH=8.0,10mM MgCl₂, 0.1M NaCl), 1μl ddH₂O and digested for 2 hours at 37°C. The reaction was stopped by placing the tubes in a water bath at 65°C for 10 minutes. The tubes were placed on ice and 1μl of loading buffer were added to them. The digested inserts were electrophoresed on 1% agarose gel (section) and visualised on a U.V. source and photographed using a Polaroid DS34 camera. 1Kb DNA ladder (Gibco BRL Life Technologies) were also loaded and used as molecular weight markers.
2.9 - PURIFICATION OF FUSION PROTEIN

2.9.1 - INDUCTION AND PURIFICATION OF FUSION PROTEIN CAPLIN

Overnight cultures of *E. coli* BL21 transformed with recombinant pGEX plasmids containing Caplin nucleotide sequence were diluted 1 in 10 into 100mls of LB medium containing 50μg/ml of ampicillin and grown for 1 hour at 37°C. Fusion protein expression was induced by addition of 0.2mM isopropyl-B-D-thiogalactoside (IPTG, Melford). The cells were allowed to grow for 3 hours at 37°C.

Cells were collected by centrifugation at 2500g for 10 minutes and gently resuspended in 10mls of wash buffer (1 x PBSA, 0.1% 2ME, 3mM EDTA and protease inhibitors (see protease inhibitors). Centrifugation was repeated and cells were lysed in lysing buffer (wash buffer + 4mg/ml lysozyme; 2% CHAPS) for 30 minutes on ice. The lysate was centrifuged at 25000g for 30 minutes and the pellet obtained was resuspended in 15mls of urea buffer (50mM Tris.HCl pH=9.5, 6M urea and protease inhibitors). The mixture was incubated at 4°C for 40 minutes and then placed onto a dialysis tube (Pierce) dialysed into 600mls of wash buffer overnight at 4°C. Dialysis was carried out with two changes of buffer to ensure that urea was dialysed out. The lysate was centrifuged at 25000g for 1 hour at 4°C. Supernatant was carefully removed by hand pipetting and placed in a 50ml polypropylene tube. 1ml of glutathione Sepharose 4B beads (Pharmacia) were added and incubation was carried out on a rotating plataform for 20mins at 4°C. The beads were collected in a column and washed with 50mls of wash buffer. The last wash was carried out with 10mls of wash buffer without AEBSF. The beads were resuspended in 1.5mls of buffer by glass pipette and placed onto a bijou. The bound material was eluted by adding 3U/ml of thrombin and allowing to incubate on a rotating plataform for 40mins at 4°C. The mixture was passed through a column and the eluted extract containing the fusion protein was collected into a bijou tube. To it 300μl of
heparin affinity chromatography gel (Bio Rad) was added and incubation was carried out on rotating plataform for further 20mins. The heparin beads were retained in the column and to the extract 20µl of 37mg/ml AEBSF were immediately added. The collected supernatant was dialysed against Imidazole buffer (10mM Imidazole, 1mM EGTA, 2mM MgCl₂, 0.1mM 2 ME, protease inhibitors).

The yield of fusion protein was calculated from the absorbance at 280nm using the Caplin protein extintion coefficient of 1.302 and further analysed by SDS-PAGE and immunoblotting.

2.10 - ACTIN PURIFICATION

Actin was purified from rabbit skeletal muscle. A protocol described by Pardee, JD & Spudich, JA was used in order to remove very small amounts of contaminants (0.2%) and obtain polymerization competent actin (Pardee and Spudich, 1982).

2.10.1 - ACETONE POWDER PREPARATION

BUFFERS

Buffer A: Phosphate-KCl solution

150ml of 2M KCl stock solution and 150ml of KH₂PO₄ stock solution were added together and diluted in water to a final volume of 1 litre.

Buffer B: 0.4% NaHCO₃ + 0.1mM CaCl₂ at 4°C

Buffer C: 10mM NaCO₃ + 10mM Na₂CO₃ + 0.1mM CaCl₂ at 4°C.

The hind leg and dorsal muscles of a rabbit were removed and immediately placed in icy water. The muscle was ground in a meat mincer previously chilled to 4°C. The minced muscle was weighted and extracted with 3 volumes of buffer A for 10 mins. The extraction
was carried out in a cold room with constant, gentle stirring at 4°C. Twelve volumes of previously chilled distilled water at 4°C were added with vigorous stirring for 5 mins. The suspension was filtered through a double layer of cheese cloth which had previously been boiled for 10 mins in distilled water and allowed to cool to 4°C. The muscle residue was suspended in 5 volumes of buffer B and stirred constantly for 30 mins in the cold room. The mixture was filtered through cheese cloth. The residue was suspended in 2 volumes of cold acetone (4°C) and stirred for 30 mins in a fume cupboard at room temperature and than filtered through cheese cloth. Acetone extraction was repeated 5 times. After the final filtration the residue was allowed to air dry on a filter paper at room temperature. Another sheet of filter paper was placed on top of the powder and allowed to dry overnight. The acetone powder was weighted and aliquoted into 5 grams fraction in sealed bags. The bags were stored with silica beads at −85°C.

2.10.11 - ACTIN EXTRACTION AND COLUMN PURIFICATION

BUFFERS

G Buffer: 2mM Tris-HCl, 0.2mM CaCl₂, 0.2mM ATP-disodium salt 0.5mM 2-mercaptoethanol and 0.005% Sodium Azide. G buffer was brought to pH=8 with concentrated HCl at room temperature before the addition of 2-mercaptoethanol, since it interferes with accurate pH determination. The buffer was prepared with stock solutions of 1M Tris-base and 0.1M CaCl₂ stored at 4°C with 0.1% sodium azide in order to prevent bacterial growth.

Wash Buffer: G Buffer, 0.6M KCl, 2mM MgCl₂ and 1mM ATP-disodium salt.

Five grams of acetone powder was extracted in 100mls of G buffer (20mls of G buffer per gram of acetone powder). The acetone powder was added to G buffer at room temperature and then left to extract for 2 hours with constant stirring at 4°C.
The extraction mixture was centrifuged at 40,000g for 30 minutes. The supernatant was decanted by hand pipetting leaving the layer just above the pellet undisturbed. The volume obtained was measured. This supernatant contained depolymerised monomeric actin.

G actin was then polymerised by slow, dropwise addition of 50mM KCl, 2mM MgCl₂ and 1mM ATP-disodium salt while stirring very gently. These were added from stocks of 3M KCl, 1mM MgCl₂ and solid ATP. The actin monomers were then allowed to form actin filaments overnight at 4°C. The KCl concentration was brought to a final concentration of 0.6M by gently adding solid KCl to the polymerised mixture with gentle stirring for 30 minutes in order to remove tropomyosin from actin filaments.

Polymerised actin (filamentous actin) was pelleted by centrifugation at 100,000g at 4°C overnight. The pellets were gently homogenised in G buffer (2mls per pellet) and dialysed for 3 days in 1 litre of G buffer at 4°C. G buffer was changed twice a day to ensure that nonhydrolysed ATP was available during the depolymerization of actin filaments.

Depolymerised actin (G actin) was further purified by gel filtration. 20mg of G actin in 8mls of G buffer were loaded onto Sephadex G150 column packed in a Pharmacia C100 x 2.6 which had been pre-equilibrated overnight with degassed chilled G buffer. The sample was applied and eluted with G buffer, run onto the column at a flow rate of 10mls/hr to maximise the resolution of the column. 10mls fractions were collected using a LKB Multirac S111 fraction collector. Each fraction was analysed by spectrophotometry at OD290nm and run by SDS-PAGE. Fractions containing actin were pooled, concentrated using sucrose, dialysed into G buffer, centrifuged at 150,000g. G actin was quickly frozen and stored at liquid nitrogen (Pollard).
Chapter 2

2.10.III - PYRENE LABELLED ACTIN

Actin was purified as described in previous section and the concentration determined using an extinction coefficient of 0.65 at 290nm for 1mg/ml.

G-actin, prepared as described in previous section, was dialysed into G buffer without 2-mercaptoethanol for 3 days at 4°C. G-actin was then diluted to a final concentration of 40μM. Pyrene-labelled actin was prepared by dissolving Pyrene iodoacetamide (Molecular Probes, Oregon USA) in dimethylsulfoxide (DMSO, Sigma) at a stock concentration of 5μM. 45μM pyrene was added dropwise to a rapidly stirring actin solution. The mixture was polymerised by addition of 100mMKCl and 2mM MgCl₂ for 16 hours in the dark at room temperature. Pyrene-labelled F-actin was centrifuged at 100,000g (beckman) for 3 hours at 4°C. The actin pellet was gently homogenised and dialysed against G buffer containing 1mM Na₃N for 32hs at 4°C. Pyrene-labelled G-actin was centrifuged at 200,000g for 3 hs and loaded on to a Sephadex G150 2.5 x 50cm column previously equilibrated with G buffer without 2-ME and allowed to run overnight at 4°C at a rate of 10mls/hour. The fractions containig pyrene-labelled G-actin were determined by OD290nm and OD344nm, pooled and analysed by SDS-PAGE.

The pyrene-actin ratio was calculated (Cooper, Collucio), 0.5mMATP added and 200μl aliquots were frozen in liquid nitrogen and stored at −85°C.

2.11 - IN VITRO functional assays

2.11.I - FALLING BALL VISCOMETRY

Low shear falling ball viscometry is an assay used to study actin filaments networks. It is particularly useful during the purification of molecules that either promote or inhibit actin
filament interactions (Pollard, 1982; MacLean-Fletcher 1980). It was used to quantify any changes in actin viscosity, caused by Caplin, in a variety of experimental conditions.

This assay uses a capillary tube (Corning, 12.7cm X 1mm) containing the sample to which a stainless steel ball (0.6mm) is added. The tube is held at 65° angle and the velocity at which the ball falls is measured (seconds per centimeter) and is inversely proportional to the viscosity of the sample (Pollard, 1982).

In all experiments the viscosity of purified actin filaments was used as control, and to minimize variability between experiments actin was cycled from monomeric to filamentous actin every three days and its viscosity was determined daily before experiments were carried out.

2.11.1.a - Concentration curve

A sample of 144μl was prepared in an eppendorf tube in which 10μM monomeric actin was polymerised by addition of 10 x buffer P (100mM Imidazole pH=7.5, 20mM MgCl₂, 10mM EGTA, 0.1M KCl) in the presence of protein Caplin at concentrations of 3.85μM, 5.8μM and 7.7μM. The buffer is added last and the sample is immediately drawn onto capillary tubes by capillary action. The tubes were sealed in the bottom with Clay Adams Seal Ease, rotated at 65°C and incubated at room temperature for periods of 0.5, 1, 1.5, 2, 2.5, 3, 4 and 12 hours. A steel ball is placed at the meniscus of the sample by hand and gently pushed through the meniscus with a thin metal wire to initiate the fall. The velocity of the ball is measured as the time the ball takes to fall 1cm intervals.

2.11.1.b - Time course

This experiment was carried out to investigate the time course effect of protein Caplin upon actin polymerization. The sample containing 10μM actin and 3.85μM protein Caplin were mixed together and polymerization was initiated by addition of 10 X buffer P. The
sample was immediately drawn onto capillary tubes and measurements were taken at 2.5, 5, 10 and 15 minutes.

2.11.I.c - Effect of calcium on Caplin-actin interactions

This experiment was carried out to investigate the effect of micromolar amounts of calcium on Caplin-actin protein interactions. Buffer P was prepared with 0.5μM or 1.5μM calcium concentrations in the absence of EGTA. The samples were added together, allowed to polymerise for 1 hour at room temperature and their viscometry was recorded. Calcium was removed by the addition of 2mM EGTA to buffer P and relative viscometry analysed as before.

2.11.I.d - Effect of Caplin on actin annealing

This experiment was carried out to investigate whether C4 L affects the annealing of pre-formed actin filaments. Actin was polymerised in an eppendorf tube upon addition of buffer P for 30 minutes at room temperature. The pre-formed filaments were broken by pipetting 5 times and 3.85μM Caplin were added to it. The test was immediately drawn to the capillary tube and the velocity to which the ball fell was recorded. Controls containing only actin were also performed.

2.11.II - FLUORIMETRY

The kinetics of actin polymerization was measured by changes in fluorescence of N-(1-pyrenyl) iodoacetamide covalently attached to cysteine 374 of column purified actin. The fluorescence intensity changes as a function of time were monitored in a fluorimeter using 366nm as the excitation and 408nm as the emission wavelengths, slits at 1.0nm at constant 25°C.
2.11.II.a - Nucleation Assay:

To determine whether protein Caplin affected the initial nucleation step of actin polymerization, 5µM G actin of which 10% was pyrene labelled was polymerised in the presence or absence of 2.3µM Caplin. Actin, Caplin and distilled water were added to a crystal cuvette to a final 400µl volume at 25°C and the reaction was initiated by addition of 10X buffer P (10mM Imidazole pH=7.5, 1mM EGTA, 2mM MgCl₂, 10mM ATP, 1mM DTT, 100mM KCl). The buffer was added to the cuvette and immediately placed onto the fluorimeter slot so that changes and fluorescence during nucleation step were recorded immediately.

2.11.II.b - Elongation Assay:

To investigate whether protein Caplin affected the elongation of preformed actin filaments, 5µM actin was polymerised in buffer P overnight in the presence and absence of protein 2.3µMCaplin. Elongation of the actin ends was observed and recorded by addition of 0.7µM actin of which 10% was pyrene labelled.

2.11.II.c - Gelsolin Assay:

To investigate whether protein Caplin effected the addition of actin monomers at the barbed end of actin filaments, 5µM actin was polymerised in the presence of 0.13µM gelsolin in the presence or absence of 2.3µM Caplin overnight in a buffer containing 10mM Imidazole pH=7.5, 50mM EGTA, 2mM MgCl₂, 1mM ATP, 1mM DTT 100mM KCl. (Weber et al., 1994). Elongation of the pre-formed actin filaments was then analysed by addition of 0.7µM actin of which 10% was pyrene labelled.
2.12 - Linear Density Sucrose Gradient

Linear density sucrose gradients were carried out to determine whether protein Caplin occur as a monomer or a polymer.

Gradients were formed from solutions containing 5% and 20% sucrose in 10mM Imidazole PH7.5, 2mM MgCl₂ and 1mM EGTA. (Martin and Ames, 1961).

Ten ml fractions were poured onto 14ml Kontron centrifuge tubes using a Biorad gradient former and left to equilibrate overnight at 4°C. Soy bean trypsin inhibitor (SBTI) Mr21Kd, ovalbumin Mr 43Kd and actin Mr43Kd were all at 0.5mg/ml in Imidazole buffer and used as standards.

Two samples were carefully loaded onto separate gradients: protein Caplin (0.5mg/ml) and protein Caplin +2ME (0.5mg/ml). The gradients were centrifuged at 150,000g for 43hs at 4°C. The tubes were then carefully removed, clamped to a retort stand, punctured at the base with a syringe needle and 1ml fractions were collected in eppendorf tubes individually labelled.

The protein concentration of each fraction was analysed by optical density at OD290 or 280nm. Fractions containing peak readings were concentrated by methanol/chloroform extraction and analysed by 12% SDS-PAGE.

2.13 – Actin binding sedimentation assay

The binding of protein Caplin to actin was analysed by a pelleting assay (Yamashiro-Matsumura and Matsumura, 1988). A 100μl samples were prepared in a airfuge tube (Beckman polyallomer centrifuge tube 5 x 70mm) containing 10μM actin polymerised in the presence of protein Caplin at concentrations of 0.96μM, 1.9μM, 3.85μm, 5.7μM, 7.7μM, 11.5μm and 15.4μM. Samples were polymerised by addition of buffer P overnight at room
temperature and centrifuged in a Beckman airfuge at 148,00g for 1 hour at 4°C. The outside edges of the tubes were marked so that the supernatant could be carefully withdrawn by hand using a drawn out Pasteur pipette. Both pellets and supernatants were suspended in an equal volume of SDS sample buffer and electrophoresed on a 12% SDS-PAGE, stained with Coomassie Brilliant Blue R250 for 1 hour. Each gel was destained in equal volume of 100mls of destain solution with constant shaking for 2 hours. To minimize variations in staining between gels, both supernatants and pellets in one series of experiments were run on the same SDS gel. The gels were scanned in a GS-670 Imaging densitometer. The data from the Model GS-670 Imaging densitometer were analysed by the Molecular Analyst™/PC image analysis software.

The densitometer was calibrated with known amounts of protein Caplin each time before experimental readings were taken.

To determine the binding constant Ka of Caplin the concentrations of bound versus free protein were plotted and a concentration curve was obtained from which the binding constant of protein Caplin was calculated.
3.1 - Background

Protein Caplin is a doublet originally identified on SDS-PAGE by a monoclonal antibody (Shapland et al., 1988). This molecule has been previously purified from human T cell lymphoma (HTCL) and at immunofluorescence level was found to be associated with vestigial actin bundles of SV40 transformed 3T3 fibroblasts. The full length cDNA encoding this protein has been sequenced and shown to be of 600 base pairs, with a calculated molecular weight of 22.3Kd. Northern blot analysis showed that Caplin is expressed as a single message of 1.44Kb.

The upper band of this doublet, Transgelin, acts *in vitro*, by gelling actin filaments. To identify the *in vitro* function and interactions of the lower protein Caplin isoform I engaged in a series of experiments in which I obtained Caplin full length cDNA sequence from normal mouse thymus and investigated its possible *in vitro* function in the cells by generating and purifying a fusion protein.

3.2 - RNA preparation

Total mouse thymus tRNA and mRNA concentrations were determined by optical density at 260nm as described in materials and methods.
3.2.1 - tRNA NUCLEIC ACID CONCENTRATION

\[ \text{OD}_{260\text{nm}} = 0.370 \]

\[ [\text{tRNA}] = \text{OD}_{260\text{nm}} \times \text{cell length} \times \text{dilution} \times 40\mu\text{g/ml} \]

\[ [\text{tRNA}] = 0.370 \times 2 \times 100 \times 40 \]

\[ [\text{tRNA}] = 2960\mu\text{g/ml} \]

\[ [\text{tRNA}] = 2.96\mu\text{g/}\mu\text{l} \]

3.2.II - mRNA NUCLEIC ACID CONCENTRATION

\[ \text{OD}_{260\text{nm}} = 0.033 \]

\[ [\text{mRNA}] = 0.033 \times 2 \times 50 \times 40 \]

\[ [\text{mRNA}] = 132\mu\text{g/ml} \]

\[ [\text{mRNA}] = 0.132\mu\text{g/}\mu\text{l} \]

3.3 - Cloning of mouse thymus Caplin cDNA

3.3.I - POLYMERASE CHAIN REACTION

RT-PCR amplifications from mouse thymus poly A\(^+\) selected mRNA using MANR and QUILrev oligonucleotides produced a single 600bp blunt-end cDNA product (figure 1). A better yield of DNA concentration was obtained using 2.5mM Mg\(^2+\) per PCR reaction.

3.3.I.a - 1% agarose gel electrophoresis of blunt-end Caplin cDNA

Two PCR control reactions were also carried out, one using HTCL cDNA and one without cDNA.
FIGURE (1). Agarose gel electrophoresis of blunt-end Caplin cDNA (A) 0.350 µg/µl Caplin cDNA; (B) 0.520 µg/µl; (C) 0.830 µg/µl Caplin cDNA; (D) 0.920 µg/µl HTCL (control); (E) no cDNA (control).

3.3.1.b - Determination of Caplin blunt-end cDNA concentration

OD260nm = 0.035 at 1.5mM Mg^{2+}

[Caplin cDNA] = OD260nm x cell length x 50µg/ml

[Caplin cDNA] = 0.035 x 2 x 100 x 50

[Caplin cDNA] = 0.350 µg/µl
3.3.11 - AGAROSE GEL ELECTROPHORESIS OF DH5ALPHA TRANSFORMED CELLS.

DH5-α E.coli cells transformed with pUC18 containing mouse thymus Caplin cDNA insert were grown and the plasmid DNA was isolated by minipreparation and further digested with EcoRI. The samples were analysed by 1% agarose gel electrophoresis (figure 2).

FIGURE (2). Agarose gel electrophoresis of pUC18-Caplin cDNA digested with EcoRI. Wells A to H contain a fragment of 600bp corresponding to Caplin cDNA.
3.4 - Sequencing of mouse thymus Caplin cDNA

3.4.1 - NUCLEOTIDE AND DERIVED AMINO ACID SEQUENCE

Double stranded sequencing of mouse thymus Caplin cDNA was carried out as described in section 2.6, allowing further translation of its amino acid sequence and database homologies search.

The complete cDNA sequence for Caplin is shown in figure (3). The ATG start codon (1-3bp) is followed by a 594bp opening reading frame (ORF) ending with a stop codon TGA at position 597-600bp.

The translated amino acid sequence is shown below the ORF and has 199 amino acids.
MOUSE THYMUS Caplin cDNA AND DERIVED AMINO ACID SEQUENCE

ATG GCC AAC AGG GGA CCT GCA TAC GGC CTG AGC GCA GAG GTG CAG 45
MAN RGPAYGLSAEV
CAO AAG ATT GAG AAG CAG TAC GAC GCG GAT CTG GAG CAG ATC CTC 90
KIETK
Q YDADL
ATC CAG TGG ATC ACC ACT CAG TGC CGC GAG GAG GAT CTG GAG CAG CCC 135
Q
15
CAO CCT GGC GTG GAC GAC GCTTTCAGAAGTGGCTCAGAAGGACGTGGGACG 180
PGRENFKWLKDGT
GTT CTG TGC AAG CT TAT ATT ATT TCA CTG TAT CCT GAG GGG CAG GGC 225
VNLKLNPSLEGQP
CCA GTA AAG AAG ATC CAG GCC TCT TCG ATG GCC TTC AAG CAG ATG 270
PKKIASASMAFKQM
GAG CAG ATC TCC CAG TCC CTG CAG GCA GCC GAG CGC TAT GGC ATT 315
Q ISEFL
AAC AAC ACG GAC ATC TCC CAG TCC CTG CAG GCC GAG CCC AAC TGG 360
NTDIFFVTDLWEGL
GCA GTA GCC AGG GAC GAT GGG CTC TTC TCT GGG GAT CCC AAC TGG 405
AVADRGGLFDGN
AAC ATG GCT TGT GTG CAG CGG ACA CTA ATG AAC CTG GGT GGG CTG 450
NMAMVTRTLMNLGGL
GTT CCT AAG AAA TCC AAG GAG AAC CT TCG GTT GTG CAG AAC GAC ACC 495
FPKKSKENEPRNFSDN
158
CAG TTG CAA GAG GCC AAG AAC GTG ATT GGG TTG CAG ATG GGC ACC 540
QLEGKNIQGLQMG
AAC CGT GTG GCA TCT CAG GCC GCC ATG ACC GCC TAT GGG ATG CCA 585
NRGASQAGMTGYGMP
CGC CAG ATC CTC TGA 600
RQIL*

FIGURE (3). Complete nucleotide and derived amino acid sequence of mouse thymus Caplin. The two oligonucleotides primers are highlighted in red; 5’ sense primer MANR and 3’ antisense QUIL. Caplin has three cysteine amino acids at positions 38,63 and 124 and they are marked in yellow. A putative nuclear localisation site is highlighted in pink. Two potential EF-Hand sites are highlighted in blue. A potential phosphorylation site is highlighted in green. The stop codon TGA is marked with (*) indicating that translation stops at this position in this reading frame.
3.5- Sequence motifs

All protein families and domain alignments were carried out using the National centre of Biotechnology information, which accesses various databases banks.

3.5.1 - CYSTEINE DISTRIBUTION

Caplin cDNA has three cysteine amino acids at positions 38, 63 and 124.

3.5.11 - PUTATIVE ACTIN BINDING DOMAINS

Caplin shares an overall sequence homology to smooth muscle basic calponin h1 (CaP h1) of 58%. The sequence identity varies within the amino and carboxyl terminals. Caplin sequence is divided into three subdomains (figure 4): a CH-domain at position 1-134 at the amino-terminus which is 43% homologous to calponin CH-domain, an actin binding site (ABS) at 152-173 which is 40% homologous to Cap h1 ABS and one calponin-like repeat (R) at 174-198 at the carboxyl-terminus which is 75% homologous to Cap h1 repeat (figure 5). Such subdomains are also found in a number of actin binding proteins including Transgelin, spectrin family of proteins (α-actinin, fimbrin, filamin, ABP120), muscle protein SM20, rat neural NP25, unc-87. The CH-domain is also found conserved in a number of proteins not known to bind actin such as VAV proto-oncogene.
FIGURE (4). Schematic representation of the molecular structure of Caplin (bottom) and Calponin h1 (top) subdomains. CH-domain (red), the actin binding site (ABS) (blue) and calponin-like repeats (R) (yellow).

<table>
<thead>
<tr>
<th>ABS Sequence</th>
<th>Homology (%)</th>
<th>Position</th>
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<tbody>
<tr>
<td>Calponin h1</td>
<td>VKYAEKQERRFEPEKLRGRNI</td>
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<tr>
<td>Caplin</td>
<td>PKKSKEKPRFSDNQLQEGKNV</td>
<td>40%</td>
</tr>
<tr>
<td>Calponin h2</td>
<td>VKYSEKQERNFDDATMKAGQCV</td>
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FIGURE (5). The actin binding site (ABS) sequence of Calponins h1 and h2 and Caplin. Comparison of the amino acid sequence indicated an overall 40% homology to both CaP h1 and Cap h2. Caplin has 13 non-conservative amino acid substitutions at 152; 154-159; 161; 163-166 and 168 residues when compared to CaP h1 and 13 non-conservative amino acids substitutions at 152; 154; 156-159; 169; 165-166; 168-169 and 171-172 when compared to CaP h2 ABS sequence.
3.5.III - POTENTIAL PHOSPHORYLATION SITES

A potential protein kinase C phosphorylation site (TNR) was found at 180-182 amino acids at the carboxyl end of Caplin cDNA.

3.5.IV - PUTATIVE NUCLEAR LOCALISATION SITE

A cluster of positively charged amino acids is found at position 152-158 (figure 6). This residue (PKKSKEN) aligns to a consensus NLS found in SV40 and polyoma large T antigens.

<table>
<thead>
<tr>
<th>NUCLEAR LOCALISATION SIGNAL</th>
<th>CONSENSUS SEQUENCE</th>
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<tbody>
<tr>
<td>RKKR</td>
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<tr>
<td>Caplin</td>
<td>PKKSKEN</td>
</tr>
<tr>
<td>SV40 large T antigen</td>
<td>PKKRRKV</td>
</tr>
<tr>
<td>Polyoma large T antigen</td>
<td>PKKARED</td>
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</table>

FIGURE (6). Putative nuclear localisation site. Caplin NLS is aligned against NLS sequences of SV40 and Polyoma large T antigens.

3.5.V - POTENTIAL EF-HAND STRUCTURE

Two sites within caplin cDNA sequence containing residues at position 23-34 (site I) and 107-118 (site II) can be aligned with the core region of the sequence motif defined by Tuffy.
& Kretsinger (1975) as the EF-hand structure (figure 7). This domain comprises seven residues proposed to be critical for the binding of calcium ions.

### 7 CRITICAL RESIDUES

**CONSENSUS SEQUENCE**

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<tr>
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<tr>
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<tr>
<td>Site II</td>
<td>TTDIFQTVDLWE</td>
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<tr>
<td>Site I</td>
<td>DADGGGDISVKE</td>
</tr>
<tr>
<td>Site II</td>
<td>DEDGSGTIDFEE</td>
</tr>
<tr>
<td>Site III</td>
<td>DRNADGYIDPEE</td>
</tr>
<tr>
<td>Site IV</td>
<td>DKNNDGRIDFDE</td>
</tr>
<tr>
<td><strong>Transgelin</strong></td>
<td></td>
</tr>
<tr>
<td>Site I</td>
<td>DEELEERLVEWI</td>
</tr>
<tr>
<td>Site II</td>
<td>KTDMFQTVDLFE</td>
</tr>
</tbody>
</table>

Where: X = ANY AMINO ACID; D=D; D’=D,N,E,Q,S,T; G=G; I=I,V; E=E

FIGURE (7). Potential EF-Hand. Table of EF-Hand structure alignment of sites present in a number of proteins.

### 3.6- Homologies

Prosite and BLAST software programs were used to search available databases and indicated significant homologies with Transgelin, rat neuronal protein NP25, Calponin multifamily (CLP), mp20, and VAV proto-oncogene. Figure (8) shows a table of Caplin
cDNA homology at nucleotide level. The homology identity was registered within regions between 0-250bp; 251-350 and 351-600bp. Figure (9) shows the percentage of overall homology between Caplin and it’s homologous proteins. Figure (10) shows the protein percentage homology based on Caplin subdomain structure.

### 3.6.1 - HOMOLOGOUS NUCLEOTIDE SEQUENCES

<table>
<thead>
<tr>
<th></th>
<th>0-450bp</th>
<th>451-519bp</th>
<th>520-597bp</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human Caplin mRNA</strong></td>
<td>90%</td>
<td>90%</td>
<td>90%</td>
</tr>
<tr>
<td><strong>Rat neuronal protein NP25</strong></td>
<td>84%</td>
<td></td>
<td>86%</td>
</tr>
<tr>
<td><strong>Chicken Transgelin mRNA</strong></td>
<td>92%</td>
<td></td>
<td>87%</td>
</tr>
<tr>
<td><strong>Human Transgelin cDNA</strong></td>
<td>80%</td>
<td></td>
<td>85%</td>
</tr>
<tr>
<td><strong>Human Transgelin mRNA</strong></td>
<td>80%</td>
<td></td>
<td>85%</td>
</tr>
<tr>
<td><strong>Human Transgelin gene</strong></td>
<td>80%</td>
<td></td>
<td>85%</td>
</tr>
<tr>
<td><strong>Human smooth muscle Transgelin mRNA</strong></td>
<td>95%</td>
<td></td>
<td>85%</td>
</tr>
<tr>
<td><strong>Human Transgelin mRNA 5’end</strong></td>
<td></td>
<td></td>
<td>95%</td>
</tr>
<tr>
<td><strong>Human Calponin mRNA</strong></td>
<td></td>
<td></td>
<td>95%</td>
</tr>
<tr>
<td><strong>H1-Calponin cDNA</strong></td>
<td></td>
<td></td>
<td>95%</td>
</tr>
<tr>
<td><strong>Equine herpesvirus 2</strong></td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PTP-TD14</strong></td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Human BAC clone GS330J10</strong></td>
<td>100%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIGURE (8). Caplin cDNA homologies. Caplin cDNA has an overall homology with Human Caplin (90%) and a high homology with calponin cDNA sequence at the 3’end of Caplin cDNA (95%).
### 3.6.11 - HOMOLOGOUS PROTEINS

<table>
<thead>
<tr>
<th>Protein</th>
<th>Overall %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Caplin</td>
<td>97%</td>
</tr>
<tr>
<td>Rat neuronal protein NP25</td>
<td>83%</td>
</tr>
<tr>
<td>Mouse smooth muscle Transgelin</td>
<td>82%</td>
</tr>
<tr>
<td>Rat smooth muscle Transgelin</td>
<td>82%</td>
</tr>
<tr>
<td>Homo sapiens smooth muscle Transgelin</td>
<td>81%</td>
</tr>
<tr>
<td>Chicken Transgelin</td>
<td>80%</td>
</tr>
<tr>
<td>Bovine Transgelin</td>
<td>73%</td>
</tr>
<tr>
<td>Human acidic calponin</td>
<td>59%</td>
</tr>
<tr>
<td>Human calponin H1</td>
<td>57%</td>
</tr>
<tr>
<td>Human smooth muscle calponin H1</td>
<td>58%</td>
</tr>
<tr>
<td>Rattus calponin</td>
<td>57%</td>
</tr>
<tr>
<td>Chicken calponin</td>
<td>57%</td>
</tr>
<tr>
<td>Pig smooth muscle calponin H2</td>
<td>58%</td>
</tr>
<tr>
<td>Human smooth muscle calponin H2</td>
<td>58%</td>
</tr>
<tr>
<td>Xenopus calponin H3</td>
<td>58%</td>
</tr>
<tr>
<td>Drosophila protein Sm20</td>
<td>56%</td>
</tr>
<tr>
<td>Mouse VAV proto-oncogene</td>
<td>54%</td>
</tr>
<tr>
<td>Rat VAV proto-oncogene</td>
<td>53%</td>
</tr>
<tr>
<td>Human transforming VAV protein</td>
<td>53%</td>
</tr>
<tr>
<td>Human VAV2 protein</td>
<td>56%</td>
</tr>
<tr>
<td>Homo sapiens VAV3</td>
<td>50%</td>
</tr>
<tr>
<td>Kinesin like heavy chain</td>
<td>47%</td>
</tr>
<tr>
<td>UNC-87 protein</td>
<td>48%</td>
</tr>
<tr>
<td>RAS GTPASE protein</td>
<td>47%</td>
</tr>
<tr>
<td>Human smoothelin</td>
<td>44%</td>
</tr>
<tr>
<td>Transcription repair coupling factor</td>
<td>43%</td>
</tr>
<tr>
<td>Fimbrin 2</td>
<td>43%</td>
</tr>
<tr>
<td>Dictyostelium fimbrin</td>
<td>40%</td>
</tr>
<tr>
<td>Human intestine plastin</td>
<td>36%</td>
</tr>
</tbody>
</table>

FIGURE (9). Protein homology. This table shows the percentage amino acid homology of Mouse thymus Caplin. The highest degree of homology being human Caplin (97%), NP25 (83), Transgelin (82%), Calponins (58%), mp20 (56%) and VAV proto-oncogene (50-54%).
3.7 - Physical properties of protein Caplin gene product

The following data was obtained using a multiprediction program, at the Ludwig Institute.

### 3.7.1 - CAPLIN MOLECULAR WEIGHT AND ESTIMATED PI.

The translated product of Caplin open reading frame is 199 amino acids in length with a calculated molecular weight of 22,391.4 KDa and an estimated PI of 8.41.

### 3.7.2 - EXTINCTION COEFFICIENT

The calculated extinction coefficient for Caplin is 1.302 (in 6M guanidium hydrochloride, 0.02M Kphos PH=6.5).
3.7.III - AMINO ACID COMPOSITION AND CHARGE DISTRIBUTION

The amino acid composition of Caplin is shown in table (1).

Table (1). Caplin amino acid composition

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Number</th>
<th>Mole percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A = Ala</td>
<td>13</td>
<td>6.533</td>
</tr>
<tr>
<td>B = Asn</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C = Cys</td>
<td>3</td>
<td>1.508</td>
</tr>
<tr>
<td>D = Asp</td>
<td>10</td>
<td>5.025</td>
</tr>
<tr>
<td>E = Glu</td>
<td>11</td>
<td>5.528</td>
</tr>
<tr>
<td>F = Phe</td>
<td>7</td>
<td>3.518</td>
</tr>
<tr>
<td>G = Gly</td>
<td>20</td>
<td>10.050</td>
</tr>
<tr>
<td>H = His</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I = Ile</td>
<td>11</td>
<td>5.528</td>
</tr>
<tr>
<td>K = Lys</td>
<td>13</td>
<td>6.533</td>
</tr>
<tr>
<td>L = Leu</td>
<td>16</td>
<td>8.040</td>
</tr>
<tr>
<td>M = Met</td>
<td>8</td>
<td>4.020</td>
</tr>
<tr>
<td>N = Asn</td>
<td>13</td>
<td>6.533</td>
</tr>
<tr>
<td>P = Pro</td>
<td>9</td>
<td>4.523</td>
</tr>
<tr>
<td>Q = Gln</td>
<td>22</td>
<td>11.055</td>
</tr>
<tr>
<td>R = Arg</td>
<td>9</td>
<td>4.523</td>
</tr>
<tr>
<td>S = Ser</td>
<td>9</td>
<td>4.523</td>
</tr>
<tr>
<td>T = Thr</td>
<td>8</td>
<td>4.020</td>
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<tr>
<td>V = Val</td>
<td>8</td>
<td>4.020</td>
</tr>
<tr>
<td>W = Trp</td>
<td>4</td>
<td>2.010</td>
</tr>
<tr>
<td>Y = Tyr</td>
<td>5</td>
<td>2.513</td>
</tr>
<tr>
<td>Z = Glx</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The Caplin polypeptide sequence is composed of 23 positively charged amino acids (Arg + Lys = 23) and 21 negatively charged amino acids (Asp + Glu = 21) resulting in a net charge of 2 positively charged amino acids (at neutral pH).

3.8 - Secondary structure prediction

The prediction of the secondary structure of Caplin was carried out by Dr Zvelebil at the Ludwig Institute for Cancer Research, London. It uses a multiprediction program which
utilises information available from a family of homologous sequences. Figures 11 and 12 contain the α-helix and β-sheets composition of Caplin respectively.

FIGURE (11). Fusion protein Caplin α helix composition.
FIGURE (12). Fusion protein Caplin β sheet composition
Chapter 3

3.9- Cloning of Caplin cDNA into pGEX fusion protein vector

The plasmid expression vector pGEX-4T-3 was chosen to allow for expression of Caplin polypeptide in frame with glutathione-S-transferase (GST, 26KDa). This vector system has a multiple cloning site (MCS 918-980) that facilitate cloning of full length mouse thymus Caplin cDNA and the cleavage and expression of Caplin as a fusion protein.

3.9.1 - CLONING LINKER SEQUENCE

Caplin cDNA obtained from PCR was cloned into an EcoRI site (938-943) of pGEX-4T-3 multiple cloning site. The blunt-end fragment of Caplin cDNA obtained by PCR was digested with EcoRI to produce cohesive ends. The recombinant fusion protein contained a fusion peptide of 4 amino acids at the NH2-terminus derived from GST linker region as demonstrated on diagram below.
PGEX-4T-3 blunt-end Caplin cDNA

5' CTG GTT CCG CGT GGA TCC CCG AAT TCC CGG 3'
3' GAC CAA GGC GCA CCT AGG GGC TTA AGG GCC 5'

5' AAT TCT GGA ATG GCC 3'
3' TTA AGA CCT TAC CGG 5'
(a) EcoRI digestion:

Dephosphorylated pGEX-4T-3

Cohesive-end Caplin cDNA
b) Ligation with T4 DNA ligase

\[
\text{thrombin} \\
5'\text{CTG GTT CCG GTG GGA TCC CCG AAT TCT GGA ATG GCC3'} \\
3'\text{GAC CAA GGC GCA CCT AGG GGC TTA AGA CCT TAC CGG5'} \\
\text{Arg Asn Ser Gly}
\]

3.9.11 - BAMHI AND ECORI RESTRICTION DIGESTIONS

Following ligation a series of restriction digestions took place in order to check for the correct orientation of Caplin cDNA insert (figure 12). Two enzymes were chosen EcoRI and BamHI. Caplin cDNA was cloned at an EcoRI site (938-943) at MCS and has no internal recognition site within its sequence and digestion with this enzyme produced 2 fragments of 600bp and 5968bp.

BamHI has a site at position 930-936 at pGEX MCS and an internal site within Caplin cDNA at position 438-443. pGEX-Caplin cDNA digestion using Bam HI produce 2 fragments of approximately 450bp and 5168bp when the insert is in the correct orientation and 2 fragments of 150bp and 5500bp when the insert is in the incorrect orientation.
FIGURE (13). Agarose gel electrophoresis of EcoRI and BamHI digestions. The upper wells contain BamHI digestions. Wells A to D contain 2 fragments of 450bp and 5168bp corresponding to the correct insert orientation and wells E to H contain 2 fragments of 150bp and 5500bp corresponding to the incorrect insert orientation. The lower wells contain EcoRI digestions. Wells A to H contain two fragments of 600bp and 5968bp.
3.10-Induction and Purification of fusion protein Caplin

Recombinant fusion protein Caplin was expressed as glutathione S-transferase fusion protein. Caplin expression was induced using 0.2mM IPTG and bacteria were lysed using the ionic detergent CHAPS. Since Caplin was found to be insoluble the bacterial pellet was resuspended in 6M urea buffer, dialysed out and then purified using an affinity chromatography matrix (Gluthathione Sepharose 4B) and cleavage of GST moiety was achieved using 3U/ml of thrombin. A further purification step was carried out using heparin affinity chromatography gel to ensure the complete removal of thrombin. During all stages of purification of Caplin a combination of protease inhibitors was present to prevent degradation (materials & methods). The yield of fusion protein from 100mls of bacterial preparations was found to be approximately 1mg/ml. Samples were analysed by SDS-PAGE and immunoblotting.
FIGURE (14). SDS-PAGE of induction and purification of Caplin. (A) bacterial lysate pellet containing induced uncle Caplin; (B) Sepharose beads pellet containing cut Caplin digested with thrombin; (C) supernatant of thrombin digested Caplin; (D) Caplin incubated with sepharose beads; (E) purified Caplin.
FIGURE (15). ECL immunoblotting of induction and purification of Caplin. (A) bacterial lysate pellet containing induced uncut Caplin, (B) Sepharose beads digested with thrombin; (C) supernatant containing cut Caplin, (D) sepharose beads with cut Caplin; (E) purified Caplin.
3.11- Actin Purification

To improve the purity of actin preparation, a series of polymerization and depolymerization G:F cycles was carried out together with a final purification step by loading samples onto a Sephadex G150 column. Rabbit skeletal muscle actin was extracted from acetone powder with a final yield of approximately 30mg of column purified actin per 5 grams of acetone powder.

3.11.1 - SDS PAGE OF COLUMN PURIFIED ACTIN

The biochemical analysis of the fractions from the eluted peak, at the level of Coomassie blue staining indicated that actin was devoid of contaminants (Figure 16 A & B).

FIGURE (16). Coomassie blue stained of total (A) and column purified actin (B)
3.11.11 - PYRENE LABELLED ACTIN

Column purified actin was labelled with N-(1-pyrenyl) iodoacetamide (Molecular probes) (Pollard, 1984). The protein concentration for pyrene actin was determined as described by Bryan and Colucio (Bryan and Coluccio, 1985).

The optical density of pyrene labelled actin was taken at 290nm (for actin) and at 344nm (for pyrene). At 290nm the OD was 0.298 and at 344nm it was 0.184.

The total actin concentration (in mM) was calculated using the formula:

\[
\text{mM Actin} = (\text{OD}_{290} - (0.127 \times \text{OD}_{344}))
\]

26.6mM

therefore,

\[
\text{mM Actin} = 0.0103
\]

The pyrene: actin ratio was calculated using an extinction coefficient of 22,000M⁻¹cm⁻¹ for pyrene with:

\[
\text{Pyrene} = (\frac{\text{OD}_{344}}{22})
\]

Actin  mM Actin

\[
\frac{\text{Pyrene}}{\text{Actin}} \text{ labelling ratio} = 0.836
\]
3.12 - *In vitro* functional assays

3.12.1 - FALLING BALL VISCOMETRY

The effects of fusion protein Caplin on the initial rate of actin polymerization and the final viscosity of polymerised actin were examined by falling ball viscometry. This technique is used to measure changes in actin viscosity under various experimental conditions. Viscosity is inversely proportional to the velocity of a ball falling through solutions containing polymerised actin in the presence or absence of Caplin.

In all experiments, column purified actin was used as control at a concentration of 10μM. Actin was polymerised by addition of buffer P and showed an apparent viscosity of 30secs/cm and gelation was never observed.
3.12.1.a - Effect of Caplin concentration

Falling ball viscometry was used to measure changes in actin viscosity in the presence of Caplin at different concentrations figure (17). These experiments show that at a concentration of 3.85μM Caplin reduces the apparent viscosity of actin by up to 25%. This effect occurred 2 hours after the initiation of actin polymerization.

FIGURE (17). Effect of Caplin concentration. 10μM actin was polymerised by addition of buffer P in the absence (dark blue) or in the presence of varying Caplin concentrations of 3.85μM (pink), 5.7μM (yellow) and 7.7μM (blue). (This experiment was repeated 5 times. ANOVA p<0.0005).
3.12.1.b - Time course of induced viscosity reduction by Caplin

Purified actin at 10μM was polymerised by addition of buffer P in the presence or absence of 3.85μM Caplin at time intervals of 2.5, 5, 10 and 15 minutes (Figure 18).

The curve indicates that the initial reduction in the rate of viscosity increase was apparent five minutes after actin polymerization was initiated. The reduction in the apparent viscosity after 10 minutes was 12% and after 15 minutes of polymerization the apparent viscosity of actin filaments was reduced 15% when compared with actin control.

FIGURE (18). Time course of Caplin. 10μM actin was polymerised by addition of buffer P in the absence (dark blue) or presence of 3.85μM Caplin (pink). (This experiment was repeated 5 times. ANOVA p<0.0005).
3.12.1.c - Effect of calcium on Caplin-actin interactions

The reduction of the apparent viscosity of actin filaments caused by Caplin is not influenced by the presence of calcium ions in the polymerization buffer (Figure 19). Actin polymerised for 1 hour by the addition of buffer P containing 1.5mM calcium or 2mM EGTA had no effect on the apparent ability of 3.85μM Caplin to reduce the apparent viscosity of actin filaments. A control experiment was also carried out in which purified 10μM actin in the absence of Caplin, was allowed to polymerise for 1 hour in the presence of 1.5mM calcium or 2mM EGTA. The apparent viscosity of purified actin was approximately 30secs/cm.

FIGURE (19). Effect of calcium ions on Caplin-actin interactions. 10μM actin polymerised by addition of buffer containing 2mM EGTA (orange) or 1.5mM calcium (green) in the presence of 3.85μM Caplin (blue and yellow respectively).
3.12.1.d - Effect of Caplin on actin annealing

The effect of Caplin on annealing of actin filaments were examined by falling ball viscometry. Ten μM actin was polymerized in an eppendorf tube for 30 minutes, in the presence or absence of 3.85μM Caplin (Figure 20). These filaments were sheared by passing through a micropipette five times and allowed to anneal in the capillary tube for 1 hour. Falling ball viscometry was used to monitor the recovery of actin viscosity in the presence or absence of Caplin. In the absence of Caplin the apparent viscosity returned to original level in 1 hour. In the presence of 3.85μM Caplin the recovery of viscosity was inhibited by almost 40%. Also a third experiment was carried out in which actin was allowed to polymerise for 30 minutes in the absence of 3.85μm Caplin. The filaments were broken and 3.85μm Caplin was added. These filaments were then allowed to anneal in a capillary tube and after 1 hour of polymerization these filaments showed a reduction in the apparent viscosity of 34%.
Chapter 3

Actin + Caplin
Actin + Caplin after shear

FIGURE (20). Effect of Caplin on actin annealing. 10µM actin was polymerised for 30 minutes in the absence (blue and yellow) or the presence of 3.85µM Caplin. The actin filaments were sheared by pipetting and the apparent actin viscosity was recorded after 1 hour annealing of actin in the absence of Caplin (blue), actin in the presence of 3.85µM Caplin (pink) and actin with 3.85µM Caplin addition after shearing of filaments (yellow).

3.12.II - FLUORIMETRY

3.12.II.a - Nucleation

This experiment showed that the addition of 2.3µM Caplin to 5µM monomeric (G) actin had no detectable effect on the rate of the onset of actin polymerization measured by the incorporation of 0.7µM pyrene labelled G actin into actin filaments (Figure 21). Actin polymerization is preceded by a lag phase in the first 40 seconds of the reaction in which monomeric actin bind to form a nuclei. This lag phase was identical when actin was incubated with or without Caplin indicating that Caplin does not affect the formation of
nuclei. The rate of pyrene actin incorporation was increased during actin polymer formation which occurred from 40 seconds up to 100 seconds and the curve obtained at this interval was identical for both samples.

This result also showed a change in the slope of the curve after 100 seconds, the exponential phase of actin polymerization, indicating that when actin was polymerised in the presence of Caplin the rate of G actin incorporation to actin polymers was reduced by 30%.

![Graph showing nucleation](image)

**FIGURE (21).** Nucleation. \(5\mu\text{M G actin was polymerised by addition of buffer P and the incorporation of 0.7\mu\text{M pyrene labelled actin was measured by fluorimetry in the absence (blue) or presence of 2.3\mu\text{M Caplin (pink).**
3.12.11.b - Elongation

In contrast to the nucleation experiment (Figure 21), the addition of Caplin to pre-formed actin filaments had a consistent effect on the incorporation of pyrene labelled G actin. Figure (22) shows that the rate of G actin incorporation was reduced by 35% in the presence of 2.3 μM Caplin. No further reduction was found when increased amounts of Caplin were added.

![Figure 22](image)

**FIGURE (22). Elongation.** 5 μM actin filaments were pre-formed by addition of buffer P in the absence (blue) and presence (pink) of 2.3 μM Caplin. The incorporation of 0.7 μM pyrene labelled actin was recorded for 900 seconds.
3.12. II.c - Gelsolin assay

To investigate whether or not the reduction in the incorporation of pyrene labelled G-actin due to the presence of Caplin occurred at the barbed or pointed ends of the actin filaments, 5µM actin filaments were pre-formed in the presence of 0.13µM gelsolin, a well defined barbed end actin binding protein, in the absence or the presence of 2.3µM Caplin and the elongation of these pre-formed filaments were analysed by addition of 0.7 µM pyrene labelled actin. Figure (23) indicates that there is no significant difference on the rate of G-actin addition in the two samples suggesting that Caplin binds at the barbed end of actin filaments.

FIGURE (23). Gelsolin Assay. 5µM actin filaments pre-formed in the presence of 0.13µM gelsolin in the absence (blue) or presence (pink) of 2.3µM Caplin.
3.13 - Linear Sucrose Density Gradient

Linear sucrose density gradients showed that Caplin remained as a monomer in the presence or absence of the reducing agent 2-mercaptoethanol (Figure 24). Furthermore, the Caplin peaks were present at an identical position to Soy bean trypsin inhibitor, a monomeric protein of 21Kda. Purified monomeric actin and ovalbumin (both with a molecular mass of 43Kda) banded in identical fractions at lower points in these gradients.

![Graph showing linear sucrose density gradient](image)

FIGURE (24). Linear Sucrose density gradient
3.14 - Actin binding Sedimentation Assay

3.14.1 - CALIBRATION CURVE

A calibration curve was obtained with known amounts of Caplin ranging from 0.96μM, 1.9μM, 3.85μM, 5.7μM, 7.7μM, 11.5μM and 15.4μM (Figure 25). Because different proteins absorb different amounts of Coomassie blue stain, known amounts of Caplin on a 12% SDS-PAGE were stained and destained in fixed volumes for a fixed period of time (Figure 26). The gels were scanned in a densitometer to determine the absorbance. The absorbance readings were plotted against varying concentrations of Caplin and the relationship was linear.

FIGURE (25). Calibration curve.
3.14.11 – SDS - PAGE

Analysis of supernatants and pellets from experiments in which Caplin ranging in concentration from 0.96µM to 15.4µM with 10µM actin in polymerising conditions showed that the amount of Caplin bound to actin increased with concentration to apparent saturation at 1.9µM. Beyond this point higher Caplin concentrations did not result in increased binding to actin filaments.

FIGURE (26). SDS-PAGE analysis of total Caplin at (A) 0.96µM , (B) 1.9µM , (C) 3.85µM, (D) 5.7µM, (E) 7.7µM, (F) 11.5µM, (G) 15.4µM.
FIGURE (27). SDS-PAGE analysis of supernatants and pellets respectively of Caplin at (A1, A2) 0.96μM, (B1,B2) 1.9μM, (C1,C2) 3.85μM, (D1,D2) 5.7μM, (E1,E2) 7.7μM, (F1,F2) 11.5μM and (G1,G2) 15.4μM.
3.14.III - DETERMINATION OF CAPLIN BINDING CONSTANT

The densitometer readings of both bound and free Caplin were plotted and a theoretical curve obtained (Figure 28). This curve shows that the apparent binding saturation occurs at 1.9 μM Caplin. The apparent binding constant of $K_a = 4 \times 10^{-5} \text{ M}^{-1}$ was estimated as the inverse of the [free Caplin] at which the binding reaches half saturation.

**FIGURE (28).** Caplin Binding constant.
3.15 - Immunofluorescence

Immunofluorescence was carried out using anti-C4 monoclonal antibody on rat embryo fibroblasts. The results showed a uniform staining throughout the cell cytoplasm, on actin filament stress fiber bundles (Figure 29).

FIGURE (29). Immunofluorescence of Caplin and Transgelin localisation on rat embryo fibroblasts.
3.15.1 - DETERGENT SOLUBILITY OF CAPLIN

Protein Caplin was removed from rat embryo fibroblasts by detergent extraction using 0.5% CHAPS. Immunofluorescence using anti-C4 monoclonal antibody shows that all detectable Caplin had been removed from the cytoskeleton (Figure 30).

FIGURE (30). Detergent solubility of Caplin. Immunofluorescence of Caplin labelling is lost in rat embryo fibroblast when extracted with 0.5% CHAPS.
3.15.1 - CAPLIN REBINDING TO PERMEBILISED CELLS

Immunofluorescence shows that when Caplin was added to detergent extracted rat embryo fibroblast cells, from which endogenous Caplin had been removed, Caplin labelling was restored (Figure 31). In these cells rebound Caplin was found on actin stress fiber bundles, where it decorates the entire length of the stress fibers.

FIGURE (31). Caplin re Binding to actin bundles in permeabilised rat embryo fibroblast cells and labelled with anti-C4 antibody. Note that the label is present along actin bundles as shown in figure 29.
Caplin is the second member of a polypeptide doublet of 22. KDa, originally identified on a SDS-PAGE by a monoclonal antibody and named PC4L (Shapland et al., 1988; Shapland et al., 1993). It is found across a variety of species in different tissues and cell types. The upper isoform of this doublet, Transgelin, acts in vitro by bundling actin filaments (Shapland et al., 1988). The lower isoform, Caplin, is localised along actin filaments by light microscopy and it’s expression is unaffected by oncogenic transformation and changes in cell shape. The aim of this thesis was to purify and identify the in vitro function of protein Caplin in normal cells.

4.1 - Sequencing of mouse thymus Caplin cDNA

The cDNA sequence of human T cell lymphoma (HTCL) Caplin was obtained previously in this laboratory (Martin Smith PhD. Thesis). In order to investigate the in vitro function of protein Caplin in normal cells, mouse thymus lymphocytes were used in conjunction with oligonucleotides derived from HTCL cDNA sequence.

4.1.1 - OLIGONUCLEOTIDE SELECTION

Based on the cDNA sequence of HTCL, two oligonucleotides were generated and used as primers for PCR and sequencing reactions. MANR (34mer sense primer 5'GGAATTCTGGAATGGCCAACAGGGGACCTGCATA3') was specific for bases coding for the NH2-terminus of HTCL cDNA containing the start codon (ATG). QUILrev (33mer
antisense primer 5’GGATTCGGGATCAGAGGATCTGCGTGGCATC3’) was specific for the 3’ end of HTCL cDNA upstream of the TGA stop codon. Both contained additional EcoRI restriction sites [underlined] (Eichinger and Schleicher, 1992). The choice of nucleotides used to generate the synthetic oligonucleotide primers took into consideration the homology at the 5’ and 3’ end of HTCL, Caplin cDNA and also the presence of an EcoRI restriction endonuclease site for cloning purposes upstream MANR and QUILrev into pUC18 (for sequencing) and pGEX (for purification) (Eichinger and Schleicher, 1992).

4.1.11 - ANALYSIS OF MOUSE THYMUS CAPLIN cDNA SEQUENCE

Double stranded mouse thymus Caplin cDNA was sequenced using MANR and QUILrev primers and found to be of 600 basepairs long. Later the nucleotide sequence of fusion protein Caplin was confirmed by automated sequencing using pGEX-4T-3 primers at the Department of Molecular Medicine, King’s College School of medicine and Dentistry.

The complete Caplin cDNA sequence consists of a start codon (1-3bp) followed by a 594bp opening reading frame (ORF) followed by a stop codon at 597-600bp.

4.1.11.a - Molecular weight and estimated PI

The 597bp ORF of Caplin encodes a 199 amino acids polypeptide with a calculated molecular weight of 22,391.4Da. This calculated molecular weight was found to be slightly higher than the one determined by SDS-PAGE in which Caplin is seen by coomassie blue staining at approximatelly 21KDa (Shapland et al., 1988). This discrepancy between the calculated and electrophoretically estimated molecular masses is likely to be due to incomplete denaturation or inconsistent association of SDS with the protein which causes the
protein to run slightly slower than the completely unfolded or detergent solubilized molecule rather than the presence of a negatively charged amino acid cluster such as in the case of demantin (Rana et al., 1993). Still, the molecular weight of mouse thymus Caplin remains ~0.5KDa less than the higher polypeptide doublet transgelin (Shapland et al., 1988).

Caplin has a calculated pI value of 8.41. This is a basic pI which is also found in other actin binding proteins such as profilin I [pI 8.4] (Gieselmann et al., 1995), demantin [pI 9.54] (Rana et al) and calponin h1 [pI 9.91] (Takahashi et al., 1991).

4.1.II.b - Amino acid composition and charge distribution

Mouse thymus Caplin has 72 hydrophobic amino acids, 95 hydrophilic amino acids and 3 cysteines, 20 glycines and 9 prolines. Analysis of the coding sequence indicated a hydrophilic protein consisting of 44 charged residues; 23 positively charged and 21 negatively charged hydrophilic amino acids confering the peptide an overall net positive charge. The polarity of the amino acid side chain is one of the forces responsible for shaping the final three dimensional structure of the proteins. Polar amino acids tend to be found on the surface of proteins whereas nonpolar amino acids avoid water by aggregating to form the water insoluble core of proteins (Chothia, 1984).

The activity of many proteins are modulated by PH environment. The cell cytoplasm has a PH ~ 7.0. Protein Caplin has three cysteine amino acids (38,63,124). Regions within a polypeptide or in separate polypeptides are, sometimes, cross-linked covalently through disulfide bonds. However, disulfide bonds are rare in intracellular proteins, due to the reducing environment of cell cytoplasm, indicating that is very unlikely that the presence of the three cysteines may affect the native folded structure of Caplin (Chothia, 1984). To investigate whether free unfolded cysteines could be used in the formation of dimers or
trimers, sucrose gradients were run in the presence and absence of DTT and showed that Caplin remains as a monomer (see section 4.4.III).

Proline and glycine are sometimes found at points on a protein surface where the polypeptide chain loops back into the protein (Anfinsen, 1973) indicating that protein Caplin should adapt such a conformation given the high number of these amino acids within Caplin polypeptide chain. Secondary structure prediction suggest that this is the case (see section 4.I.II.c).

4.I.II.c - Secondary structure prediction

Alpha helices and beta sheets are the major internal supportive elements in proteins. The secondary structure occurs when stabilizing hydrogen bonds occur between certain residues and the polypeptide backbone folds into either a spiral geometric arrangement such as α helix or an extended polypeptide geometric arrangement, the β strand. Lateral associations of strands produce β sheets.

The use of a prediction program, based on the amino acid content of protein Caplin, indicates that the secondary structure of protein Caplin consist of a start turn composed of glycine and proline followed by a α helix (10-34), β strand (35-40), β sheet (55-68), α helix (74-102), β sheet (104-116), β strand (123-130), β sheet (170-178) and ends with a β strand.

The long α helix domain is a feature found in many actin binding proteins. For example, Ezrin and band 4.1 are soluble proteins that contain a long α helix at their carboxyl terminal end involved in cytoskeleton binding (Algrain et al., 1993). Profilin has a α helix followed by a β sheet in the core of the molecule which participates in the binding of actin (Machesky & Pollard, 1993). Smooth muscle calponin h1 also has a similar secondary structure where α
helical regions are interspersed with β sheet and β strand structures (Takahashi and Nadal-Ginard, 1991).

This result provides an idea as to the α helix and β sheets content of protein Caplin. Future studies using X-ray crystallography would be required in order to access the functional significance and validity of this secondary structure.

It is well known that the presence of same motif in different proteins with similar functions clearly indicates that evolution reuses certain combinations of secondary structures rather than devising new ones. Many proteins interact with actin. Functional diversity comes from variation in primary and secondary structures outside homologous region domains in different proteins (Stradal, 1998). Transgelin and Caplin are examples of isoforms that share high homology at structural level but differ in the way they regulate actin polymerization (Shapland et al., 1993).

4.1. II.d - Putative Nuclear localization signal

Transport between the nucleus and cytoplasm involves both stationary components and mobile factors acting in concert to move macromolecules through the nuclear pore complex (NPC). The transport of proteins and RNAs >20-60 KDa through the NPC is an active, bidirectional, energy-requiring process (Kolher et al., 1999). The best-characterized protein import pathway is the nuclear localization signal-dependent pathway. The nuclear localization signal (NLS) is a region found in many proteins characterised by the presence of a cluster of basic amino acids (RKKR). The process of actively transporting nuclear proteins through nuclear pores via NLS can be divided in two steps: (a) targeting to the pores and (b) translocation through the pores (Yoneda, 1997). Targeting involves the formation of a heterodimeric complex consisting of nuclear protein with NLS site and importin α (or α-
karyopherin) and β (or β-karyopherin). Importin-α specifically binds to NLS while importin-β recognizes the importin-α subunit accounting for the interaction with the nuclear pore complex (NPC) (Xiao et al., 1997). Translocation into the nucleus is terminated by the disassembly of the importin α/β complex and both subunits return to the cytoplasm (Gorlich et al., 1997). This process is mediated by the small GTPase Ran. Although the molecular mechanisms underlying nuclear import is unclear, it appears that Ran’s GTP cycle drives translocation into the nucleus via the binding of cytoplasmic RanGDP to the NPC followed by nucleotide exchange and GTP hydrolysis. The binding of RanGTP to importin-β disassembles the importin α/β complex at the nuclear side of the NPC, thereby terminating translocation (Gorlich et al., 1997). Thus, low RanGTP levels in the cytoplasm allow importin-α to bind to importin-β, the high RanGTP concentration in the nuclear compartment dissociates the importin α/β complex. Recently an entire class of potential Ran targets have been identified which compete with RanGTP binding site of importin-β, therefore bringing about another level of nuclear import regulation (Gorlich et al., 1997).

Protein Caplin has a putative nuclear localisation site (152-158) rich in lysine residues. The high amino acid homology of this site with the NLS consensus sequence raises the possibility that Caplin may provide means for altering nuclear structures in cell, since caplin is seen, at fluorescence level, to be present in the cell nucleus.

Supervillin, an F-actin binding protein, contains a functional NLS and has been implicated in signal transduction to the cell nucleus. Overexpression of chimeras containing the NLS sequence results in the formation of large nuclear bundles of F-actin suggesting that supervillin may contribute to the cytoarchitecture in the nucleus (Wulfkuhle et al., 1999). Other actin binding proteins which localize to the nucleus have been observed. Protein Mbh1 (Cap G) sequence has a NLS (134-147) rich in argenine and lysine residues which may play a
role in nuclear architecture through potential interactions with actin (Prendergast and Ziff, 1991a). In nonerythroid nucleated cells, an isoform of protein 4.1 named 4.1H was found in the nucleus. This isoform has a cluster of basic amino acids (KKKR) generated by alternative splicing of exon 13 and 16 sequences. Substitution of the KKKR with uncharged amino acids completely abrogated the nuclear targeting indicating that the presence of this basic amino acid cluster are necessary for nuclear import of 4.1H. This finding was the first demonstration of differential nuclear targeting by the inclusion of an alternative domain among naturally occurring protein 4.1 isoforms (Luque et al., 1998).

While the NLS is absolutely necessary for nuclear import, this mechanism may be regulated by phosphorylation. Nuclear import of simian virus SV40 large tumor antigen is regulated by phosphorylation of CcN motif, which comprises phosphorylation sites for casein kinase II (CKII) and cyclin-dependent kinase 2 (Cdc2) together with the NLS (Xiao et al., 1997). Phosphorylation of the CKII site accelerates the rate of nuclear import, whereas phosphorylation at the Cdc2 site reduces nuclear transport. The mechanisms underlying this regulatory pathway is not fully understood, but it appears that in vitro, phosphorylation of CKII site increases the affinity of the NLS with importin α/β while indirect evidence suggests that Cdc2 site phosphorylation may increase the affinity of SV40 T antigen for a cytoplasmic anchor protein (Xiao et al., 1997). Although sequence alignment indicates high homology between Caplin and SV40 T antigen NLS sites, it is very unlikely that Caplin nuclear transport could be mediated by phosphorylation as there are no CKII or Cdc2 sites within caplin cDNA sequence.
4.1.II.e - Potential EF-hand sites

Many calcium binding proteins have regions of homology and contain from two to eight copies of a calcium binding domain known as EF-hand (Moncrief et al., 1990). This functional and evolutionarily conserved domain consists of 29 amino acids arranged in a characteristic helix-loop-helix motif responsible for the binding of calcium ions. The loop domain consists of 12 amino acids, of which 7 residues are critical for calcium ion binding (Moncrief et al., 1990). The 7 critical residues have a carboxyl group which are precisely spaced so to coordinate the binding of the calcium ion (Heizmann and Hunziker, 1991).

Analysis of Caplin homologous peptide sequence indicates the presence of two possible EF-hand structures, one near the N-terminus (23-34) and another at C-terminus (108-118), both containing aspartic acid residues which generate a loop. Although the loop region shared some sequence homology with EF hand sequence, the predicted Caplin secondary structure of this region has a helix-loop conformation only, suggesting that Caplin function does not require calcium. Indeed, by falling ball viscometry, it was shown that Caplin does not require calcium in order to reduce actin viscosity (see section 3.12.I.c). This result is also in agreement with the known fact that some EF-hands do not have the ability to bind calcium; they are easily recognized as homologous but lack the characteristic secondary structure due to folding (Moncrief et al., 1990). This result is further verified with the fact that Transgelin, which has similar EF-hand sequences, does not require calcium in order to cross link actin filaments (Shapland et al., 1993).

The EF-hand-containing family of proteins are thought to have evolved from a single ancestral EF-hand gene which during evolution duplicated generating proteins with distinct structural and functional features. Through mutations in the EF-hand loop, some domains have lost the ability to bind calcium. (Heizmann and Hunziker, 1991) This may be the case for Caplin as sequence alignment of the critical EF-hand consensus sequence shows that at
site I (N-terminus) caplin has only two out of the 7 critical residues whereas at site II (C-terminus) only 3 amino acids have been conserved.

4.1.11.7 - Potential phosphorylation site

Data base analysis of Caplin cDNA sequence indicates a possible protein kinase C (PKC) phosphorylation site at position 180-182. This site is composed of a threonine, asparagine and an arginine amino acids.

Protein kinase C is a 77KDa enzyme that phosphorylates serine, and threonine residues in a calcium regulated manner. At physiological calcium concentration level (0.1 μM), PKC is inactive and found in the cytosol (Perez-Moreno et al., 1998). Upon an increase in calcium concentration, PKC becomes active and is located at the vicinity of the cell membrane. Vinculin and α-actinin are actin binding proteins found at adherens junction, whose function is modulated by PKC (Bakolitsa et al., 1999). Vinculin and α-actinin are substrates of PKC phosphorylation which is known to play a central role in the assembly of the epithelial junctional complex \textit{in vivo} and \textit{in vitro} (Perez-Moreno et al., 1999). \textit{In vitro}, addition of calcium to monolayer cultures of epithelial cells increases phosphorylation of serine and threonine residues of vinculin and α-actinin by PKC causing the accumulation of both proteins at cell-cell border (Perez-Moreno et al., 1998). The subcellular redistribution of both proteins was further found to be sensitive to PKC inhibitors, indicating that PKC regulates the assembly of epithelial junctional complex acting through the cytoskeleton via actin binding proteins (Perez-Moreno et al., 1999).

Analysis of the Caplin sequence motif combined with further evidence obtained from \textit{in vitro} functional studies indicates that it is unlikely that the function of Caplin is regulated by PKC phosphorylation. Caplin possible homologous site contains only one amino acid
(threonine) that is phosphorylated by PKC, whereas in the case of vinculin and \( \alpha \)-actinin, two amino acids (serine and threonine) are phosphorylated. In addition, PKC activity is regulated by calcium concentration and \textit{in vitro} studies showed that calcium has no effect on the binding of Caplin to F-actin. Falling ball viscometry (see 4.4.1.c) has shown that calcium has no effect on the ability of Caplin to reduce the viscosity of actin filaments.

Furthermore, the localization and expression of Caplin along actin filaments throughout the cytoplasm, by light microscopy, is unaffected by oncogenic transformation or changes in cell shape (Martin Smith PhD. Thesis). It may be reasonable to infer that upon cellular stimuli, Caplin distribution within cells remain the same, indicating that Caplin distribution and function does not require a regulatory mechanism for activation and translocation of this protein to different subcellular compartments, as seen in the case of vinculin distribution and its role at the assembly of adherens junction in cultured epithelial cells (Perez-Moreno et al., 1998).

4.1.II.g - \textit{Calponin subdomain homology}

Sequence analysis revealed a partial sequence identity (58\%) between the calponin (CaP) family of F-actin binding proteins and the Transgelin family of F-actin proteins.

The calponin family contain three genetic variants, a smooth muscle basic variant (Cap h1), a neutral variant (h2) and an acidic variant that is particularly enriched in the brain. All variants share a high percentage homology identity along the length of the molecule (70\%) (Gimona and Mital, 1998; Stradal et al., 1998). The CaP sequence is divided into three subdomains based on the data available for the Cap h1 variant: an amino-terminus calponin homology (CH) domain (1-134); an actin binding site (ABS) (142-163) and three 29-residue tandem repeats (164-275) and the carboxyl-terminus (276-305). When Caplin amino acid
sequence was aligned against Cap h1 amino acid sequence, the same subdomain structure was found. Caplin has a CH-domain at the amino-terminal end (1-134); an ABS domain (152-173) followed by one calponin like repeat (174-198) at the carboxyl-terminal.

Caplin CH-domain has 43% sequence identity with Cap h1 CH-domain. The CH-domain is a sequence motif of about 100 amino acids, termed the calponin homology domain, which has been suggested to confer actin binding to a variety of cytoskeletal and signalling molecules (Stradal et al., 1998). The involvement of the calponin CH-domain in actin binding was investigated by transfection of recombinant Cap h1 and Cap h2 constructs into cultured fibroblasts and assayed for their association with F actin by co-sedimentation (Gimona and Mital, 1998). The authors concluded that the single CH-domain does not confer actin binding per se, as recombinant mutants of both Cap h1 and Cap h2 containing only the CH-domain sequence (h1/1-127 and h2/1-127 respectively) failed to co-sediment with F actin. Also, there was no decoration of actin stress fibers when these constructs were transfected to fibroblast cells. It is, therefore, reasonable to conclude that it is very unlikely that Caplin CH-domain solely, which has only 43% identity to CaP h1 CH-domain, is responsible for binding to F actin.

Caplin has an ABS subdomain at 152-173 residues 40% homologous to CaP h1 ABS (142-163) and 40% homologous to CaP h2 ABS. Mutated Cap h1 constructs lacking the CH-domain (h1/131-297) and a construct containing only the three calponin-like tandem repeats (h1/164-275) was shown to be associated with stress fibers. However, sedimentation assays were not performed (Gimona and Mital, 1998). Full length Cap h2 (h2/1-305), which has an inactive ABS sequence (lacks a Lys156-identified as the residue most crucial for actin binding), was found to co-sediment with F actin and to be associated with stress fibers. CaP h2 ABS has a cluster of several non-conservative amino acid exchanges affecting the charge of residues. CaP h2 construct lacking the calponin-like repeats (R domain) (h2/1-161) failed
to both co-sediment and decorate stress fibers whereas a construct lacking both the ABS
domain and the entire carboxyl-terminus (h2/1-127) failed to decorate stress fibers. Although
co-sedimentation assay was not determined for h2/1-127 construct, these findings
demonstrated that CaP h2 ABS domain does not confer \emph{per se} actin binding either \emph{in vitro}
(co-sedimentation) or in cultured cells.

Although the amino acid identity of Caplin ABS varies along the length of both CaP h1
and CaP h2 ABS subdomains, the overall homology remains at 40\% for both. Sequence
alignment of Caplin ABS and CaP h1 and CaP h2 ABSs showed, however, that homologous
amino acids are located at different positions within CaP h1 and CaP h2. When compared to
CaP h1, Caplin has a cluster of homologous amino acids at the carboxyl-terminal of ABS
domain (169-173) whereas no such cluster is found when Caplin ABS domain is compared to
the ABS domain of CaP h2. Also, CaP h1 has a lysine residue at position 156 of the CaP h1
ABS domain which has been defined as the residue most crucial for actin binding (Gimona
and Mital, 1998). CaP h2 did not conserved this amino acid within it’s ABS amino acid
sequence where instead, a threonine residue is found. Caplin has an equivalent glutamine at
position 166 and since a lysine has been reported to be essential to actin binding it is
reasonable to conclude that Caplin ABS domain is functionally similar to CaP h2 ABS
domain and, therefore, Caplin does not use this domain for the binding to actin filaments.

Caplin has a calponin-like repeat subdomain (R domain) of 24 residues which extends
from 174 to the end of the molecule (199). This region was found to be highly homologous
(75\%) to the first calponin-like repeat (R domain) extending from 164 to 188 of calponin
molecule. CaP h1 constructs lacking the carboxyl-terminal up to the middle of the second
repeat (h1/1-228) still decorates stress fibers and co-sediments with actin, whereas a CaP h2
construct lacking the entire R domain (h2/1-163) and a construct containing only the first 7
amino acids of the first repeat (h2/1-186) fail both to bind to stress fibers and to co-
polymerise with actin. CaP h1 and CaP h2 constructs containing only the three tandem repeats (h1/164-275 and h2/164-275 respectively) were found to decorate stress fibers, and although co-sedimentation assay was not performed in both, it indicates that the carboxyl-terminal tandem repeats of calponin R domain harbour an independent actin binding motif (Gimona and Mital, 1998).

Taking into consideration (a) the high sequence homology of 75% between Caplin and CaP R domains; (b) the structural similarity of ABS domains of Caplin and CaP h2; and (c) the ability of Calponin tandem repeats to decorate actin stress fibers in fibroblasts, it is suggestive that Caplin may use the 24 amino acids residues at Caplin R subdomain as an actin binding domain to bind to filamentous actin. Furthermore, it is known that Transgelin interacts with actin filaments via a cluster of positively charged amino acids at the carboxyl terminal of it's cDNA sequence (Shapland et al., 1993).

Unfortunately these CaP constructs experiments failed to have constructs with the entire first calponin-like repeat (first R domain only). It would be very interesting to investigate the functional significance of Caplin R domain using constructs containing only the R domain (174-198) for the binding of Caplin to actin filaments.

The presence of similar domains within families of actin binding proteins is a feature of evolutionary significance. The maintenance of some critical domains (such as CH domain) allows classification of members within the same family, whereas diversification is the result of evolutionary fitness to adapt to specific functions carried in cells that have evolved higher functions (such as signalling domains). Nature employs a highly economical strategy in which a relatively small number of fundamental modules perform specific catalytic and recognition functions, and are then modified, shuffled and combined to form a spectrum of proteins able to carry out the broadest possible array of biological functions. The ABPs are an example of such regulatory proteins (Puius et al., 1998). This result indicates that Caplin
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is one of this regulatory proteins that evolved to interact with F-actin as a result of modular design variation.

4.2 - Database sequence homologies

Prosite and BLAST software programs were used to search available databases. Significant homologies were found between Caplin amino acid sequence throughout the sequences of NP25, Transgelin, Calponin family, sm20, and UNC87. Partial homology was found at the amino terminal end of VAV proto-oncogene, Ras GTPase, Fimbrin 2.

Further analysis based on Caplin subdomain structure revealed specific homologies at each domain. Most of these proteins contain a CH domain. The CH-domain has been suggested to confer actin binding to a variety of cytoskeletal and signalling molecules (Stradal et al., 1998). Proteins can be divided into phylogenetic groups according to the number of CH-domains present within their sequence (Stradal et al., 1998). Transgelin, calponin and VAV proteins have a single CH-domain whereas fimbrin has two.

4.2.1 - TRANSGELIN

Analysis of transgelin amino acid content based on the modular subdomain structure, indicates a 63% homology at CH-domain, 60% at the ABS domain and 80% at R domain. This high homology throughout the amino acid sequence is not surprising since protein Caplin is the lower molecular weight of this peptide doublet, originally identified by monoclonal antibody, which constitutes the transgelin multifamily of actin proteins (Prinjha et al., 1994; Shapland et al., 1993; Shapland et al., 1988). The highest domain homology found
between Caplin and transgelin is at the carboxyl-terminal R-domain (80%) indicating that this might be the site transgelin uses to cross link actin filaments, as transgelin lacks the lysine residue (it has a glutamine) known to be responsible for the binding of actin to the ABS domain. Indeed, it is known that a small cluster of positive residues (154-161) at the carboxyl end of transgelin molecule is responsible for the binding to actin (Prinjha et al., 1994). This result further emphasizes the modular domain redundancy among actin binding proteins. Nature utilizes modular redundancy in a way that instead of shuffling pre-existing domains, with well defined functions, it has evolved variants to carry out specific catalytic and recognition functions to best fit the vast different biological functions of the cell.

4.2.11 - NEURONAL PROTEIN 25

NP25 cDNA sequence is 71% homologous to Caplin amino terminal CH-domain, 47% homologous to ABS domain and 90% homologous to Caplin carboxyl terminal R domain. Little is known about the function of this protein. Immunocytochemistry and in situ hybridization demonstrated that NP25 is a protein expressed solely in the central nervous system, localized in the central amygdaloid nuclei and in the granule layer of cerebellum (Ren et al., 1994). The remarkable degree of amino acid homology and structural similarity between NP25 and Caplin infer that they may represent a family of proteins. Indeed, as already discussed previously, the presence of the CH domain is a feature of evolutionary significance (Stradal et al., 1998).

The amino acid identity of the ABS domain varies, and like Caplin, NP25 has a glutamine rather than a lysine residue at position 166, the amino acid known to be essential for the binding to actin filaments via the ABS domain (Gimona and Mital, 1998).
The high homology found at the carboxyl terminal R domain may represent an essential structural or functional sequence of this family of proteins. Although the direct binding of NP25 to actin filaments has not been established yet, the cytoplasmic distribution of this protein coupled to the high homology to Caplin may indicate that NP25 binds to actin cytoskeleton and may play a particular important role in the function of specific neuronal systems. This observation would further support the possibility that Caplin uses the carboxyl terminal R domain for the binding to actin filaments.

4.2.III - VAV FAMILY

The VAV family of proteins showed an overall ~50% sequence homology to Caplin sequence. Analysis based on subdomain structure shows homology between residues 56-143 of Caplin and residues 37-126 of VAV corresponding to the CH domain. A variety of signal transducing molecules such as IQGAP and VAV, contain a CH-domain (Stradal et al., 1998) However, single CH domain containing proteins fail to bind to actin, suggesting a subordinate role for this motif in actin binding (Kranewitter and Gimona, 1999; Gimona and Mital, 1998) Indeed, mutant CH-deleted Vav and full length Vav constructs localize throughout the cytoplasm but fail to colocalize with F-actin demonstrating that the CH domain is not a critical determinant for the cellular localization of Vav (Kranewitter and Gimona, 1999).

VAV is a guanine nucleotide exchange factor for Rho family GTPases (Kranewitter and Gimona, 1999). The specific mechanism by which Vav activates Rho GTPases is not well defined, but Vav's transforming activity is achieved by regulating the nucleotide-bound state of Rho-GTP and Rho-GDP, thereby modulating GTPase activity. Although GTPases themselves can not modulate the conformation of actin filaments, they cause rearrangement of the actin cytoskeleton by mobilizing actin binding proteins to specialized sites within the cell.
leading to the formation of lamellipodia, stress fibers and filopodia (Kranewitter and Gimona, 1999). Deletion of the CH domain of Vav enhances the formation of Rho-dependent stress fibers in NIH 3T3 cells suggesting a regulatory role for the CH domain in Vav and an actin cross-linking or bundling protein as a downstream effector molecule of Vav-mediated pathways (Kranewitter and Gimona, 1999). Taking these finding into consideration it might be possible that Vav is an upstream effector molecule within the signalling pathway of small GTPases involved in actin cytoskeleton rearrangement and caplin is one the downstream effector molecules, responsible for capping the barbed ends of actin filaments upon extracellular stimuli. Nonetheless, it is also possible that the homology found between Vav and Caplin domains is the result of evolutionary pathway due to modular redundancy rather than functional significance.

4.2.1 V - SM20

Smooth muscle protein 20 (sm20) is a *Drosophila* calcium regulated F-actin binding protein found to have an overall 56% homology to Caplin amino acid sequence. When compared to Caplin at the level of subdomain structure it showed a 39% homology at CH-domain, 43% at the ABS domain and 65% at the R domain. Further analysis showed that at the ABS domain, protein sm20 has a glutamine at position 169 instead of a lysine residue, which is the amino acid shown to be required for the binding to actin. Given the low amino acid homology at CH domain and that the CH domain *per se* is neither sufficient nor necessary to mediate actin binding (Gimona and Mital, 1998), it is very unlikely that sm20 binds to actin filaments via it's amino terminal end. The highest amino acid identity between Caplin and sm20 lies within the R domain, indicating that the carboxyl terminal of sm20 may be involved in the binding to actin filaments. If this is the case, this result further supports the
hypothesis that the R domain of Caplin may be responsible for the binding of this protein to actin filaments.

**4.2.V - UNC-87**

The Unc87 gene has been identified in *C. elegans* and has been shown to be associated with impaired motility and abnormal muscle organization (Goetinck and Waterston, 1994). Electron microscopy of this mutant bodywall muscle shows patches of thick and thin filaments instead of normal interdigitation of the two sets of filaments. The UNC-87 gene product protein was sequenced and analysis shows that it contains seven copies of a 23 residues tandem repeats (240-424) at the carboxy-terminal (Goetinck and Waterston, 1994). When UNC-87 amino acid sequence was compared to Caplin based on the subdomain structure, it showed a 39% homology at the CH-domain, 26% homology at the ABS domain and 83% at R-domain. Further analysis of Caplin R domain (174-196) showed that 19 out of the 23 residues of UNC-87 (240-263) are identical. Immunohistochemistry indicates that UNC-87 protein is localized to the I-band of *C. elegans* bodywall muscle. The I-band is the region of sarcomere containing actin thin filaments where they do not overlap with thick filaments (Goetinck and Waterston, 1994), indicating that UNC-87 binds directly and preferably to actin filaments. The high homology between both proteins R-domains and the low homology at both CH and ABS domains in UNC-87, suggest that Caplin may bind to actin filaments via it’s R-domain. This finding is also in agreement with the high homology found between Caplin and calponin (Cap h2/166-275) R-domains as discussed before.
4.2.VI - FIMBRIN

Fimbrin is a calcium sensitive cross-linking protein found in many species. It can be classified into two subgroups of two CH-containing proteins, according to the position of this domain at the amino-terminus or middle of the protein sequence (Stradal et al., 1998). Fimbrin contains two actin binding sites which consist of two tandemly repeated CH domains and bundling of actin filaments involve the interaction of two CH domains (Hanein et al., 1998). Despite the overall structural conservation of unique CH-domain fold, the first and the second CH-domains are disparate, representing a unique protein module (Stradal et al., 1998). Sequence analysis show sequence homology between Caplin (9-120 residues), and fimbrin (101-216 residues) of 35%. At modular structural level these amino acids correspond to the CH domain of Caplin and the first tandemly repeated CH domain of the first actin binding domain of fimbrin.

The fact that bundling of actin filaments by fimbrin requires the interaction of the two tandemly repeated CH domains (Hanein et al., 1998) and low homology with Caplin further support the view that the binding of Caplin to actin filaments is very unlikely to involve the amino terminal of this molecule. It is still possible, however, that the presence of CH domain is due to evolutionary sequence divergence.

4.2.VII - RAS GTPASES

Ras GTPase protein has an overall 47% sequence homology. Analysis based on Caplin subdomain structure shows a 42% identity between residues 15-122 of Caplin and residues 36-145 of Ras indicating that homology occurs between the CH-domains of Caplin and at the amino terminal end of Ras. There are four Ras proteins (H-Ras, N-Ras, K-Ras4A and K-
Ras4B), which are functionally distinct from one another. The Ras family of small GTPases are regulators of the cell signalling pathway (Reuther and Der, 2000). Functional diversity is now known to be a function of the carboxyl end of these proteins, which is involved in the association with the plasma membrane (Reuther and Der, 2000). The amino terminal end is highly homologous and interacts with effector proteins in the cytosol.

A novel Rac and Cdc42-binding protein has been identified, which contains a CH domain and binds directly to and cross-links actin filaments called IOGAP1 (Bashour et al., 1997; Fukata et al., 1997). Like Ras, Rac and Cdc42 are small GTPases known to stimulate formation of microfilament-rich lamellipodia and filopodia, however, the mechanisms underlying this process are still obscure (Bashour et al., 1997). Taking that Caplin regulates the dynamics actin filaments, it is therefore, possible that Ras regulates cell signalling by directly linking signal transduction molecules to the actin cytoskeleton via an association with F-actin stimulating the formation of such cytoskeletal structures allowing for a rapid response of the cell cytoskeleton. Nonetheless, it is still possible that the presence of CH module is the result of evolutionary divergence, rather than functional significance.

4.3 - Cloning of fusion protein Caplin

The optimal supply for purifying Caplin is lymphocytes since it is the only PC4 isoform found in these cells. However, the maximum yield of this protein obtained from ~10^9 HTL cells was found to be ~2µg. This meant that I had to generate a fusion protein to obtain purified Caplin protein at amounts necessary to carry out the functional assays in this thesis.

To allow for reversible insertion into a bacterial cloning vehicle, the double stranded blunt-end mouse thymus cDNA was given cohesive ends by EcoRI digestion. Primers
MANR and QUILrev contained the recognition site for EcoRI which is not present within protein Caplin cDNA and occurs once in the bacterial plasmid pGEX-4T-3 MCS at position 938-943.

The cDNA of mouse thymus Caplin protein was subcloned into the prokaryotic expression vector pGEX-4T-3 using unique EcoRI and BamHI sites. The EcoRI site was generated for cloning purposes and the BamHI site was chosen in order to check for correct orientation of the insert as Caplin cDNA has only one internal site at 438-443.

This vector was chosen because it allowed frame 3 ligation of Caplin cDNA into an EcoRI site. Upon thrombin digestion this construct encoded a recombinant fusion protein with a fusion peptide of 4 extra amino acids (R-N-S-G) at the amino-terminal of Caplin cDNA sequence.

### 4.3 - Expression and purification of fusion protein Caplin

Recombinant fusion protein was generated by appropriate modifications of Smith & Johnson original protocol (Smith and Johnson, 1988).

Bacterial expression was achieved by growth to OD600=0.6, inducing bacterial expression using 0.2mM IPTG for 3 hours. Attempts to use higher concentrations of IPTG for longer periods of time (in order to increase Caplin expression) resulted in increased protein degradation verified by SDS-PAGE analysis. The cells were lysed in the presence of 2%CHAPS and lysozyme and fusion protein (FP) Caplin was shown to be insoluble by SDS-PAGE analysis. The formation of insoluble protein in the bacterial cells is one of the several difficulties associated with the synthesis and purification of FPs, FPs may be rendered insolubility even when the native state protein is soluble (Smith and Johnson, 1988; Olsen and
FPCaplin was solubilised in 6M urea buffer at 4°C for 40 minutes to minimise exposure to this strong denaturant. Urea was dialysed out and to ensure complete removal, two changes of dialysing buffer took place. FPCaplin was absorbed to GST column and a series of washes was carried out to ensure complete removal of unwanted material. The last wash, however, was carried out using wash buffer without AEBSF: AEBSF inactivates serine proteases reversibly and it's presence affected thrombin activity. Fusion protein was cleaved while still attached to GST beads to reduce protein degradation, and the thrombin concentration used was enough to produce 1mg/ml yield and to avoid cleavage within the recombinant protein itself. Although Caplin does not have an internal thrombin recognition sequence, experiments have been reported in which in order to increase thrombin efficiency higher concentrations of thrombin were used which led to a broadening specificity of thrombin resulting in FP degradation (Olsen and Mohapatra, 1992). Heparin affinity chromatography and addition of AEBSF to final buffer ensured the complete removal of thrombin from cleaved Caplin sample. Purified Caplin was verified free of contamination and degradation by SDS-PAGE and western blot staining.

4.4 - In vitro functional assays

4.4.1 - FALLING BALL VISCOMETRY

Falling ball viscometry (FBV) was performed to test the effect of Caplin on the apparent viscosity of actin filaments. This assay was designed on the principle that actin filaments have a higher viscosity than actin monomers because filaments are large and asymmetric. Furthermore, longer filaments have higher viscosity than shorter filaments (Cooper and
All experiments in this thesis were carried out using column purified actin to avoid any contaminants which was frozen in liquid nitrogen as the G form, stored in aliquots at -85°C and spun in a microfuge before using in all functional assays.

4.4.1.a - Effect of Caplin concentration on actin filament viscosity

Falling ball viscometry showed that the final steady state apparent viscosity of actin filaments in the presence of 3.85μM Caplin was reduced to 25% compared to the actin control apparent viscosity. Increasing concentrations of Caplin did not enhance the reduction effect, indicating that at 3.85μM Caplin reached saturation.

Also analysis of the concentration curve obtained over a period of two hours showed a higher shear rate for actin in the presence of 3.85μM Caplin whereas, the shear rate of purified actin control was lower indicating that in the presence of Caplin the viscosity of actin filaments is reduced, as the shear rate varies inversely proportional with viscosity (Pollard, 1982). Because the reduction in the apparent viscosity is observed in polymerized filaments it is possible that the reduced viscosity effect may be due to the presence of shorter filaments.

A similar curve is observed in the apparent viscosity of actin filaments in the presence of actophorin (Maciver et al., 1991) and radixin (Tsukita and Hieda, 1989) where the final steady state viscosity is reduced.

4.4.1.b - Time course of Caplin

This experiment was designed to investigate the time course of Caplin during actin polymerization. The polymerization of actin monomers into filaments is divided into four steps and is characterized by a sigmoidal curve (Cooper and Pollard, 1982). The exponential curve is preceded by a lag phase corresponding to monomer activation and nucleation.
(≈2mins), followed by an exponential phase corresponding to the elongation of actin filaments (≈10mins) leading to a plateau when actin polymerization reaches a steady state. Although the time frequency of actin polymerization varies according to the level of actin purification, upon addition of buffer P actin monomers are activated and nucleated (≈2mins) into oligomers which are elongated (10mins) till a steady state is reached (Cooper and Pollard, 1982).

Upon buffer addition, the curve obtained shows that the initial effect of reduction of the apparent viscosity occurred at 5 minutes after actin polymerization was initiated. After 10 minutes and 15 minutes the reduction in the apparent viscosity was 12% and 15% respectively. These results indicate that in the presence of Caplin, the reduction effect on the apparent viscosity of actin filaments, caused by the increase rate at which the ball falls through the capillary tube, is observed during the elongation of actin polymers as the reduction in the apparent viscosity is seen 5 minutes after polymerization is initiated and increases with time.

A similar curve is seen during the polymerization of actin filaments in the presence of radixin (Tsukita and Hieda, 1989) where after ≈4 minutes the shear rate of actin polymerization in the absence of radixin is lower (therefore, higher viscosity) than in the presence of radixin and this effect is observed with time.

Based on the similarity between the curves obtained by both Caplin and radixin, it is indicative that the reduction on the apparent viscosity of actin filaments by Caplin may be due to the binding of Caplin to the ends of actin filaments. Binding to the ends of filaments would block elongation, therefore generating shorter filaments, hence increasing the shear rate at which the ball falls through the capillary tube leading to the reduction in the apparent viscosity of actin filaments.
4.4.1.c - Effect of calcium on Caplin-actin interaction

The effect of calcium on the ability of Caplin to reduce the apparent viscosity of actin filaments was investigated by FBV. This experiment also contained controls of both actin polymerized with and without EGTA in the presence or absence of Caplin. The results obtained showed that actin alone polymerized with or without EGTA showed the same apparent viscosity in both samples. When actin was polymerized with and without EGTA in the presence of Caplin, it showed an equal reduction in the apparent viscosity of actin filaments in both samples when compared to actin controls, indicating that calcium has no effect in the ability of Caplin to cause a reduction in the apparent viscosity of actin filaments.

The effect of calcium on actin filament severing activity of fusion protein adseverin was investigated by viscometry and showed that the presence of calcium promoted the reduction of the final viscosity of polymerized actin. Actin alone, polymerized in the presence or absence of EGTA showed the same apparent viscosity in both samples. The apparent viscosity of actin polymerized in the presence of adseverin with EGTA showed the same apparent viscosity as actin controls whereas, actin polymerized in the presence of adseverin without EGTA showed a dramatic reduction on the apparent viscosity of polymerized actin filaments compared to the actin controls (Nakamura et al., 1994).

This comparative analysis indicates that Caplin reduces the apparent viscosity of actin filaments in a calcium independent manner. Similar results were obtained in viscometric analysis of purified human and mouse platelet heterodimeric actin filament capping protein (CP). In resting cells, 98% of actin filament barbed ends were found to be capped. Purification and viscometric analysis of fractions containing CP showed that reduction in viscosity was independent of calcium (Hartwig et al., 1996).

This result is also in agreement with the finding that Caplin protein does not have an EF hand structure for the binding of calcium ions (as discussed in 4.1.II.e).
4.4.I.d - Effect of Caplin on actin annealing

This assay was designed to investigate whether the reduction in the apparent viscosity of actin filaments caused by Caplin observed by FBV was due to the binding of Caplin to the ends of actin filaments, based on the principle that the rate of filament annealing is high and dependent on the concentration of filament ends, therefore the higher the number of free filament ends the higher is the rate of annealing. To test this hypothesis doublet samples were used in which the viscometry was measured at two stages: First at steady state and secondly after mechanical shearing when filaments were allowed to anneal for 1 hour (as described in 2.11.I.d).

The curve obtained showed that at steady state, actin polymerized in the presence of Caplin reduced the viscosity of actin filaments. After shearing, the results showed that both actin in the presence and absence of Caplin retained the original apparent viscosity; i.e., actin alone recovered the original steady state viscosity by annealing the short filaments created by shearing whereas actin in the presence of 3.85 μM Caplin recovered the original steady state viscosity which was already reduced due to the presence of Caplin during actin polymerization.

In a further experiment in which actin was polymerized in the absence of Caplin and addition of this protein was carried out after shearing, the resulting curve obtained showed a reduction in the apparent viscosity of filaments, similar to the reduction observed when actin filaments were polymerized and sheared in the presence of Caplin.

This result suggests that the reduction in the apparent viscosity caused by Caplin may be due to the binding of Caplin to the ends of actin filaments, therefore, preventing the annealing of sheared short filaments. This may account for the reduction in the apparent viscosity seen, when actin is polymerized in the presence of Caplin (binding occurring during elongation of actin polymer) or when the addition Caplin is carried out after shearing when a greater
number of filament ends are created, due to the presence of shorter filaments, therefore allowing for the binding of Caplin to newly created filament ends.

Capping protein, CapZ, reduces the length of actin filaments by binding to the barbed ends regulating the mechanical properties of the cytoplasm. The cytoplasmic network provided by shorter filaments was found to be more fluid and less elastic (Pollard et al., 1999).

**4.4.11 - FLUORIMETRY**

The effect of Caplin on the rate and extent of actin polymerization was analysed by fluorimetry using pyrene iodoacetamine-labelled actin monomers. The basis of this assay is that fluorescence intensity of pyrene actin is much greater for polymeric than monomeric actin. The rate of actin polymerization depends on the concentration of free actin monomers and filament ends. Actin binding proteins can alter polymerization kinetics by numerous mechanisms including nucleating filament assembly, binding to monomers and preventing the incorporation into filaments or by binding to filament ends and blocking monomer addition.

Pyrene labelled actin was used at a concentration of 0.7\(\mu\)M so that it did not affect the critical concentration of the barbed (0.12\(\mu\)M) and pointed (0.7\(\mu\)M) ends of actin filaments (Gaerntner et al., 1989).

**4.4.II.a - Nucleation**

Caplin does not nucleate the polymerization of actin filaments. Analysis of the curve at the onset of monomeric actin polymerization, in the presence and absence of Caplin showed that the rate of actin nucleation is exactly the same for actin alone and actin nucleated in the
presence of Caplin. In the absence of a nucleating protein, actin polymerization is preceded by a lag phase which is necessary for the spontaneous formation of actin oligomers from monomers. This lag phase is showed within the first 40 seconds and is shown to be the same for actin alone and actin nucleated in the presence of Caplin.

In the presence of nucleating protein such as ponticulin, actin polymerization occurs immediately, each nucleating protein produces a new filament at the time of addition of actin monomers to nucleating protein causing the lag phase before the onset of polymerization to be much shorter than that observed for actin alone (Luna et al., 1993; Hitt and Luna, 1994).

This result also suggested that by not nucleating actin polymerization, Caplin requires filamentous actin for binding recognition. Indeed, this seems to be the case as analysis of the curve obtained for actin alone and actin polymerized in the presence of Caplin after 40 seconds of polymerization showed that the presence of Caplin reduced the incorporation of actin monomers to exponentially growing actin filaments. This result is also in agreement with FBV's assays, which indicated that the presence of Caplin reduced the apparent viscosity of actin filaments.

### 4.4.II.b - Elongation

The rate at which pyrene labelled actin is incorporated into pre-formed actin filaments is 35% reduced in the presence of Caplin indicating that Caplin blocks the addition of actin monomers during elongation step of actin polymerization. This is indicative that Caplin binds to the ends of actin filaments therefore preventing monomer additon and further elongation of actin filaments.

During elongation, a pool of monomeric actin is available for addition at the pointed or barbed end. As addition of G actin proceeds, the F actin pool increases and G pool decreases
till the concentration of G actin reaches a critical concentration in which no further addition occurs resulting in no further increase of filament length. Because the association/dissociation constants of barbed end is ten times higher than the pointed end it creates a steady state flux of molecules through the filament named treadmilling. The critical concentration at the two ends are different and so at steady state there is a net addition at the barbed end which is balanced by net loss at the pointed end (Pollard & Mooseker, 1981). In this experiment, the addition of 0.7 μM pyrene-labelled actin was chosen so it was sufficiently low to not induce spontaneous nucleation and sufficiently high to allow monomer addition without affecting the critical concentration at the pointed end (0.7 μM) or barbed end (0.12 μM). When pyrene-labelled actin was added to pre-formed actin filaments, it generated an exponential curve up to 600 secs followed by a plateau. The exponential curve reflects the addition of labelled G-actin to the barbed end of pre-formed actin filaments whereas, the plateau indicates the steady state of actin polymerization. When pyrene-labelled actin was added to actin filaments that had been pre-formed in the presence of Caplin a similar shape of curve was observed; however, the rate by which actin monomers were added to the end of filaments was 49% reduced during the first 600 secs and 35% at steady state. This decrease of actin polymerization is an indication for a capping reaction because proteins that either nucleate or sever actin filaments accelerate the incorporation of actin monomers into filaments because these proteins produce new pointed ends (Cooper and Shafer, 2000; Weber 1999).

Analysis of tensin on the kinetics of actin assembly showed a very similar curve. Tensin reduces the rate at which pyrene-labelled monomeric actin was incorporated to pre-formed actin filaments in the presence of tensin by 50% (Chen et al., 1994). It was further suggested that the reduction effect on the rate of addition of monomers to filaments was the result of
blocking sites of addition, and that this limited inhibition resulted from moderate affinity of
tensin to the barbed filament ends.

In order to investigate whether the 35% reduction on the rate of monomeric addition
caused by Caplin was due to low affinity of this protein to the ends of the filaments or to the
binding of Caplin to pointed rather than barbed ends; considering the difference in
association/dissociation rates at the barbed and pointed ends, the following experiment was
carried out in which gelsolin was used to block filament kinetics at the barbed end.

**4.4.11.c - Gelsolin assay**

The fluorimetry curve analysis showed no significant difference on the rate of
monomeric actin addition of gelsolin capped pre-formed filaments in the presence or absence
of Caplin, suggesting that Caplin binds to the barbed end of actin filaments.

To investigate the possibility that the reduction on monomeric addition caused by Caplin
was due to the binding of this protein to the point end of filaments, gelsolin capped actin
filaments were generated (Fowler et al., 1994). Gelsolin has a high binding constant(Ka=10^{11}
M^{-1}) reaching saturation at low concentrations.

Capping the barbed filament end restricts monomer-polymer exchange entirely to the
point end and therefore significantly increases the critical concentration at the point end
(Weber, 1999). This effect is seen here by the presence of an exponential curve. Although
both curves showed similar rates of monomer incorporation, capping of the barbed end by
gelsolin generated a shift in monomeric addition to the pointed end. Because addition at the
pointed end is ten fold less than the barbed end, actin polymerization takes longer to reach
steady state, generating an exponential curve in which a plateau is obtained after 800 secs of
polymerization.
Because both samples (in the presence or absence of Caplin) showed no significant difference in the rate of monomeric incorporation, it is reasonable to conclude that reduction in actin polymerization by Caplin is due to the binding of this protein to the barbed end of actin filaments. If both ends of filaments were capped, a reduction in the rate of polymerization should have been seen in the sample containing Caplin although this would not be highly different as barbed end filaments have higher rates than pointed ends.

This result also indicates that the affinity of Caplin for barbed ends is much lower than of Gelsolin. Gelsolin has a high binding affinity therefore causing reduction of monomer-polymer exchange higher than 90%. Because the reduction on actin polymerization caused by Caplin is 35%, it is indicative that the affinity for barbed ends is much lower and the capping effect caused by Caplin is 'leakier' than by gelsolin.

4.4.III - ACTIN BINDING SEDIMENTATION ASSAY

The binding constant of mouse thymus Caplin was estimated at \( K_a = 4 \times 10^5 \, \text{M}^{-1} \), at a molar ratio of one 22KDa protein to 5 actin molecules. This binding constant was derived from a binding curved obtained from co-sedimentation of actin polymerized in the presence of various concentrations of Caplin. Analysis of SDS-PAGE gels of supernatants and pellets, showed that a greater amount of Caplin was found to be in the supernatant fraction.

The binding constants of actin binding proteins range from high affinity (\( K_a = 10^6 \, \text{M}^{-1} \)) and low affinity (\( K_a = 10^4 \, \text{M}^{-1} \)) (Yamashiro-Matsumura & Matsumura, 1988) indicating that Caplin has a low affinity to actin filaments. Indeed, this low binding affinity is very much in agreement with the results from fluorimetry experiments in which it was shown that the presence of Caplin causes a 35% reduction on the rate at which polymers are elongated. In
other words, the low affinity of Caplin, indicates that in the presence of this protein only 35% of the filaments are capped.

The critical concentration reflects the binding constant of monomer for polymers that react with monomers only at their ends; this binding constant is the same for both ends (Weber, 1999). At steady state, G-actin concentration is close to the critical concentration of the barbed end. The barbed end predominates because the rate constants are ten times higher at the barbed end than the pointed end, hence the critical concentration of the barbed end is lower than for the pointed end. Capping the barbed end of filaments restricts monomer-polymer exchange entirely to the point end therefore increasing the concentration (Cooper and Shafer, 2000). If capping is higher than 90% such as in the case of gelsolin, the critical concentration increases, whereas, if capping is lower than 90% the critical concentration increases relatively little because the barbed end on-rate constant is so much higher than the pointed end, that a relative small fraction of free barbed ends can keep the steady state G-actin concentration at low level (Weber, 1999). This is very indicative that when Caplin is bound to the barbed end of actin filaments there are still free ends to carry out the addition of monomers to the fast growing ends, hence capping activity of 35%.

A large number of barbed end capping proteins exist in cells. Most important among them appears to be CapZ (Cooper and Shafer, 2000; Weber, 1999). Muscle CapZ has a binding constant of $K_a = 3.5 \times 10^9 \text{ M}^{-1}$ (Shaffer & Cooper, 1996) and like Caplin, this CP-β1 muscle isoform, regulates actin assembly by capping the barbed end during elongation of actin filaments in a calcium independent manner. In contrast to Caplin, CP-β1 also nucleates actin polymerization in vitro at a rapid rate. However, filaments nucleated by capping protein are capped at the barbed ends. A non-muscle isoform isolated from human polymorphonuclear leukocytes (PMN CapZ) has been found to strictly bind to the barbed end
of actin filaments independently of calcium which has no nucleating activity (Maun et al., 1996). Aginactin is also a barbed end capping protein that inhibits the rate and final extent of actin polymerization in a calcium independent manner, without affecting nucleation. This 70KDa protein has a calculated binding of $K_a = 2.7 \times 10^9 \text{M}^{-1}$ which is within the range of higher affinity capping proteins such as gelsolin family of barbed end capping proteins (Sauterer et al., 1991).

The fluorimetry and binding assay findings suggest that Caplin is able to decrease the rate of actin polymerization and this limited inhibition results from the low affinity of Caplin to the barbed end of actin filaments. Therefore, Caplin regulates actin assembly by binding to the barbed end of actin filaments changing the kinetics of filament growth.

### 4.4.4 LINEAR SUCROSE DENSITY GRADIENT

Linear sucrose gradients showed that Caplin remains as a monomer in the presence or absence of 2-ME with a peak at the same position of soy bean trypsin inhibitor, a monomeric protein of 21KDa. Furthermore, controls of actin and ovalbumin showed peaks at the same position at lower points in these gradients corresponding to 43KDa.

Based on the fact that the viscosity and density of sucrose gradients at 4°C gives a linear migration of biological molecules and, therefore, the ratio of distances travelled during centrifugation will be always constant, this assay was used to investigate whether Caplin remained as a monomeric protein irrespective of presence or absence of reducing agents, as cDNA analysis revealed the presence of three cysteine residues. This result indicates that the three cysteines present within Caplin's cDNA sequence are not involved in the formation of disulphide bonds with the SH groups of a second Caplin molecule.
4.4.V - IMMUNOFLUORESCENCE

Rat embryo fibroblast cells were chosen because Caplin is the only isoform of the transgelin family present in these cells; therefore, any staining present after probing with anti-C4 antibody should indicate solely the distribution of Caplin. This *in vitro* immunofluorescence rebinding assay was developed (Shapland et al., 1993) and carried out to visually investigate the binding activity of Caplin.

At the resolution of the light microscope, Caplin was found to be distributed along the lengths of actin filaments indicating that Caplin is an actin filament binding protein. The intensity of anti-C4 antibody fluorescence is generally consistent along the actin filaments and staining was also noted within the nuclei of these cells indicating a possible nuclear localization for Caplin in rat embryo fibroblasts.

A wide range of studies indicate the presence of actin in the nucleus, although very little is known about its role in the nucleus (Rando et al., 2000). Purification of nuclear actin is generally met with the objection that actin is an exceedingly abundant cytosolic protein hendering contamination to different protocols. Evidence indicating that actin can be found in the nuclei of intact cells comes from studies exploring the presence of two putative nuclear export sequences found in actin. An actin expression vector lacking these sequences show the presence of mutated actin in the nuclei of transiently transfected cells. Furthermore, cells treated with leptomycin B, a cell permeable nuclear export inhibitor, showed the presence of phalloidin-stained actin rods in their nuclei without the breaking down of the nuclear membrane (Rando et al, 2000).

Many actin binding proteins have been shown to be localized to the nucleus by immunofluorescence microscopy but unfortunately the nuclear role of these proteins remain to be elucidated. Although some of them are small enough to enter the nucleus passively such
as in the case of thymosin, profilin and cofilin, larger proteins such as supervillin and Gap G have also been shown to be present in the nucleus (Rando et al., 2000). Caplin has a molecular weight of 22KDa, therefore within the limits of the molecular weight cutoff for passive nuclear entry. It may also be possible that the presence of a putative nuclear localization signal could be involved in the presence of Caplin in the nuclei of rat embryo fibroblast cells.

Caplin was fully removed by detergent extraction from the cytoplasm cytoskeleton, indicated by the lack of actin filament staining; although staining is still seen at nuclei level. This may be due to the biochemical nature of the nuclear matrix which requires higher salt for solubilization.

The labelling of actin filaments was restored upon addition of Caplin to detergent extracted rat embryo fibroblasts, indicating that Caplin binds directly to actin fibers. These data reinforce the biochemical and functional studies data and showed that Caplin rebound specifically to actin filaments in a calcium independent manner.

Furthermore, this study is also consistent with the existence of functional domain evolution. Whereas Transgelin, the higher molecular weight isoform, cross-links actin filaments, Caplin caps the barbed-end of actin filaments. It indicates that this family of actin binding proteins evolved fundamental modular domains to perform specific catalytic and recognition functions within the cell.
4.4.VI - MODEL FOR CAPLIN INTERACTION WITH ACTIN FILAMENTS

FIGURE (32). Schematic representation of Caplin binding and capping the barbed end of actin filaments. Caplin binds directly to actin filaments regulating actin polymerization by capping the barbed ends and preventing monomer addition during elongation of actin filaments, thus reducing the viscoelasticity of filamentous actin in the cell cytoplasm.

4.4.VII - FUTURE EXPERIMENTS

Caplin amino acid sequence analysis revealed the existence of a subdomain structure homologous to the Calponin family of F-actin binding proteins, consisting of an amino
Chapter 4

terminal calponin homology (CH) domain (1-134), an actin binding site (ABS) domain (152-173) followed by one calponin-like repeat (174-198) at the carboxyl-terminal. The CH domain has been identified in a variety of actin binding proteins and signalling molecules responsible for directly linking signal transduction molecules to the actin cytoskeleton via an association with F-actin (Puius, 1998; Stardal et al., 1998)

It will be of great interest to define which of Caplin's domains is responsible for the binding to filamentous actin. Structural and functional analysis of each calponin subdomain has suggested that the binding of Caplin to the barbed ends of actin filaments may involve the calponin-like repeat at the carboxyl end (Gimona and Mital, 1998). To test whether or not this domain is responsible for the binding of Caplin to actin filaments, a recombinant Caplin construct lacking the entire calponin-like repeat (Caplin 174-199) will be expressed as GST-fusion protein, and used for co-sedimentation assays with skeletal muscle F-actin as described for Calponin (Gimona and Mital, 1998). The involvement of this domain in the direct binding to actin stress fibers will be further analysed using the immunofluorescence re-binding assay described in section 2.4 (Shapland et al., 1993). I will detergent extract rat embryo fibroblasts to remove all endogenous Caplin and Transgelin and carry out re-binding experiment using purified recombinant constructs with and without this domain.

These protocols, in conjunction with generation of further constructs, will be used to investigate the functional significance of the CH (1-134) and ABS (152-173) domains. As previously discussed (section 4.1.II.g), the presence of a single CH domain alone may not account for the binding to actin filaments *per se* (Gimona and Mital, 1998). However, it's presence in variety of signalling proteins such as the VAV family of Rho GTPases and Ras GTPases indicates a function as a downstream effector linking extracellular stimuli to cytoskeletal arrangements (Kranewitter and Gimona, 1999) (section 4.2.III and 4.2.VII). Although Caplin distribution is not restricted to specific cytoskeletal structures such as
lamellipodia (it is found along stress fibers throughout the cytoskeleton as shown by immunofluorescence, section 4.4.V), it will be interesting to investigate whether the presence of the CH domain in Caplin has some functional significance in controlling and/or modifying lamellipodia formation and/or stability. This could be investigated by microinjection of recombinant Caplin protein with and without the CH domain to serum starved fibroblasts and analysing, by immunofluorescence, the effect of these constructs on stress fiber formation following the addition of lysophosphatidic acid to cells (Machesky and Hall, 1996).

It would be also interesting to find the crystal structure of Caplin, since proteins that share modular structures are known to have evolved to adapt to specific functions (Puius et al., 1998). Information obtained from Caplin crystals could further define the regions involved in the binding and capping of this protein to the barbed ends of actin filaments, since the crystal structure of molecules such as gelsolin and CH domain of Calponin family has been elucidated (Burtnick et al., 1997; Carugo et al., 1997).

Caplin has a potential nuclear localization signal that could be involved in signal transduction of this molecule to the cell nucleus (section 4.1.II.d and D. Lawson personal observation). This possible additional function in the cytoarchitecture of the nucleus, as well as the cytoplasm, could be investigated using antisense oligonucleotides or by microinjection and overexpression of constructs lacking the potential NLS amino acid region (152-158) (Pestonjamasp et al., 1997).
Many families of proteins are known to be associated with the actin cytoskeleton where they play a variety of roles such as controlling filament length and interactions.

I have investigated the *in vitro* function of Caplin, a member of the Transgelin multigene family of actin associated proteins and found that: Caplin cDNA obtained from normal thymocyte cells has a molecular weight of 22,391.4Da and an estimated PI of 8.41. Analysis of Caplin cDNA sequence indicated the presence of a possible nuclear localisation signal, which raises the possibility that Caplin may provide means for altering nuclear filamentous actin in the cell, since at immunofluorescent level, Caplin was found to decorate the nucleus of rat embryo fibroblast cells.

Analysis based on sequence alignment with the calponin family of actin binding proteins indicated that Caplin shares the same modular structure consisting of an amino terminal CH domain followed by an ABS domain and a carboxyl terminal R domain. The structural and functional similarities of each modular domain suggest that the binding of Caplin to the ends of actin filaments is very likely to involve the carboxyl end of Caplin.

Cell permeabilisation assay and immunofluorescence has also shown that Caplin binds directly to actin filament. The *in vitro* functional assays showed that monomeric Caplin controls the length of actin filaments by capping them at their barbed end with a binding constant of $K_a=4\times10^5\text{M}^{-1}$. Falling ball viscometry has shown that Caplin reduces the apparent viscosity of actin filaments by 35%, and that the reduction effect was independent of calcium. Fluorimetry also demonstrated that Caplin has no effect on the nucleation of actin filaments.
Expression of fusion protein was achieved with modifications to the single-step purification protocol described by Smith & Johnson (Smith and Johnson, 1988).

E.coli BL21 cells were transfected with GST-Caplin constructs and the expression of fusion protein was achieved using 0.2mM IPTG for 3 hours (figures 14 and 15).

Prior to Caplin fusion protein induction and purification, I also carried out the purification of fusion protein Transgelin, as the in vitro function of this isoform was known and, therefore, could be used as a control for my future experiments on Caplin. Transgelin fusion protein induced in the presence of 0.5mM IPTG for 4 hours was found to be soluble, without the formation of inclusion bodies, and caused the gelation of actin filaments by falling ball viscometry (data not shown).

Expression of Caplin fusion protein at 0.5mM IPTG for 4 hours indicated the presence of many degradation products by SDS-PAGE (figure A.1.a). In order to reduce degradation products and optimise the production of non-degraded Caplin protein, I carried out inductions using 0.1mM, 0.2mM and 0.5mM IPTG for four hours (Pestonjamasp et al., 1995; Smith and Johnson, 1988). SDS-PAGE (figure A.1.a) and immunoblot analysis (not shown) showed significantly higher degradation when induced with 0.5mM IPTG compared to inducing with 0.2mM and 0.1mM IPTG. There was no significant difference in the presence of degradation products between cells induced with 0.1mM and 0.2mM IPTG. To further optimise expression of Caplin protein a time course of expression after induction using 0.2mM IPTG
was carried for 1h, 3hs and 4hs (figure A.1.b)(see also figure 2A in Smith and Johnson, 1988). Coomassie blue stained SDS-PAGE showed increased yield of fusion protein expression with time (fig A.1.b). However, the increase in bacterial expression was accompanied by an increase in formation of aggregates and degradation. Based on SDS-PAGE (figure A.1.b) and immunoblot analysis (not shown) and in order to minimize the exposure of Caplin fusion protein to the reducing systems of the bacteria and consequent denaturation (Schein, 1989), 3 hours was chosen as the optimal time for Caplin fusion protein induction.

FIGURE A.1.a - SDS-PAGE Commassie blue stained analysis of IPTG concentration at 0.1mM (A1), 0.5mM (A2) and 0.2mM (A3).
FIGURE A.1b - SDS-PAGE Coomassie blue stained time course of 0.2mM IPTG induction at 1 hour (A1), 4 hours (A2) and 3 hours (A3).

**A.2 - Production of soluble protein**

The production of soluble recombinant Caplin fusion protein was limited by the formation of inclusion bodies, which caused increased insolubility. Insolubility of fusion proteins is a problem often faced when eukaryotic proteins are expressed in bacteria and the formation of inclusion bodies is not directly related to the production rate nor the mass of the protein undergoing expression (Marston, 1986; Schein, 1989; Schein 1990). There are many ways of increasing the production of soluble protein (Marston, 1986; Schein, 1989, Schein 1990). The easiest way to reduce formation of inclusion bodies is to reduce the temperature at which the bacteria is growing (Schein, 1989). When cultures of BL21 cells were grown at
30°C fusion Caplin protein remained insoluble by SDS-PAGE and immunoblot analysis of both supernatant and pellets (not shown). It has been suggested that the amino acid content of proteins may correlate with solubility (Marston, 1986). Gelsolin carboxyl and amino terminals have shown different solubilities when expressed as fusion proteins (Schein, 1989). The soluble carboxyl terminal has one cysteine residue whereas, the amino terminal contains four. Furthermore, the carboxyl terminal has four repeats of negatively charged residues. Caplin has three cysteines and no cluster of negative amino acids within its sequence, which could account for its insolubility. Proline content may also correlate with solubility, since proteins with high proline content seem to be more insoluble than proteins with a lower number of proline residues (Schein, 1989; Schein, 1990). Caplin has nine proline residues within its sequence, which may account for the insolubility of this molecule. This dictated the use of 6M urea, a denaturant that allows proteins to correctly refold after removal by dialysis (Marston, 1986; Schein., 1990). The solubilization of fusion Caplin protein was achieved in 6M urea (pH=9.5) at 4°C for 40 minutes to minimize exposure of fusion Caplin to the high PH environment (Marston, 1986; Schein., 1990). Removal of urea and the refolding of soluble fusion Caplin was achieved by dialysis as described in section 2.9.1.

Cleavage of fusion protein Caplin to remove the GST fragment was achieved in a single step purification using thrombin while fusion protein Caplin was still attached to Sepharose beads (Guan & Dixon, 1991). Removal of the GST moiety (26KDa) was necessary due to the size of this fragment compared to Caplin (22KDa) and its possible steric effect in the binding to actin at in vitro functional studies. This purification step takes advantage of the high efficiency specific protease recognition site located at position 918-935 of pGEX-4T-3, and also minimize further steps of protein purification and hence, degradation (Smith & Johnson, 1988; Guan and Dixon, 1991). No similar recognition sequence was found within Caplin peptide sequence (figure 3). Optimal cleavage efficiency was achieved using 3U/ml of
thrombin for 40 minutes at 4°C (figures 14 and 15). The use of higher thrombin concentration resulted in higher recovery but increased protein degradation (not shown).

Thrombin was removed using sepharose beads and in these experimental conditions approximately 1mg/ml of non degraded, soluble Caplin recombinant protein was obtained from 100mls cultures as shown by SDS-PAGE and immunoblot analysis (figures 14 and 15).

B- Actin polymerization curve

![Steady-state](Steady-state)

![Elongation](Elongation)

![Activ/nucleation](Activ/nucleation)

**FIGURE B-** Sigmoidal curve representing the four steps of actin polymerization: monomer activation, nucleation, elongation and steady state.


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