The role of ADF/Cofilin family proteins in the acquisition and maintenance of cell polarity during fibroblast migration

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For Mum, Dad and Sarah

Abstract

To migrate normally, a cell must establish morphological polarity and continuously protrude a single cell margin, termed the lamellipodium, polarised in the direction of migration. Previous data from our laboratory showed that actin filament disassembly was necessary for protrusion of the lamellipodium during fibroblast migration but was not required for non-polarised lamellipodial protrusion in non-migrating cells. DNase I staining of actin monomer levels in the lamellipodium showed that this was because actin monomer was highly limiting in the lamellipodium of polarised migrating cells. As ADF/cofilin (AC) proteins are essential for the catalysis of filament disassembly in cells, their role in polarised cell migration was assessed. The spatial distribution of AC and inactive, phosphorylated AC (pAC) was compared in the lamellipodium of polarised migrating cells. AC, but not pAC, localised to the lamellipodium. Adenoviral-mediated gene transfer was used to manipulate AC activity levels in cells. Locally maintaining active AC at the leading edge was required for maintaining cell polarity during fibroblast migration. When pAC was forced into the lamellipodium by introduction of a constitutively active form of LIM kinase, cells lost both their morphological polarity and their ability to migrate. This polarity loss could be prevented by expression of a non-phosphorylatable form of AC. Furthermore; AC activity was necessary for the acquisition of morphological polarity. Fibroblasts polarised in a distinct series of sub-steps. The first step in polarity acquisition was organisation of actin from a circumferential organisation to an oriented array. This was required to specify position of the cell tail. Both jasplakinolide treatment and introduction of either constitutively active LIM kinase or dominant negative AC blocked formation of oriented actin bundles; actin remained circumferentially oriented and the cell failed to polarise. Blocking AC and actin filament disassembly did not affect later steps in acquisition of polarity. Stabilisation of the cell tail was dependent on myosin II. Blocking myosin using either methyl-blebbistatin or Y-27632 produced abnormally crescent-shaped cells as the tail encroached into the cell body. Microtubules were not required for polarity acquisition, however blocking microtubule dynamics led to de-stabilisation of the lamellipodium and a loss of migratory capability.

THE ROLE OF ADF/COFILIN FAMILY PROTEINS IN THE ACQUISITION AND MAINTENANCE OF CELL POLARITY DURING FIBROBLAST MIGRATION

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Finally I would like to thank my family. I promise I'll stop being a student now...

Abbreviations

AC	actin depolymerising factor/cofilin
ADF	actin depolymerising factor
CEF	chick embryo fibroblasts
DNase I	deoxyribonuclease I
G-actin	actin monomer
GFP	green fluorescent protein
F-actin	filamentous actin
LIMK	LIM-domain containing protein kinase
LIMK EE508	constitutively active LIMK
LP	lamellipodium
PAK	p21-activated protein kinase
pAC	phosphorylated ADF and phosphorylated cofilin
ROCK	Rho kinase
VDBP	vitamin D binding protein
XAC	Xenopus ADF/cofilin
XAC A3	constitutively active XAC
XAC E3	pseudophosphorylated XAC

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Chapter I: Introduction

1.1 The importance of polarised cell migration

Many types of eukaryotic cell have the capacity to migrate along a substratum and cell migration plays an important role in both normal physiological behaviour and disease. Unicellular organisms use migration as a means to hunt for food, development of an embryo requires a whole series of tightly regulated migration events for tissue and nervous system formation, the immune response involves migration of leukocytes and wound healing requires migration of several cell types including fibroblasts. Aberrant cell migration occurs following oncogenic transformation, and leads to metastasis of cancer cells and invasion of surrounding tissue.

There are many distinct types of polarity that exist in cells and polarity is a feature of most cell types. Productive, normal, cell migration requires the cell to become morphologically polarised, in other words to have a clearly distinct front and back shape that is maintained throughout the migration process. Although the molecular mechanisms that underlie morphological polarity are not particularly well understood, it is generally agreed that the actin cytoskeleton is a key player in regulating cell shape changes and provides the driving force needed to move a cell. There are many different types of actin organisation in cells. Formation and correct organisation of these actin filament networks to create morphological polarity is vital for the generation of a polarised cell shape. While the precise details of polarity acquisition and maintenance are likely to differ between cell types, the essential principles will remain the same. This thesis examines how the spatial control of actin filament assembly and disassembly regulates how a cell that intends to migrate initiates its polarised morphology, and how that polarity is subsequently maintained, enabling continuous migration to occur.

In this Introduction I first provide a brief overview of the background to cell migration studies and some of the key points in the discovery of the

cytoskeleton. As I am interested in the organisation of actin to form a polar shape, the actin cytoskeleton and the major proteins that regulate its dynamics are dealt with in detail with particular focus on actin filament disassembly and the ADF/cofilin (AC) family of actin disassembling/severing proteins. Finally, the current status of morphological cell polarity work is reviewed.

1.2 Cell migration

While many scientists had observed the behaviour of cells under the microscope, Michael Abercrombie was the first to apply scientific principles of controlled, quantitative analysis to this study (Dunn and Jones 1998). One aspect of his work examined the locomotion of fibroblasts in tissue culture. In a series of five papers (Abercrombie *et al.* 1970c, a, b, 1971, 1972), Abercrombie carefully examined the mechanism of locomotion using time-lapse imaging and electron microscopy. The results laid the foundation for all subsequent cell migration studies, including the characterisation of the leading edge of motile cells (Abercrombie *et al.* 1970b,c), the extreme tip of which was termed the lamellipodium (Abercrombie, 1980), and the identification of what later would be known as focal contacts (Abercrombie *et al.* 1971).

Subsequent work has made it clear that the cell is made up of regions that are morphologically different from each other. These are the lamellipodium, lamella, cell body, and tail (Harris 1994). The distinction between the lamellipodium and the lamella is not always made. In this work the term lamellipodium describes the short, thin, actin-rich band at the extreme leading edge of the cell. The lamella is the region immediately behind the lamellipodium that is of intermediate thickness and reaches to the cell body. This is the thickest region of the cell and contains the nucleus and the majority of the organelles. The tail can be either wedge-shaped or more drawn out, depending on the cell type and the behaviour of a particular cell, for example a keratocyte has a very wedge-shaped tail while a fibroblast's tail can either be wedge-shaped or elongated.

Today, the process of cell migration is generally divided into cell motility stages along spatio-mechanical lines (fig. 1.1. For reviews see (Lauffenburger and Horwitz 1996; Mitchison and Cramer 1996; Sheetz *et al.* 1998). Forward movement of the lamellipodium is the first stage in motility and is termed protrusion. More is known about the mechanism of this stage than any of the others. Adhesions must form in order to translate protrusion into movement of the cell along the substrate. Following formation and adhesion of the protrusion, the nucleus and cell body are translocated forwards in a less well-understood second stage. The third and final stage is to break down cell adhesions at the rear of the cell and retract the tail. The coordinated movement of these stages results in net forward movement across the substratum.

1.3 Chick embryo fibroblasts

From Abercrombie onwards, fibroblasts in culture have proven a popular tool for the study of cell motility. In this work, primary fibroblasts from chick embryo hearts are used to study polarisation and polarity during cell migration. Chick embryo fibroblasts (CEF) are a well-characterised system for cell motility studies that change their polarity and migration capabilities according to the length of time they spend in culture. This behaviour has been extensively characterised for an explant-based culture set-up in which chick heart explants are left to adhere and from which fibroblasts migrate outwards (Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b). These culture conditions enable the comparison of polarised migrating and non-polarised non-migrating cells of the same cell type. Any differences seen can be related back to the migration or polarity status of the cell. Overall morphological polarity in fibroblasts is composed of a number of individually polarised regions: the formation and maintenance of a dominant protrusion, orientation of the actin bundles within the cell body in the direction of migration and the retraction of the edge opposite to the protrusion to form the cell tail. Non-polarised protrusion occurs in non-migrating cells. Here, protrusive behaviour is not restricted to a single region of the cell margin, but instead occurs randomly around the entire cell periphery. In this case it is not possible to distinguish a cell front and rear.

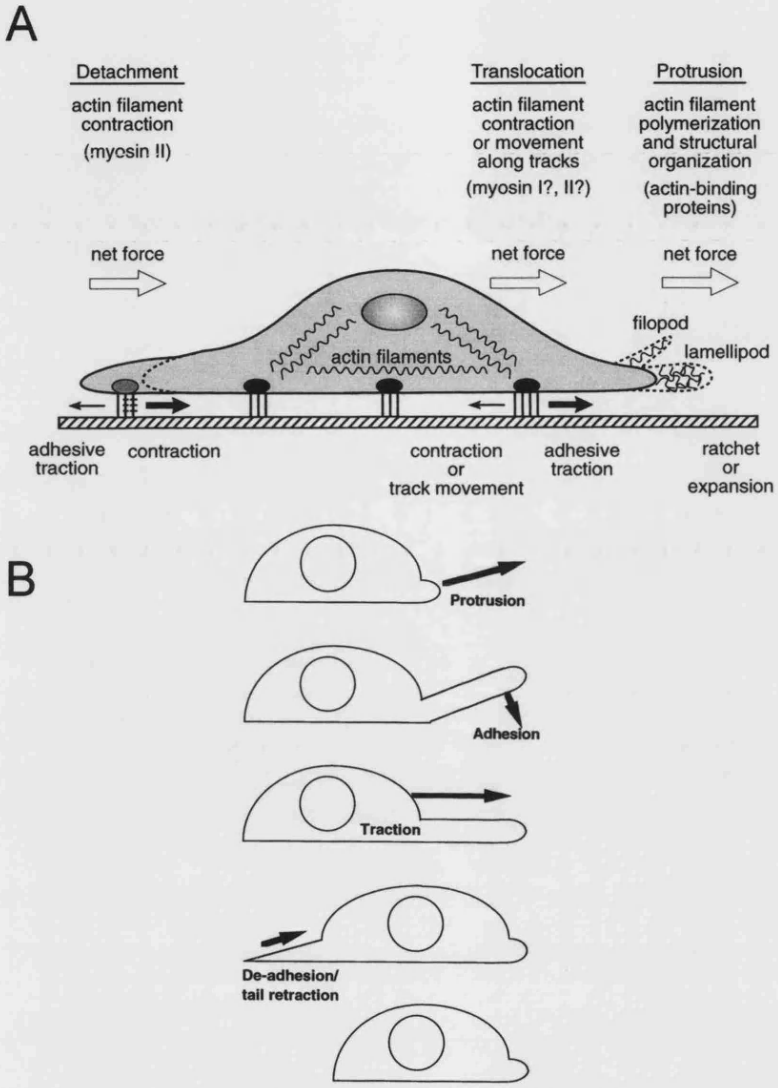

Figure 1.1: The stages of locomotion of a migrating cell. (A) Shows a vertical section through a locomoting cell, including the contacts between the cell and the substratum and some of the general morphological features of the cell including the lamellipodium and a filopodium. (B) The sub-steps of cell locomotion that lead to complete cell translocoation on a two-dimensional substrate. (A) taken from (Laufenburger & Horwitz) 1996, (B) taken from (Mitchison & Cramer 1996).

CEF exhibit spontaneous polarisation and migration. In an explant culture, fibroblasts grow out from the explant in a polarised fashion and migrate constitutively without the need for application of exogenous growth factors or chemoattractant. The first fibroblasts to migrate out of the explant do so rapidly (0.5-1μm per minute, (Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b) and at this point have a single well-spread thin lamellipodium (Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b) and lack focal adhesions (Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b) After 48 hours in culture there is a decrease in the rate of migration accompanied by a change in morphology from a "fan" or "kite" shape to a more polygonal and less polarised shape (Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b). By around 4 days after plating, most of the cells have an orthogonal morphology with multiple lamellipodia and are non-migrating (Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b). This progressive loss of polarity correlates with the appearance of focal contacts, focal adhesions (Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b) and stress fibres (Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b). While lamellipodium protrusion is not abolished in the non-migrating cells, this occurs randomly around the cell periphery and is highly transient with multiple retraction events (Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b).

In dissociated culture, cells spontaneously polarise in a random direction and then migrate with the same speed and morphology as fibroblasts cultured in an explant-based system (H. Dawe, unpublished observations and see Chapter V).

The molecular details of the cell motility stages that together comprise cell migration have been the subject of intensive and ongoing research, which mainly focuses on the molecules that make up the actin cytoskeleton. While its main role is in organelle movement and mitosis, the microtubule cytoskeleton also plays a role in cell migration and morphological polarity. Nevertheless, actin force-generating mechanisms remain the major source of power to move adherent cells forward across a substratum and the actin cytoskeleton is the main focus of this thesis. The next section provides some background

information on the discovery of the three components of the cytoskeleton: actin, microtubules and intermediate filaments.

1.4 The discovery of the cytoskeleton

The idea that living tissue could consist of minute fibres has been around for centuries (Frixione 2000) but it is only in the last 50 years that biochemists and microscopists have come together to define the properties of the series of proteins that make up what we now call the cytoskeleton. The earliest surviving drawing of the cytoskeleton comes from an 1844 paper by Robert Remak (Frixione 2000) on the morphology of certain nervous structures. This illustration (fig. 1.2) shows a crayfish ganglion as being composed of a fine array of fibres that surround what is obviously the nucleus and extend up the axon. Debate raged throughout the latter part of the nineteenth century and the first few decades of the twentieth century as to the veracity of this and other observations, and it was not until around 1930 that the theoretical necessity of a supporting cellular framework of protein, or "cytosquelette" was recognised (Frixione 2000).

It was muscle research and the study of cilia and flagella that lead to the identification and biochemical characterisation of some of the major constituents of the cytoskeleton. As early as the nineteenth century it had been suggested, and subsequently repudiated, that muscles could work using a system of sliding rods made up of a protein isolated from muscle and named "myosin" (Frixione 2000). During the early 1940s this idea was revisited and it was discovered that myosin was in fact two separate proteins, myosin and a second component that was named "actin" (Frixione 2000). Further work revealed that myosin itself was composed of two distinct subunits, heavy and light "meromyosin" (Szent-Gyorgyi 1953). Analysis of the structure of muscle using thin-section electron microscopy showed a series of overlapping thick and thin filaments (Hanson and Huxley 1955) and this lead to the suggestion that the two sets of filaments could slide over each other (Huxley and Niedergerke 1954, Huxley and Hanson 1954), and subsequently to the sliding filament

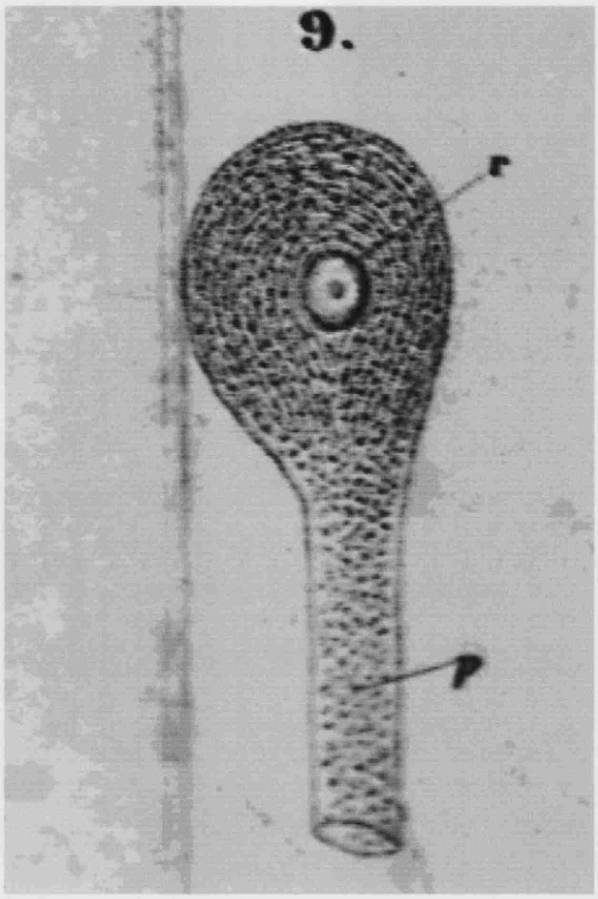

Figure 1.2: 19th century drawing of the morphology of a crayfish ganglion cell. A drawing from Robert Remak's 1844 paper on nervous cell morphology. The picture clearly shows a fine array of fibres that surround the nucleus and extend down the axon. According to Frixione (2000) this is one of the first known representations of the cytoskeleton. Taken from (Frixione, 2000).

model of muscle contraction that we know today. Further ultrastructural analysis demonstrated that the heavier of the two meromyosins could decorate actin filaments, producing a distinctive arrowhead structure with the points and barbs all arranged in the same direction along the length of each filament. This provided a means of reliably recognising actin filaments in cells. Actin filaments were soon found in a wide variety of non-muscle cells and eventually the connection was made between actomyosin contractility and motility in non-muscle cells (Pollard and Weihing 1974) and also between actin polymerisation or re-organisation and cell shape changes (Tilney *et al.* 1973; Tilney 1975).

Concomitant with these discoveries, studies on the mechanism of movement of cilia and flagella identified a second set of proteins that could generate a motile response. Electron microscopy had revealed that cilia contained a distinctive set of structures apparently consisting of a circle of nine fibres, each of which was in itself a doublet, with a central pair of single fibres (Fawcett and Porter 1954). The fibres came to be called "microtubules", and the major constitutive protein was isolated and named "tubulin" (Shelanski & Taylor 1967, Mohri 1968). Microtubules too, were soon seen in almost every cell type and their sub-cellular distribution relative to other cellular components suggested that they could facilitate intracellular motility (Porter & Tilney 1965).

Following the identification of the actin and microtubule cytoskeletons, a third class of cytoskeletal polymers were identified that were of an intermediate size between actin filaments and microtubules (Ishikawa *et al.* 1968; Cooke 1976). The main function of these "intermediate filaments" is to maintain the structural integrity of the cell, and their molecular architecture and functions in cells have been the subject of recent reviews (see (Strelkov *et al.* 2003) and (Fuchs and Weber 1994) for examples). As intermediate filaments are not a part of this work, they will not be dwelt upon further.

Actin, myosin and tubulin were soon joined by a host of other interacting proteins. Observation of actin filaments (Lazarides and Weber 1974) and microtubules (Fuller *et al.* 1975; Weber *et al.* 1975) confirmed the early idea of a skeleton responsible for the maintenance of cell shape. This rather static view

of the cytoskeleton has been gradually dispelled by new techniques such as the development of tools to examine cytoskeleton structure and dynamics. These have enabled the discovery of some of the molecular mechanisms that allow the cytoskeleton to constantly rearrange both itself, and other cellular organelles (see (Machesky and Schliwa 2000)). Today the cytoskeleton is understood as a complex network of highly integrated and coordinated processes. Research into exactly how the molecular mechanics of the cytoskeleton are regulated has taken the last three decades, and is still continuing. As this thesis focuses particularly on the actin cytoskeleton, the role of microtubules will be put aside for the moment and in the next sections the structure of actin (section 1.5), its organisation within migrating cells (section 1.7) and the ways in which actin assembly and disassembly is controlled at the molecular level (section 1.8) are examined in more detail.

1.5 Actin structure

Actin exists in two forms in cells: monomeric globular "G"-actin and polymeric filamentous "F"-actin. In cells actin is highly dynamic and assembles and disassembles readily. These processes are essential for life: blocking either actin filament assembly or disassembly with drugs results in death of the cell. The formation of actin filaments is fuelled by a large cellular pool of actin monomer, which is maintained at a very high concentration (8-250μM (Rosenblatt *et al.* 1995), much higher than the so-called "critical concentration", the concentration at which spontaneous polymerisation occurs (0.2μM, (Pollard 1986)). As a consequence, actin monomer is prevented from spontaneous assembly in cells (Zigmond 1993). Each actin filament consists of an oriented double helix. Filaments are polarised, with a rapidly growing barbed end named for the characteristic arrowhead shape seen on myosin decorated filaments, and a slower growing pointed end **(Chen *et al.* 2000)**. In vivo actin filaments elongate from free barbed ends and shrink from the pointed ends and under steady state conditions, the rate of elongation equals the rate of shrinkage. Therefore the length of the filament remains the same. This process is called treadmilling (Chen *et al.* 2000). Each actin monomer is bound to an adenine

nucleotide, probably in association with magnesium as magnesium is at a much higher level than calcium within the cell (Pollard *et al.* 2000). Filaments assemble from ATP-actin and over time there is a slow, irreversible hydrolysis of the ATP to ADP+ inorganic phosphate and then ADP (Pollard and Weeds, 1984). This is accompanied by a change in the conformation of the actin filament (Belmont *et al.* 1999). New actin filaments are nucleated from trimers of actin monomer (Pollard *et al.* 2000). This is a highly unfavourable reaction compared with elongation of pre-existing filaments, therefore pure actin poorly initiates new filament formation and in cells nucleation factors are required.

1.6 Myosin

Actin filaments often function in conjunction with molecular motors of the myosin family. The name myosin encompasses a superfamily of proteins that may carry out a variety of functions within cells. Each myosin has a similar structure, being formed from head, tail and neck regions, and all myosins are predicted to bind actin filaments. The similarity ends there, however, with some myosins composed of monomers, others dimers, and others (only myosin II to date) filamentous. Family members implicated so far in cell motility are from groups I, II, V and VI (Cramer 1999a). Myosin contains an ATPase activity that is used to generate mechanical force, with all myosins except myosin VI being directed towards the barbed ends of actin filaments (Wells *et al* 1999, reviewed by Cramer 2000).

Non-muscle myosin activity is regulated by phosphorylation. The serine/threonine kinases ROCK I and II can phosphorylate the myosin regulatory light chain (MLC), causing increased actomyosin contractility and increased cell adhesion (Alblas *et al.* 2001). Myosin phosphatase is also a downstream target of ROCK II, which phosphorylates and inhibits the myosin binding subunit of the phosphatase, leading to increased phosphorylation of MLC (Alblas *et al.* 2001; Fukata *et al.* 2001) and therefore increased myosin activity.

Historically, the role of myosin has been thought of as to generate contractile force by sliding actin filaments over each other within the actin network. The only myosin known to be capable of achieving this is myosin II, as to date it is the only family member that forms filamentous structures with actin but there is the possibility that myosin I may also be able to perform this role. Future work may show that other myosins are also able to cross-link actin structures. Other myosins such as class V myosin appear to function as short-range transporters of organelles within the cell (reviewed by (Wu *et al.* 2000)). Finally, myosins including myosin IX and myosin I appear to be capable of regulating assembly of actin filaments by inactivating Rho and modulating the activity of the Arp2/3 complex, respectively (reviewed by (Wu *et al.* 2000)). In terms of myosin-based polarised cell locomotion, contractile mechanisms as described for muscle may be less important as these generate symmetric force (Cramer 1999a). Modified forms of contractile force where there is a source of polarity for movement may, however, theoretically exist in cells ((Mitchison and Cramer 1996), (Cramer 1999a)). Myosin force driving generated by cargo transport, on the other hand, is polarised as the cargo is transported in a single direction. The organisation of the actin filaments within specific cellular regions is likely to play a significant role in determining what myosin activity occurs.

1.7 Organisation of actin in migrating cells

Once formed, actin filaments can be organised in a variety of different ways to create a variety of different populations of actin bundle, which form structures of differing stability in different cellular locations and have different functions in cell motility. Muscle sarcomere structure has long been defined, but it is beyond the scope of this introduction and will not be described here. In addition, the actin structures found in non-migrating cells will not be covered in depth. Instead, this section focuses on some of the higher order actin structures found in migrating cells. In non-migrating serum-starved fibroblasts the Rho family of small GTPases controls the formation of some of the different actin structures in cells: Rho promotes stress fibre formation, Rac promotes lamellipodia and Cdc42 induces filopodia formation and the action of the Rho

GTPases has recently been reviewed (Hall and Nobes 2000; Etienne-Manneville and Hall 2002). The regulators of actin structures in migrating cells are less well understood. Evidence from migrating macrophages has demonstrated that directed migration is regulated by the Rho family (Jones *et al.* 1998; Ridley *et al.* 1999) and in this thesis data are presented that demonstrate AC controls actin bundles in migrating fibroblasts.

More is known about actin structures at the cell edge of migrating cells than in the cell body. There are known, commonly observed bundles in the cell body termed "stress fibres", but these only occur in non-migrating cells and are inhibitory to cell migration (Byers *et al.* 1984). Therefore they will not be described here except to state that they have sarcomeric-like organisation with alternating polarity within the bundles (fig. 1.3A) (Cramer 1999a). In addition there are other (radial) actin bundles with distinct organisation to stress fibres that are found in the cell body of *Drosophila* nurse cells (Guild *et al.* 1997). Again, these are not migrating so will not be described here. The many actin structures found in different cell types have been described in a detailed review (Cramer 1999a).

Actin organisation at the cell edge

Cortical actin is important in both migrating and non-migrating cells, where it maintains cell shape. It has sarcomeric-like organisation that is less well-organised than that found in muscle, but forms criss-cross actin bundles networks in cells. In migrating fibroblasts, parallel and perpendicular bundles cross to form an actin mat that underlies the plasma membrane (Cramer *et al.* 1997), generating equal and opposite tension over the entire cell surface. Three possibilities have been suggested for the function of these bundles: first that they may make a minor contribution to the generation of motile force for the cell body during fibroblast migration, second that since these bundles are found close to the plasma membrane, they may be responsible for the generation of cortical tension to maintain cell shape during migration and third that they may contribute to the mechanism of tail retraction (Cramer *et al.* 1997).

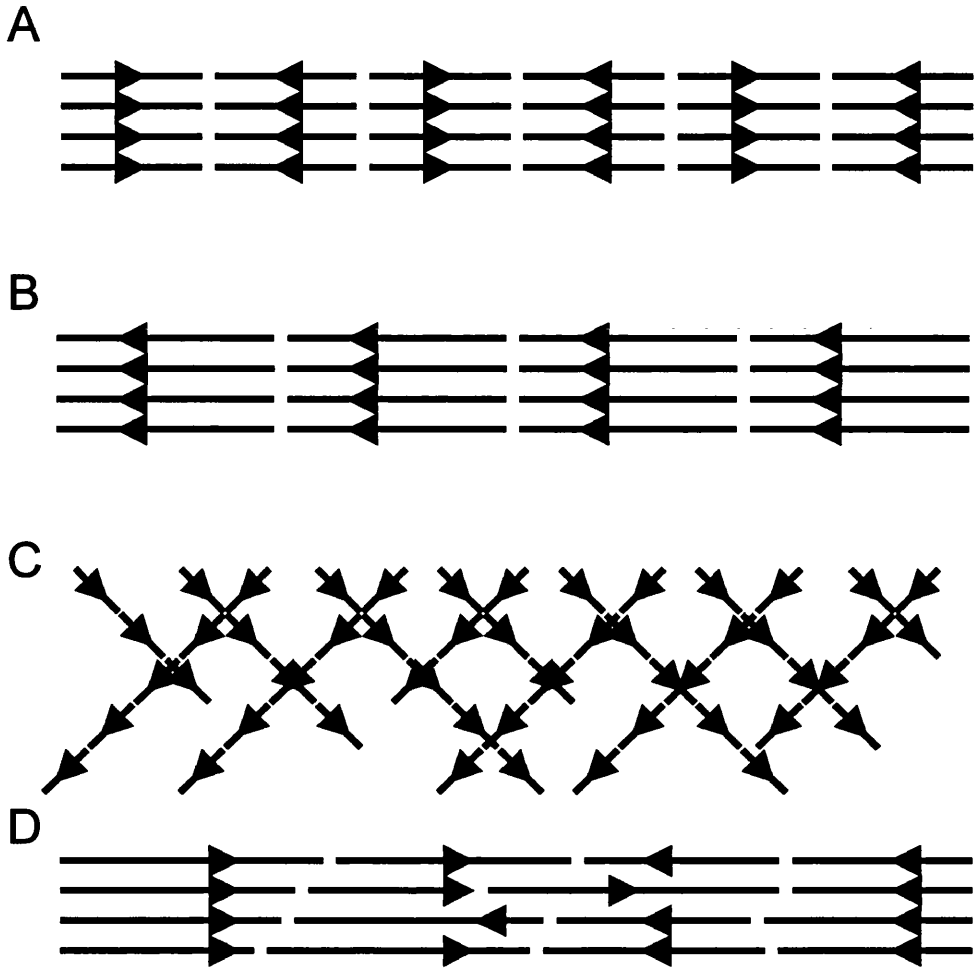

Figure 1.3: Actin filament organisation in cells. (A) to (D): Simplified diagrams of actin polarity in four different actin networks. The polarity of the actin filaments within each bundle or meshwork is shown by arrowheads. The point of each arrowhead denotes the slow growing pointed end, and the rear of the arrowhead denotes the fast-growing barbed end. (A) Actin bundles with alternating polarity are found in stress fibres and the cortical actin bundles of migrating fibroblasts as well as in muscle sarcomeres. (C) The longitudinal bundles of the cell body of a migrating fibroblast show graded polarity. Within the graded polarity bundle, polarity varies according to cell position and length of the fibre. (B, D) Bundles with uniform polarity are found in filopodia and microspikes (B) and also within the actin meshwork that makes up the dentritic brush of lamellipodia (D). ((A) adapted from Cramer et al 97, (D) adapted from Cramer 1999a)

Filopodia

Filopodia and microspikes are actin protrusions that either protrude beyond the lamellipodium or are contained within it, respectively. In terms of structure, they consist of parallel actin bundles with uniform polarity, in other words all the barbed ends point in the same direction (Small *et al.* 1978; Lewis and Bridgman 1992) and see fig. 1.3B). Filopodia are thin extensions of up to 0.2um in diameter and 20μm in length that are formed from a core of parallel bundled actin filaments covered by the plasma membrane (Bailly *et al.* 1998; Jones *et al.* 1998) while microspikes are actin bundles contained within the lamellipodium that may be precursors to filopodia (Small *et al.* 2002b). Like the branched networks of the lamellipodium (see below), filopodia do not require anchorage to the substratum to form (Small *et al.* 1999b). Once formed, filopodia are very stable; the half-life of actin filaments within a filopodium is at least 25 minutes (Mallavarapu and Mitchison 1999). The function of filopodia is thought to have to do with sensing of guidance cues and the extracellular matrix (Bailly *et al.* 1998).

Branched filaments of lamellipodia

A dense network of heavily branched actin filaments is found within lamellipodia where it forms a structure termed the dentritic brush (Svitkina and Borisy 1999). This has been most extensively studied in fish and *Xenopus* keratocytes (Small *et al.* 1995; Svitkina *et al.* 1997; Svitkina and Borisy 1999). The highest filament density tends towards the front of the lamellipodium, gradually decreasing with distance from the leading edge and the barbed ends point towards the plasma membrane (Svitkina *et al.* 1997; Svitkina and Borisy 1999). Within the dentritic brush the actin filaments are highly cross-linked with numerous branches (Svitkina *et al.* 1997) that grow out at a 70 degree angle (Mullins *et al.* 1998) from the side of other "mother" filaments, forming Y-junctions (fig. 1.3C). Each actin filament within this array is fairly short (Cramer 2002) and turnover is rapid, as little as 0.5-3 minutes (Wang 1985; Theriot and Mitchison 1991, 1992). The shortness and stiffness of the actin filaments pushing up to the leading edge allows force to be applied to the plasma membrane without resulting in filament breakage, thus enabling protrusion of the lamellipodium to occur (Mogilner and Oster 1996).

It should be noted here however, that highly branched filaments are not the only actin structures found within lamellipodia. Motile fibroblasts also contain much longer (l20μm) and less branched actin filaments (Cramer 2002), distinct from actin contiguous with filopodia, that extend through the lamellipodium. The function of these filaments is less clear than the highly branched dentritic brush of keratocytes but changes in filament length and/or branching may be important in initiation of cell migration from a stationary state (Cramer 2002).

Actin organisation in the cell body

As mentioned above, less is known about the organisation of actin filaments within the cell body of migrating cells, however information from fibroblasts, keratocytes and growth cones has begun to shed some light on the structural organisation of actin filaments within this region.

Graded polarity bundles

Graded polarity bundles are the most abundant actin structure found in locomoting fibroblasts (Cramer *et al.* 1997). They are stable structures with a slow turnover rate (Cramer *et al.* 1997) that are found in the lamella, cell body and tail and consist of long overlapping actin bundles with an average length of 13μm, although they can be much longer at up to 30μm (Cramer *et al.* 1997). Unlike stress fibres graded polarity bundles do not have a sarcomeric structure. Instead, polarity changes along the length of each actin bundle, with the polarity of the bundle itself determined by its proximity to the front of the cell; the closer to the front, the more barbed ends face forward ((Cramer *et al.* 1997)and see fig. 1.3D). As yet little information is available on how a graded polarity bundle is formed, however it is known that actin incorporates preferentially into the filament ends closest to the leading edge (L. P. Cramer, personal communication). The distribution of actin bundling proteins along these bundles has been analysed and, while fimbrin does not localise at these sites, a-actinin does, suggesting that a-actinin may play a role in the bundling of actin filaments to form graded polarity bundles ((Cramer *et al.* 1997) and L. P. Cramer,

personal communication). The identification of these bundles on the ventral surface of migrating fibroblasts has led to the suggestion it is highly likely that they, in conjunction with myosin, may be responsible for the generation of actin-based motile force to drive forward movement of the cell body during migration (Cramer *et al.* 1997).

Actin organisation in other migrating cell types.

Keratocytes are rapidly migrating cells that, outside of the lamellipodium, contain actin organisation distinct from that found in fibroblasts. Outside of the lamellipodium, most of the actin consists of an oriented dense filament meshwork found in the transition zone between the lamellipodium and cell body. Also present in this region, and increasing in amount with increasing proximity to the cell body, are arc-shaped actin bundles oriented parallel to the leading edge (Svitkina *et al.* 1997). Growth cones contain a single lamellipodium region attached to the long axon. They contain two populations of actin filament within the lamellipodium: long actin bundles that radiate from the leading edge, and a branched network of shorter filaments that fill the volume of the lamellipodium. The polarity of the actin within these two networks differs. The majority of the barbed ends of the long filaments are oriented toward the leading edge, while the orientation of the shorter filaments is more random (Lewis and Bridgman 1992). Growth cones also contain filopodia and these are oriented as described above.

Each of these different actin structures is formed and maintained only under certain conditions and in certain cell types; however single cells have the capacity to possess more than one at any given time. To ensure that formation and turnover of each individual actin network is rigorously controlled, the cell has a host of actin binding proteins. Each filamentous structure is in dynamic equilibrium and small alterations in the activity of a single actin binding protein can rapidly cause re-organisation of the actin filaments to perform different functions (Bamburg and Wiggan 2002).

It is well known that actin assembly is required for protrusion of the lamellipodium and for the other sub-steps of cell migration and morphological

polarity. The molecular details of how the actin cycle is regulated by various important actin-binding proteins are well studied. Also recognised is how actin assembly alone provides the driving force for protrusive motility. Within cells, formation of the dentritic brush at the leading edge of lamellipodia has been the best-studied actin structure. The specific focus of this thesis is the role of actin filament disassembly. In order to fully understand the importance of this process it is useful to see how actin disassembly is integrated into the actin cycle. The next section therefore provides an overview of the current state of our knowledge on the molecular regulation of the actin cycle as a whole.

1.8 Molecular control of actin assembly and disassembly

The vast majority of the enormous amount of work that has gone into understanding the molecular regulation of actin dynamics has focussed on events at the cell margin. Based upon knowledge of the minimal requirements for self-sustaining actin assembly and disassembly in cells (Loisel *et al.* 1999), a model for the assembly and disassembly of actin at the leading edge has been proposed (Pollard *et al.* 2000).

The dendritic nucleation model

"Dentritic nucleation" is the initiation of new actin filaments from a nucleus of three actin monomers, and the initiation of new actin branches at Y-junctions from pre-existing mother filaments (Pollard *et al.* 2000; Pantaloni *et al.* 2001). The dentritic nucleation model is shown in figure 1.4. This section describes the model in the numerical order provided in the figure.

Step one proposes that in the absence of free barbed ends the unpolymerised actin monomer pool remains stable by the prevention of spontaneous filament assembly and capping of barbed ends (Pollard *et al.* 2000). It may be supposed that, because only ATP-actin is competent to polymerise, it would be advantageous for the cell to maintain its actin monomer pool as ADP-actin, thus preventing assembly. This is not the case, however, as the majority of actin in

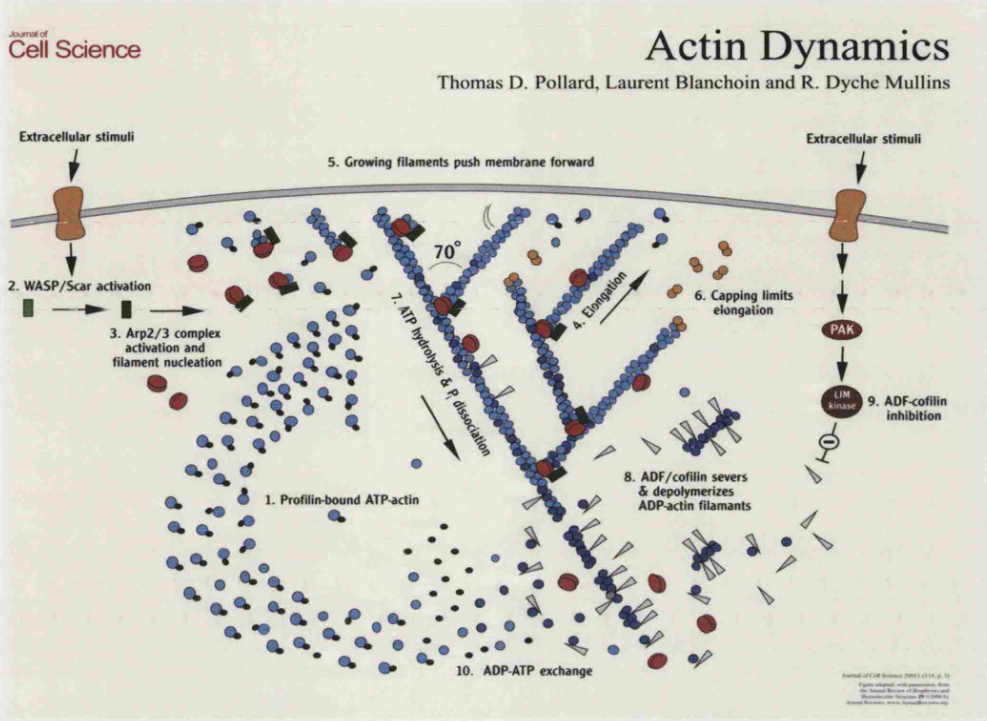

Figure 1.4: The dentritic nucleation model for actin dynamics at the leading edge of motile cells. Dentritic nucleation is a multi-step process. Profilin (black) maintains the actin monomer pool (light blue). On stimulation by extracellular signals, intracellular pathways are activated that lead to the activation of WASP/Scar proteins (green). These then bring actin monomer and Arp2/3 complex (red) together and new filament growth is initiated as a branch at a 70° angle from the side of an existing filament. The new filament extends rapidly from the barbed end and as it grows the membrane is pushed forwards. Capping protein (yellow) binds the barbed end to halt filament growth. ATP hydrolysis within the filament occurs within seconds and the gamma phosphate is released. This promotes debranching and enables AC (grey) to bind to ADP-actin subunits (dark blue) and catalyse filament disassembly. AC activity is regulated by many factors, including phosphorylation at serine 3 which is mediated by PAK and LIMK and inactivates AC by preventing actin binding. Profilin catalyses nucleotide exchange to regenerate ATP-actin for another round of polymerisation. (Taken from Pollard et al 2001).

cells is in the ATP-bound form (Rosenblatt *et al.* 1995). Therefore, cells have monomer sequestering proteins. In protozoa, slime moulds and fungi profilin is the major binding protein for ATP-actin monomer (Pollard *et al.* 2000). In vertebrate cells the main sequestering protein for ATP-actin monomers is thymosin B-4 (Safer *et al.* 1990). Profilin is also present, and competes with thymosin for ATP-actin binding (Carlier *et al.* 1993; Vinson *et al.* 1998; Kang *et al.* 1999) and can act as a shuttle to transport monomer to the barbed ends of actin filaments (Pantaloni and Carlier 1993). If the barbed ends of actin filaments were free, filament elongation would quickly deplete this assembly-competent monomer pool and so the concentration of free barbed ends is held at a low level by capping protein (Schafer *et al.* 1998). Capping protein does not bind the pointed ends of actin filaments, but it is likely that these are capped by Arp2/3 complex (Machesky and Insall 1998).

WASp/Scar proteins are a protein family that interact with the Arp2/3 complex and activate it (Weaver *et al.* 2003). The precise nature of the external stimuli that activate WASp/Scar proteins (step2) is not well established, but can include signalling via integrins, 7-pass transmembrane receptors and receptor tyrosine kinases (Pollard *et al.* 2000). Downstream events include signalling via the Rho family of small GTPases (Machesky and Insall 1999). It has been postulated that since relatively little WASp/Scar may be present in cells and their activation is required for all subsequent steps in the model, activation of WASp/Scar proteins may be the limiting step in actin polymerisation (Pollard *et al.* 2000). Proteins of the Ena/VASP family bind to both actin monomer and F-actin, and are thought to act as connectors that link signalling pathways to organisation of the actin cytoskeleton. The action of these proteins has been recently reviewed (Machesky 2000; Reinhard *et al.* 2001; Cramer 2002; Kwiatkowski *et al.* 2003).

It is generally accepted that the Arp2/3 complex, a complex of seven proteins first identified in Acanthamoeba (Machesky *et al* 1994) and highly conserved from yeast to mammals (Welch *et al.* 1997a); Machesky and Way 1998; Machesky and Insall 1999), regulates actin filament assembly at the leading edge in addition to its role in actin-driven rocketing motility of pathogens

(Mullins *et al.* 1997; Machesky and Way 1998; Machesky and Insall 1999; Mullins and Pollard 1999; Svitkina and Borisy 1999; Insall *et al.* 2001). WASp/Scar proteins directly bind the Arp2/3 complex to activate it and stimulate nucleation of new actin filaments from actin monomer (Machesky and Insall 1998) (step 3). This process provides free barbed ends for elongation. Actin filaments act as co-activators of Arp2/3 complex and this promotes filament branching (Pollard *et al.* 2000), producing the Y-junctions observed by electron microscopy at the leading edge (Svitkina *et al.* 1997; Svitkina and Borisy 1999). Other ways of producing free barbed ends include uncapping, possibly a mechanism that regulates filopodium elongation (Mallavarapu and Mitchison 1999), and severing of actin filaments by gelsolin or AC, which appears to be a major mechanism in platelet activation (Pollard *et al.* 2000).

Rapid elongation of the new filament (step 4) is the next step and occurs primarily at the barbed end. The reconstitution of pathogen rocketing motility using a minimal set of proteins (Loisel *et al.* 1999) definitively established that no myosin motor activity is required for protrusive motility, therefore how does actin polymerisation alone drive forward movement (step 5)? The idea that actin polymerisation provides the force to push the plasma membrane was first proposed by Tilney et al (Tilney *et al.* 1981) and was finally demonstrated experimentally *in vitro* in 1999 (Miyata *et al.* 1999), however the exact mechanism whereby actin polymerisation generates force is not well understood. The "elastic Brownian ratchet model" (Mogilner and Oster 1996) and its extension the "tethered ratchet model" (Mogilner and Oster 2003) propose that the actin filaments act like springy wires that bend due to thermal energy, allowing actin monomer to assemble into the filament between the end of the actin network and the plasma membrane. Subsequent straightening of the filament pushes the membrane forwards. For this to be the case, the actin filaments need to have a critical length and stiffness, be anchored to the substratum or cross-linked, and have an optimal angle to the membrane of 45 degrees. All of these have been shown to hold true (Small *et al.* 1995; Svitkina *et al.* 1997; Blanchoin *et al.* 2000a; Cramer 2002). Despite this evidence, it is not clear that actin polymerisation is the sole mechanism of protrusive behaviour and others, such as lipid flow from membrane recycling (Bretscher

and Aguado-Velasco 1998) may play a part. Finally, unlike protrusion of the leading edge, other types of motility including cell body translocation and tail retraction are myosin dependent (Mitchison and Cramer 1996).

Filaments are only able to elongate for a relatively short period as capping protein rapidly blocks polymerisation (step 6) by tightly binding to the barbed ends (Pollard *et al.* 2000); therefore new barbed ends must be produced at the same rate to that of capping (Pollard *et al.* 2000). This requires continuous activation of Arp2/3 complex as the activated complex is incorporated into the nascent actin filament. Capping protein is abundant in cells and enriched in Arp2/3 complex-containing lamellipodia (Schafer *et al.* 1998). The function of capping is probably to bias actin polymerisation, through nucleation and elongation of new filaments, to the extreme leading edge where force is required (Borisy and Svitkina 2000). Barbed ends elsewhere would compete for monomer and deplete the monomer pool, reducing the likelihood of effective protrusion.

The intrinsic hydrolysis of ATP-actin to ADP-actin with the release of inorganic phosphate is thought to target filaments for disassembly (step 7). As actin filaments extend using ATP-actin, a gradient of ATP-actin, ADP.P_i-actin and ADP-actin will be formed along the filament from the barbed to the pointed end. This is an effective way of marking filaments for disassembly as the ACs bind ADP-actin with greater affinity than the ATP or ADP.P_i bound forms (Bamburg 1999). Disassembly and severing by AC proteins (step 8), mediated by PAK and LIM kinase (step 9) can then occur. These are the subject of this work and are discussed in detail below. Disassembly results in newly released ADP-actin monomer, which must be regenerated as ATP-actin to be assembly-competent (step 10). Profilin competes with AC for binding to ADP-actin and catalyses nucleotide exchange (Maciver *et al*, 1991; Nishida, 1985; Blanchoin and Pollard 1998). This process both regenerates the pool of assembly-competent actin monomer, and releases AC to facilitate another round of depolymerisation.

Molecular control of actin dynamics in the cell body

The mechanism of the actin assembly and disassembly cycle elsewhere in the cell remains less well understood. In comparison to the in-depth molecular model detailed above, information about the molecular control of actin dynamics within the cell body is limited and largely restricted to studies of the structural organisation of actin and its turnover time within various cellular regions (see above). The molecular regulation of assembly and disassembly of these networks is not at all clear. It is likely that many of the actin binding proteins that regulate assembly of the dentritic brush will also be involved in actin dynamics in the rest of the cell; however there must be important differences to account for the huge difference in turnover rate and structural orgnaisation.

One such difference may be in how the actin filaments are nucleated. It is now known that the Arp2/3 complex is not the only actin nucleator. Since the Arp2/3 complex promotes filament branching and the actin networks of the cell body are not typically branched, alternative actin nucleators are likely. The other known option to the Arp2/3 complex is the formins, which were recently shown to have actin nucleating activity (Pruyne *et al.* 2002; Sagot *et al.* 2002) and promote the assembly of unbranched actin filaments (Wallar and Alberts 2003).

1.9 Bundling and stabilisation of actin filaments

Once polymerised, actin filaments are further organised by being bundled together and stabilised. This is facilitated by a variety of different actin binding proteins that appear to act sequentially to form higher order structures (Bartles 2000). The combination of actin bundling proteins present varies greatly between cell types. In leukocytes, cross-linking proteins include fimbrin, a-actinin and calpactin (Friedl *et al.* 2001). Talin is another cross-linking protein that is proposed to facilitate lamellipodia-cell matrix interactions by binding to B-integrins (Adams 2002). In Dictyostelium pseudopodia, coronin and ABP120 are thought to cross-link actin filaments (Friedl *et al.* 2001). Finally, the highly ordered parallel actin bundles found in brush border microvilli are formed by

the combined actions of three bundling proteins: villin, fimbrin and small espin (Bartles 2000).

Given that actin will hydrolyse to the ADP-bound form over time and that AC is extremely efficient at disassembling ADP-actin, how do the stable filaments of the cell body survive in the cytosol? Two main, and interrelated, mechanisms are possible: regulation of AC activity, which is dealt with in detail below, and actin binding of proteins such as tropomyosin, which acts as a stabilising protein.

The majority of actin microfilaments within cells contain a rod-like tropomyosin polymer that is inserted into the a-helical groove of the actin (Gunning *et al.* 1998). Tropomyosin exists in both muscle and non-muscle forms and is encoded by a multigene family, of which 4 genes are currently known (Pittenger *et al.* 1994), generating more than 20 different tropomyosin isoforms by alternative splicing (Pittenger *et al.* 1994). In non-muscle cells, tropomyosin stabilises actin filaments by preventing both severing and depolymerisation from the pointed end (Bernstein and Bamburg 1982; Broschat 1990) and the region of rapid actin turnover at the leading edge of EGF-stimulated adenocarcinoma cells shows very low levels of various tropomyosin isoforms (DesMarais *et al.* 2002), suggesting that these filaments are available for AC-mediated disassembly. Tropomyosin may have additional roles than the prevention of filament disassembly since in vitro it inhibits Arp2/3 complex nucleated filament assembly (Blanchoin *et al.* 2001). The function of the large number of non-muscle tropomyosin isoforms is not well understood. Recent data suggests that, while some isoforms prevent AC binding to actin, others may facilitate this interaction to differentially regulate morphological changes and cell migration (Bryce *et al.* 2003). Further work will be needed to determine the precise relationship between individual tropomyosin isoforms and actin turnover.

In contrast to actin filament assembly and lamellipodium protrusion, there are several important issues for cell migration and morphological polarity that are less well understood. These include the role of actin filament disassembly and

AC proteins in cell migration and morphological polarity, the source of the actin monomer used to fuel actin assembly, and the mechanism of acquisition and maintenance of cell polarity during migration. These issues are the focus of this thesis and therefore the remainder of this introduction will deal with the background to AC proteins (section 1.10), the role of actin filament disassembly in polarised protrusion (section 1.11) and morphological polarity (section 1.12).

1.10 The ADF/cofilin family

Experiments have shown that the rate of actin turnover in cells is more than 100 fold faster than that measured in vitro (reviewed by (Zigmond 1993)). Catalysis of actin disassembly must therefore occur in cells. Initially put down to the combined actions of many actin binding proteins fulfilling various functions, this is now known to be carried out by the AC family, essential proteins that act as the prime catalysers of filament disassembly in cells (Carlier *et al.* 1997; Lappalainen *et al.* 1997; Rosenblatt *et al.* 1997). These proteins promote rapid actin turnover in vivo (Lappalainen and Drubin 1997) and consist of many family members, spread across all eukaryotes (Bamburg 1999).

The first family member to be identified came from actin-depleted embryonic chick brain extracts and was observed to cause an increase in the actin monomer pool (Bamburg *et al.* 1980). This function led to it being named actin depolymerising factor, or ADF. A second protein with actin depolymerising activity were isolated from starfish eggs (Mabuchi 1981) and named depactin. The first mammalian ADF was isolated from bovine brain extracts in 1983 (Berl *et al.* 1983) and other family members soon followed: destrin (destroys F-actin) from porcine brain (Maekawa *et al.* 1984; Nishida *et al.* 1984) and kidney (Nishida *et al.* 1985), cofilin (forms cofilamentous structures with actin) from porcine brain (Maekawa *et al.* 1984), and actophorin from *Acanthamoeba castellani* (Cooper *et al.* 1986). Since 1990, many more family members have been identified by cDNA cloning, including yeast cofilin from *Saccharomyces cerevisiae* (Moon *et al.* 1993), twinstar from *Drosophila melanogaster*

(Edwards *et al.* 1994; Gunsalus *et al.* 1995) and XAC1 and XAC2 from *Xenopus laevis* (Abe *et al.* 1996).

AC structure and mode of action

Each AC family member is between 13-19 kDa and despite considerable sequence diversity each contains highly conserved regulatory regions (fig. 1.5A). Structural analysis of destrin (Hatanaka *et al.* 1996), actophorin (Leonard *et al.* 1997) and yeast cofilin (Fedorov *et al.* 1997) has demonstrated that family members share a similar three-dimensional topography, being composed of a structural motif termed the ADF homology domain (Lappalainen *et al.* 1998) that consists of a central six-stranded β-sheet in between two pairs of α-helices (fig. 1.5B). Mutagenesis studies on yeast (Lappalainen *et al.* 1997) and chicken (Kusano *et al.* 1999) cofilin have demonstrated that actin binds towards the N-terminus of AC, although other residues throughout the protein are needed for interaction with actin (fig. 1.5A, asterisks) and further support for this was provided by identification of a regulatory serine (Ser3 in mammals and insects, Ser6 in plants) that abrogates F-actin binding when phosphorylated (Agnew *et al.* 1995) (see below). AC has a greater affinity for ADP-actin than ATP-actin (Carlier *et al.* 1997). Two actin subunits interact with AC during F-actin binding (McGough *et al.* 1997) and AC binding reduces the degree of twist in the actin filament by 4-5 degrees per subunit without altering the subunit length (McGough *et al.* 1997). One consequence of this conformational change may be to increase the distortion of the filament, making it more likely to fragment and increasing the rate of monomer dissociation from the pointed end (McGough *et al.* 1997). In addition, AC also disrupts lateral actin:actin contacts in the filament, promoting unwinding of the actin helix, fraying of the ends, and increased branching (McGough and Chiu 1999).

The precise mechanism whereby AC disassembles actin filaments has been controversial. The complete picture is not yet clear, but it is understood that AC can depolymerise actin by two mechanisms: severing (Maciver 1998) filaments to provide increased free filament ends that can disassemble where conditions

A

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S.cer. Cof1 **MSRS**GVA**VADESLTAFNDLKL*********GKKYKFILFGLNDAK********TEIVVKETSTDPS**
A.cas. Acto ****MS**GIA**VSDDCVQKFNELKL*********GHQHRYVTFKMNASN********TEVVVEHVGGPNAT*
H.sap. Des1 ***MAS**GVQ**VADEVCRIFYDMKVRKCSTPEEIKKRKKAVIFCLSADKKCIIVEEGKEILVGDVGVTITDP
M.mus. Cof1 ***MAS**GVA**VSDGVIKVFNDMKVRKSSTPEEVKKRKKAVLFCLSEDKKNIILEEGKEILVGDVGQTVDDP

S.cer. Cof1 *******YDAFL*EKLP**END**CLYAIYDFEYEIN*****GNEGKRSKIVFFTWS*PDTA*PVRSKMVYASS
A.cas. Acto *******YEDFK*SQLP**ERD**CRYAIFDYEFQVD*******GGQRNKITFILWA*PDSA*PIKSKMMYTST
H.sap. Des1 *******FKHFV*GMLP**EKD**CRYALYDASFETK*******ESRKEELMFFLWA*PELA*PLKSKMIYASS
M.mus. Cof1 *******YTTFV*KMLP**DKD**CRYALYDATYETK*******ESKKEDLVFIFWA*PENA*PLKSKMIYASS

S.cer. Cof1 KDALRRALNG******VSTDVQGTDFSEVS******************YDSVLERVSRGAGSH**********
A.cas. Acto KDSIKKKLVG******IQVEVQATDAAEIS******************EDAVSERAKKDVK**********
H.sap. Des1 KDAIKKKFQG******IKHECQANGPEDLN******************RACIAEKLGGSLIVAFEGCPV*****
M.mus. Cof1 KDAIKKKLTG******IKHELQANCYEEVKD*****************RCTLAEKLGGSAVISLEGKPL*****
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B

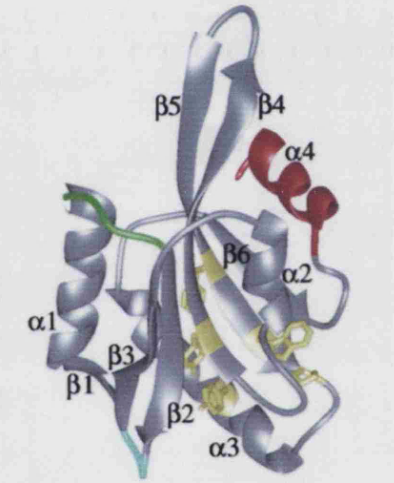


Figure 15: Features of the ADF homology domain. (A) Sequence alignments of ADF homology domains from yeast cofilin, actophorin, destrin and muscle cofilin. Black asterisks indicate positions filled by residues in other AC family members. The red asterisks indicate residues in yeast cofilin that are required for interaction with actin (Lappalainen et al 1997). The large arrow shows the major regulatory serine and the small arrow denotes the serine needed for 14-3-3ζ binding. (B) Ribbon model of the yeast cofilin structure. Insertions in mammalian cofilins are shown in green and blue, and red represents a region that is divergent in members of the twinfilin family. Yellow shows highly conserved residues for protein stability and folding. (A) and (B) adapted from (Lappalainen et al 1998)

are favourable, and increasing the rate of pointed end disassembly (Carlier *et al.* 1997). The extent to which each of these contributes to actin disassembly remains contentious. Much evidence has been accumulated that demonstrates AC-mediated actin severing (see (Pollard *et al.* 2000)). The degree of severing depends on both the amount of AC bound to the actin and time, but is low in relation to the amount on AC bound (Blanchoin and Pollard 1999). AC is thus said to have weak severing activity. AC promotes pointed end disassembly by around 30-fold (Carlier *et al.* 1997). This enhancement of the rate of disassembly may not be sufficient to account for the actual *in vivo* rates and other factors may be involved. Profilin acts in synergy with AC to further increase the rate of filament turnover (Didry *et al.* 1998). Another candidate is the accessory protein actin interacting protein 1 (Aip1), a conserved protein that localises to regions of rapid actin dynamics in *Dictyostelium* (Konzok *et al.* 1999) and interacts with both actin and AC to enhance the rate of filament turnover (Okada *et al.* 1999; Rodal *et al.* 1999). The mechanism is as yet unknown, but Aip1 does not have any significant severing activity of its own (Okada et al 99). One hypothesis is that it may aid AC binding to actin filaments (Pollard *et al.* 2000).

Isoform expression and tissue distribution

Although single-celled organisms express only a single AC member, most organisms possess more than one AC; for example vertebrates express ADF, muscle cofilin, and non-muscle cofilin, which have differing expression patterns during development (Bamburg 1999). The expression patterns of ADF and cofilin differ across tissues and cultured cell lines. While cells may express both ADF and cofilin, one or the other tends to be more abundant (Maciver and Hussey 2002). This is normally cofilin (Maciver and Hussey 2002); however in chick fibroblasts the opposite holds true and these cells contain approximately 95% ADF (J. Bamburg, personal communication).

Regulation

Regulating AC in cells is essential in order to prevent aberrant actin filament disassembly. As many different actin organisations are found within a single cell (see above) and the dynamics of each of these must be separately controlled, AC activity is required to be tightly regulated within each spatial region.

AC activity is known to be regulated in several different ways to spatially and temporally modulate actin dynamics. Possibly the principal mechanism regulating AC activity in cells is phosphorylation on a single conserved serine residue close to the N-terminus (Agnew *et al.* 1995) (fig. 1.5A, large arrow). Phosphorylation renders AC inactive in *in vitro* depolymerisation assays (Morgan *et al.* 1993) but it can be dephosphorylated and reactivated (Agnew *et al.* 1995) in cells by the phosphatase Slingshot (Niwa *et al.* 2002). Phosphorylation does not change the conformation of AC, but instead creates a repulsive charge that inhibits actin binding (Blanchoin *et al.* 2000b). Dephosphorylation occurs rapidly, however it has been demonstrated that this need not necessarily coincide with any change in the global ratio of phosphorylated to non-phosphorylated AC (Meberg *et al.* 1998). The half-life of the phosphate on AC decreases from around 5-7 minutes to less than 2 minutes on stimulation with EGF (Meberg *et al.* 1998). This suggests that the total net phosphorylation of AC may not be as important as the rate of phospho-cycling.

To date, two families of protein kinases are known to carry out phosphorylation of AC on ser3: LIM kinases, of which there are currently two members, were the first to be identified (Arber *et al.* 1998; Yang *et al.* 1998) and remain the best studied. More recently TES kinases (currently TESK1 and 2) were found to carry out the same reaction, but while TESK1 is cytoplasmic, TESK2 is predominantly nuclear (Toshima *et al.* 2001a; Toshima *et al.* 2001b). Plant ADF is not phosphorylated by LIMK1 in vitro (Bamburg 1999). Therefore plants may have evolved alternative kinases to carry out this phosphorylation event. One possibility is a calmodulin-like domain protein kinase that has been

found to phosphorylate ser6 in plants (Allwood *et al.* 2001; Smertenko *et al.* 2001). LIM kinases are ubiquitously expressed (Bamburg and Wiggan 2002) and are effectors of the Rho family of small GTPases. Rac and Cdc42 activate PAK (Manser *et al.* 1994), which activates LIMK1 (Edwards *et al.* 1999) and Rho activates ROCK (Matsui *et al.* 1996), which can activate LIMK2 (Maekawa *et al.* 1999). TESK1 kinase activity, however, is not stimulated by either ROCK or PAK (Toshima *et al.* 2001a; Toshima *et al.* 2001b).

In many cell types AC activation by dephosphorylation occurs swiftly in reaction to a stimulus (reviewed by (Moon and Drubin 1995; Theriot 1997)); for example neutrophils rapidly dephosphorylate AC and translocate it to actively motile membrane regions within 30 seconds of stimulation (Suzuki *et al.* 1995; Heyworth *et al.* 1997; Nagaishi *et al.* 1999), and EGF-stimulation of adenocarcinoma cells causes a burst of AC activity at the cell margin (Chan *et al.* 2000; Zebda *et al.* 2000). Therefore, AC dephosphorylation must be tightly regulated. Restricting AC activity to a single region of the cell margin by closely controlling the level of AC phosphorylation at this site might provide an effective means of providing rapid actin dynamics in one cellular location, thus enabling polarisation of the cytoskeleton and productive migration. Regulation of AC dephosphorylation is less well understood. Dephosphorylation occurs via a recently identified AC phosphatase called slingshot (Niwa *et al.* 2002). The upstream signalling that leads to this event are unclear, but it would make sense if slingshot were regulated by the Rho GTPases, as this would allow coordinate regulation of AC through both the kinases and phosphatase, however to date this has not been demonstrated. Binding of phosphorylated AC to 14-3-3zeta shields the phosphate group from exposure to other proteins, thus stabilising pAC against dephosphorylation and increasing the pool of inactive AC (Gohla and Bokoch 2002). These experiments were, however, carried out at high 14-3-3zeta levels and whether pAC shielding by 14-3-3zeta occurs under physiological conditions remains to be verified. An important residue within AC for 14-3-3zeta binding is a single serine at position 24 that is found in AC proteins in higher organisms including destrin and muscle cofilin 1 (fig. 1.5A, small arrow, J. Bamburg, personal communication). Some of the known

signalling components controlling the phosphorylation status of AC on ser3 in animal cells are summarised in figure 1.6.

A second way of controlling where AC-catalysed actin filament disassembly can occur is to spatially regulate AC distribution within the cell. This is predicted to occur in cells as the rates of actin turnover vary in different cellular regions, being most dynamic at the leading edge. As a consequence, more AC activity is likely to be required at the leading edge than in other cellular regions. ADF and cofilin appear to be enriched in regions of rapid actin turnover such as ruffling membrane edges of lamellipodia (Bamburg and Bray 1987; Yonezawa *et al.* 1987), while to date the phosphorylated form has not been shown to be localised to a particular cellular region (Bamburg 1999). Within the lamellipodium, AC appears to be depleted from the first 0.5μm of highly motile keratocytes, while non-migrating fibroblasts AC is evenly distributed throughout lamellipodia (Svitkina and Borisy 1999). Whether this difference reflects a cell-type specific difference in distribution or is related to the migration capability of the cell remains to be determined.

AC contains a nuclear localisation signal (Matsuzaki *et al.* 1988) and under conditions of stress AC and actin collect in the nucleus as highly stable rod structures (Nishida *et al.* 1987). The function of the rods, which can also be induced in the cytosol (Bershadsky *et al.* 1980; Iida and Yahara 1986) is unknown, but may be a form of regulation by sub-cellular compartmentalisation that exists to conserve ATP in times of stress by reducing actin dynamics (Daniel *et al.* 1986; Bamburg 1999). Recent data suggests that cofilin can also be localised to mitochondria in an early step in apoptosis induction (Chua *et al.* 2003). Thus, AC proteins are not only restricted to the cytosol.

It has been reported that AC and tropomyosin binding to actin are mutually exclusive (Bernstein and Bamburg 1982; Nishida 1985), presumably by affecting AC's ability to induce a change in the twist of the actin filament (McGough *et al.* 1997). This is an oversimplification as multiple tropomyosin isoforms exist in cells and only some of these compete with AC for actin binding (Ono and Ono 2002; Bryce *et al.* 2003). As different tropomyosin

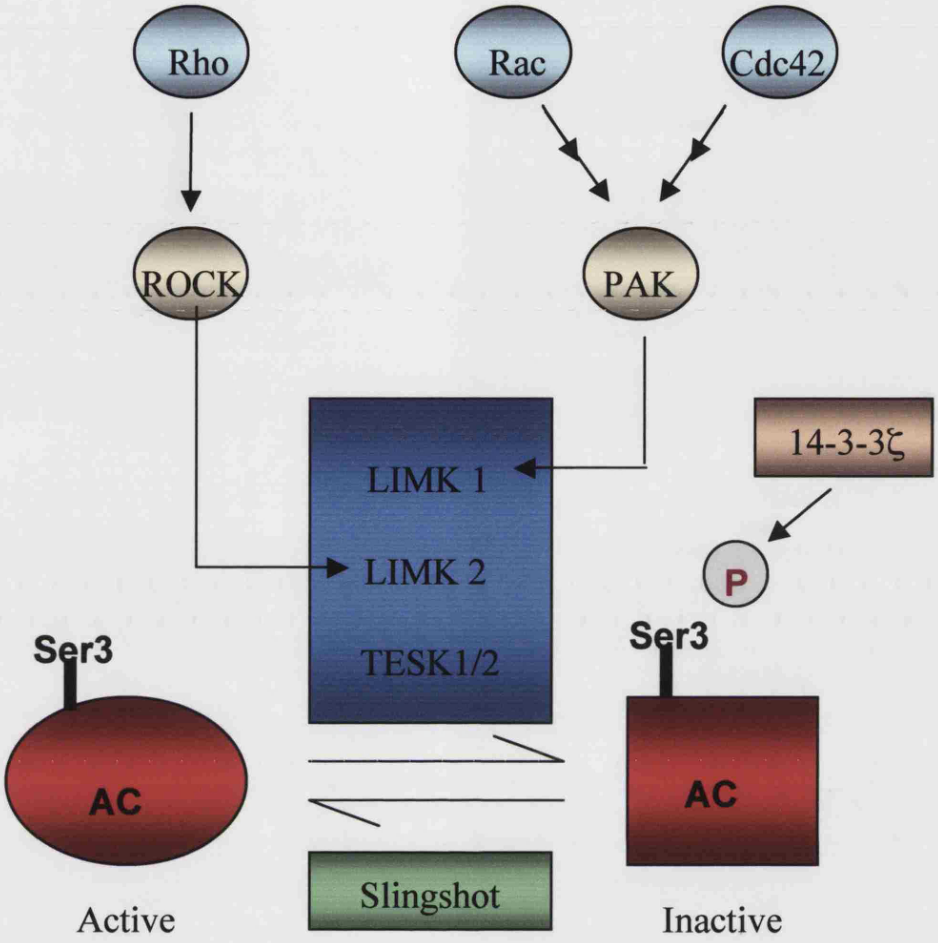


Figure 1.6: Signalling pathways regulating AC phosphorylation. Phosphorylation of serine 3 renders AC inactive (red square) and is known to be mediated by four different protein kinases (blue box): LIMK1 and 2, and TESK1 and 2. LIMK1 and 2 are activated by phosphorylation by the Rho family GTPases (light blue ovals) via ROCK or PAK (cream ovals) Upstream regulators of TESK1 and 2 are unknown, but TESK is not regulated by either ROCK or PAK. 14-3-3ζ (peach box) binds phosphorylated AC and protects it from dephosphorylation by Slingshot (green box). The regulation of Slingshot is unclear, but may involve PKB/Akt (cream oval). Abbreviations: AC, ADF/cofilin; TESK1/2, testicular protein kinase 1/2; LIMK1 and 2, LIM kinase 1 and 2; ROCK, Rho-associated kinase; PAK, p21-activated protein kinase

isoforms localise to different cell regions in neurones (Weinberger *et al.* 1996) it is likely that tropomyosin can regulate which actin filaments are available for turnover.

Since one way that AC disassembles actin is by increasing end-wise depolymerisation from pointed ends, capping of the pointed ends will inhibit AC activity. This is achieved by actin binding proteins such as spectrin and the Arp2/3 complex (Bamburg 1999).

The majority of AC proteins show pH dependence in their ability to depolymerise actin, with enhanced depolymerising activity at pH 8.0 than at pH 7.0 (reviewed by (Carlier *et al.* 1999)). Additionally in Swiss 3T3 fibroblasts, the distribution of ADF and cofilin is pH regulated. In these cells, ADF is found co-localised more with G-actin and less with F-actin at pH 7.1-7.4 compared with pH 6.8 while cofilin does not change its distribution over this pH range (Bernstein *et al.* 2000). The precise role that pH plays in the regulation of AC in vivo has not been determined, but one noteworthy point is that large pH changes are likely to occur close to the membrane at the leading edge of cells, where high levels of ion exchange activity by transporters such as the Na^+/H^+ pump may occur.

AC activity can be regulated by phosphatidylinositol 4-phosphate (PtdIns(4)P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (Yonezawa *et al.* 1990). Binding of these lipids to a region that overlaps with the actin-binding domain of AC (Van Troys *et al.* 2000) inhibits AC's ability to bind actin (Yonezawa *et al.* 1991; Kusano *et al.* 1999). It has been suggested that one function of PtdIns(4,5)P_2 binding may be to localise AC to the membrane, where rapid actin turnover, as well as PtdIns(4,5)P_2 metabolism, is often needed (Maciver and Hussey 2002).

The relative contribution of each of these mechanisms to the regulation of AC activity in cells is uncertain and the ways in which AC is regulated in different cellular locations are poorly understood It has been generally assumed that phosphorylation is probably the key regulator in cells, although how this is

spatially controlled is not clear, while tropomyosin is expected to be crucial for the stabilisation of actin filaments in the cell body. As discussed above, pH and phosphoinositide binding may be important at the leading edge. Also unknown is how far the exact means of AC regulation will be conserved between cell types, or between cells that perform different functions; for example migrating versus stationary cells. Tightly regulating the activity of proteins such as AC, which play central roles in biological processes, is extremely important. Aberrant regulation of AC is likely to severely disrupt actin dynamics and to date has been implicated in a variety of disease states. AC function and involvement in disease are detailed in the next two sections.

Function of AC in cells

Within cells the AC proteins have multiple functions, which have in the main been elucidated from studies where AC activity has been either enhanced or compromised. Each of these processes is dependent on actin dynamics.

Genetic studies that result in null forms of AC have demonstrated that AC members are essential proteins. In *Saccharomyces cerevisiae* disruption of the yeast cofilin gene is lethal and cells fail to divide (Iida *et al.* 1993; Moon *et al.* 1993), suggesting a cytokinesis defect. The role of AC in cytokinesis was confirmed using a mutation in the *Drosophila* AC twinstar that gives a defective form of the protein without generating a null (Gunsalus *et al.* 1995) and by studies where LIMK1 overproduction (and hence AC inactivation) results in multinucleated cells (Amano *et al.* 2002). In addition to its part in cytokinesis, AC is needed for cell migration (Maciver and Weeds 1994; Chen *et al.* 2001), muscle development (Abe *et al.* 1989; Ono and Benian 1998; Ono *et al.* 1999) and phagocytosis (Nagaishi *et al.* 1999).

In 1999, the minimal requirements for actin filament assembly were determined by taking advantage of the ability of certain pathogenic micro-organisms including *Listeria monocytogenes* and *Shigella flexneri* to hijack the host cell cytoskeleton and form actin "comet tails" that allow the pathogen to move

around the infected cell in a rocket-like movement. These organisms by-pass the normal extracellular signalling cascades that trigger actin polymerisation and use their own proteins to start actin assembly using a simplified version of the cell's own motility apparatus (Theriot *et al.* 1992; Welch *et al.* 1997a; Welch *et al.* 1998; Egile *et al.* 1999). During infection, this motility allows the pathogen to travel between cells without exposure to the immune system or to leave the cell completely (Machesky 1999). Carlier and colleagues successfully reconstituted formation of an actin comet tail and rocketing motility of pathogenic bacteria in a defined in vitro system (Loisel *et al.* 1999) using only 4 highly conserved proteins. One of these was AC, providing further evidence that these proteins are not only essential for life, but are essential for cell movement. Precisely why AC is needed for cell migration is unclear. One explanation, given in this work, is that AC activity is required to initiate and maintain morphological cell polarity as is necessary for normal cell migration.

ACs in disease

AC proteins have been implicated in a variety of pathological conditions. These include neurodegeneration, ischaemia, cancer and the genetic disease Williams Syndrome. This section reviews the state of our knowledge about each of these in turn. More tenuous links have been made between aberrant AC activity and inflammation, infertility and immune deficiency. These have been recently reviewed (Bamburg and Wiggan 2002).

Much of the work on the pathology of aberrant AC activity concerns neurodegeneration. Hirano bodies are a characteristic of aging brain tissue and are prevalent in the brains of patients with Alzheimer's disease (Bamburg and Wiggan 2002). They are rod-shaped aggregates of filaments (Schochet and McCormick 1972) that contain both actin (Goldman 1983) and abundant AC (Maciver and Harrington 1995). Rod structures that contain AC and actin have been found in the hippocampus and frontal cortex of the brains of Alzheimer's patients (Minamide *et al.* 2000) and these somewhat resemble Hirano bodies in their appearance and composition (Bamburg 1999). Initially thought to be an

effect of neurological damage rather than a cause of the degeneration itself (Maciver & Harrington 95), the observation that neurite function is disrupted by AC/actin rods (Minamide *et al.* 2000) may mean a more major role for the rods in the development of neurodegeneration than has previously been thought.

In the proximal tubule cells of kidney, ischemia results in disruption of the apical membrane apical cytoskeleton and a decrease in ATP levels (see (Sutton and Molitoris 1998) for a review). Following ischemia there is a huge loss of apical membrane by blebbing into the lumen of the proximal tubule (Bamburg and Wiggan 2002). Ischemia activates AC by causing its dephosphorylation and the activated AC localises to the apical cytoskeleton (Schwartz *et al.* 1999). It has been proposed that AC-mediated loss of the apical cytoskeleton is one of the events that allow the blebbing to occur (Bamburg 1999). The lost membrane results in formation of protein aggregates that block the tubule, leading to reduced glomerular filtration and acute kidney failure (Bamburg and Wiggan 2002).

Williams syndrome is a complex disease caused by a chromosomal deletion (Martindale *et al.* 2000) that leads to mild mental retardation (Rosenblatt and Mitchison 1998) and severe visuo-spatial cognition problems that have been traced to a defect in neuronal pathfinding caused by a defect in LIMK1 (Frangiskakis *et al.* 1996; Bellugi *et al.* 1999). As LIM kinase and AC play a role in neuronal pathfinding by mediating the response to repulsive guidance cues (Aizawa *et al.* 2001), impaired AC regulation might lead to incorrect neuronal migration.

While a direct role for AC in cancer has not been found, any molecule that can cause increases in the rates of cell division or cell migration has the potential to play a part in the development of malignancy, and altered AC levels have been found in ovarian cancers (Martoglio *et al.* 2000). Cell transformation requires cytoskeletal changes that decrease the cell's dependence on adhesion for growth. This occurs by inhibition of Rho signalling by the MAP kinase kinase MEK, resulting in AC dephosphorylation and activation (Pawlak and Helfman 2002). A role for cofilin has also been demonstrated in the formation of large

numbers of new barbed ends that are needed for enhanced cell migration in tumour cells (Ichetovkin *et al.* 2002).

1.11 The importance of actin filament disassembly for polarised protrusion

Prior to 1999, the role of actin filament disassembly was largely perceived as a rather constitutive process that occurred merely to replenish the cellular actin monomer pool, and so enable on-going actin filament assembly. The two classes of proteins that catalyse actin disassembly had been identified as AC and gelsolin (reviewed by (Welch *et al.* 1997b)) and it was known that AC proteins were primarily responsible for the catalysis of actin filament disassembly in cells (Carlier *et al.* 1997; Lappalainen and Drubin 1997; Rosenblatt *et al.* 1997).

In 1999 it was shown for the first time that filament disassembly is necessary for lamellipodium protrusion. Furthermore, the requirement for actin filament disassembly differed between polarised migrating cells that protrude a single lamellipodium, and cells that were depolarised and non-migrating, but were still able to actively protrude multiple lamellipodia (Cramer 1999b). This paper sought to identify the source of the monomer supply for a migrating cell, distinguishing between utilisation of actin monomer released from cellular stores, and actin monomer released from recent actin filament disassembly.

When primary migrating fibroblasts were treated with jasplakinolide, lamellipodium protrusion was blocked in 1-5 minutes. This was distinct from the situation in non-migrating fibroblasts. In these cells there was a delay in the block in lamellipodium protrusion implying that in migrating cells, but not in non-migrating cells, actin filament disassembly is necessary lamellipodium protrusion (Cramer 1999b). Since migrating cells are polarised while non-migrating cells are not, one hypothesis is that on-going actin filament disassembly directed at a single spatial location would contribute towards maintaining cell polarity. The reasons why a migrating cell has an absolute requirement for on-going disassembly in order to protrude a lamellipodium

remain unclear. One possibility is that lamellipodium protrusion uses direct recycling of recently disassembled actin monomer in preference to monomer released from cellular stores (fig. 1.7) (Cramer 1999b). Another is that there is simply not sufficient monomer present in the lamellipodium to provide enough fuel for on-going protrusion. This work predicts that AC activity is needed to catalyse the continuous actin filament disassembly and that this is required for maintenance of morphological cell polarity. Further evidence for a role for AC in cell polarity comes from a large-scale yeast two-hybrid screen for molecules involved in polarity that placed AC within an integrated network of signalling proteins and effectors that underlie the development of cell polarity (Drees *et al.* 2001).

Tools for studying actin disassembly and AC proteins in cells

Jasplakinolide

The discovery and development of various drugs, both natural and synthetic, and most recently from high-throughput screening strategies (reviewed by (Peterson and Mitchison 2002)), which affect specific components of the eukaryotic cytoskeleton has been central to cytoskeletal research. Amongst these reagents, jasplakinolide has proven a useful tool for studying actin filament disassembly in cells.

The product of marine sponges of Jaspis species, jasplakinolide specifically stabilises the actin cytoskeleton. Phalloidin, which also stabilises actin filaments (Small *et al.* 1999a), is not useful for studies of actin filament disassembly as, unlike jasplakinolide (Cramer 1999b) it is not cell permeant. It is known that both *in vitro* with purified actin and in cells that jasplakinolide both blocks actin disassembly (Bubb *et al.* 1994; McGrath *et al.* 1998; Cramer 1999b) and induces actin assembly (Bubb *et al.* 1994; Lee *et al.* 1998; Shurety *et al.* 1998; Cramer 1999b). Until recently, jasplakinolide has not been useful to specifically block disassembly in cells due to its additional filament assembly-inducing activity. Recent work shows, however, that the two activities are separable in cells by dose (time and concentration) and that the assembly-inducing activity

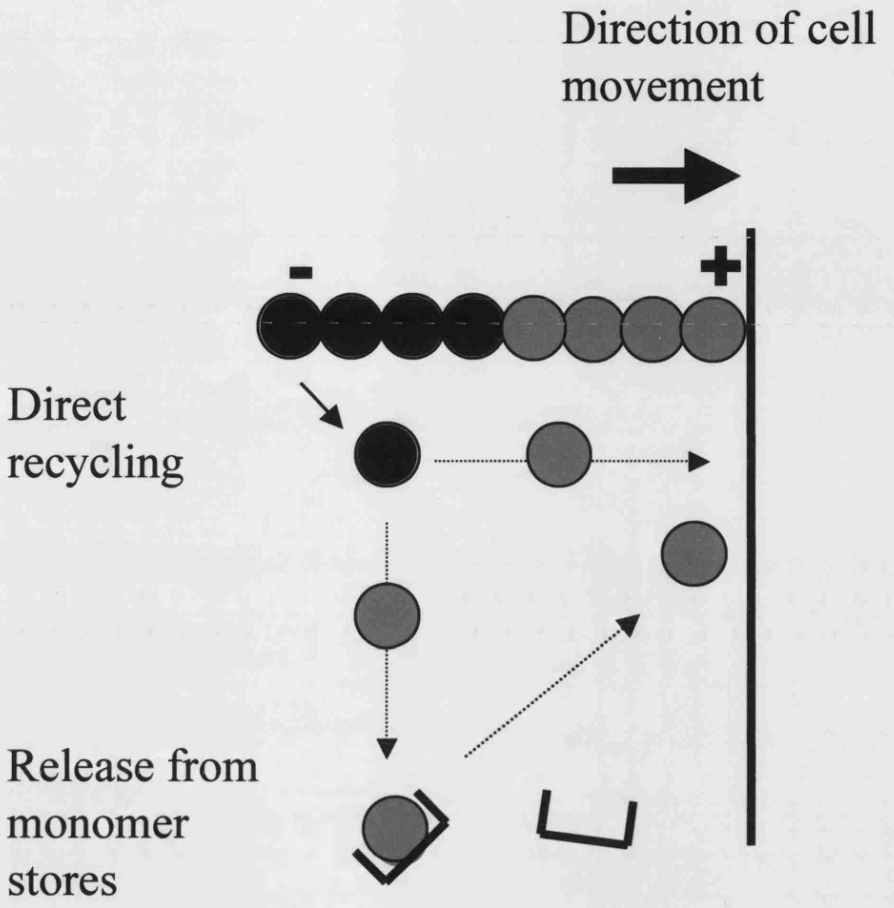


Figure 1.3: Models for actin monomer supply to the lamellipodium during polarised migration. Two possibilities for the source of actin monomer during migration are shown in this diagram. In the first, ADP-actin monomer (black circles) is released by disassembly, undergoes nucleotide exchange to regenerate ATP-actin (grey circles), and is directly recycled to the barbed end to undergo another round of polymerisation. In this scenario the rate of lamellipodium protrusion is closely related to the rate of filament disassembly. In the second, ADP-actin is disassembled and ATP-actin regenerated by nucleotide exchange as before, but the monomer is sequestered into cellular stores (grey circle in box) until needed, whereupon it is released for polymerisation. Here there is less dependence on filament disassembly and the role of disassembly is to replenish the monomer pool. (Adapted from Cramer 1999).

in cells is weak. If cells are treated at low doses (0.5-1μM for up to 45-60 minutes (Cramer 1999b)), jasplakinolide specifically blocks actin filament disassembly without much effect on actin polymerisation and thus these are the most useful for studies on actin filament disassembly.

Spatial measurement of actin monomer in cells

There have been few studies of the spatial distribution of G-actin in cells and these have been limited by the reagents that are available. Two proteins not normally found within the cytosol can bind to actin monomer: vitamin D binding protein (VDBP) and deoxyribonuclease I (DNase I). When these are labelled they can be used as probes for G-actin in cells, however in-depth information on their specificity and the populations of G-actin that they bind has been lacking. Labelled VDBP has been used as a useful probe for G-actin, revealing an intriguing series of punctate structures in the cell (Cao *et al.* 1993); however the methodology used may only see a select population of the G-actin in cells. While many groups use DNase I staining to look at monomer in cells, this molecule can also be an excellent probe for F-actin and the pointed ends of actin filaments. Whether DNase I binds G- or F-actin is fixation-dependent. A specific and sensitive means of probing G-actin levels with DNase I has been developed using a formaldehyde fixation procedure that contains no methanol contamination (Cramer *et al.* 2002). This procedure is very sensitive for cellular G-actin (Cramer *et al.* 2002) and thus is a reliable method of quantifying G-actin levels in various spatial locations. These methods are used in Chapter III alongside jasplakinolide treatment to determine why actin filament disassembly is required for polarised protrusion.

Expression of exogenous and mutant proteins

The use of green fluorescent protein (GFP) tags on proteins that are then introduced into cells has revolutionised the study of actin dynamics as it is now possible both to track movement of individual proteins around the cell in response to stimuli and also to measure the rates and location of both actin assembly and disassembly *in vivo* as a function of time. Alongside this, the expression of mutant forms of proteins that affect their function, such as rendering them constitutively active, or dominant negative, has led to many

insights into protein function and protein interactions in cells. AC proteins can be made constitutively active or dominant negative by means of mutating the regulatory serine at position 3. Mutation to glutamate (negatively charged, mimics phosphorylation) greatly reduces the ability of AC to bind actin, and hence its ability to catalyse actin filament disassembly. Mutation to alanine (neutral amino acid, mimics the non-phosphorylated serine) renders the protein constitutively active, as it can no longer be regulated by phosphorylation. Manipulation of AC activity in this manner has led to advances in our understanding of AC function in cells (Meberg and Bamburg 2000). More recently, further important sites for AC function have been identified and a mutant that distinguishes between the depolymerising and severing activities of AC has also been generated (J. Bamburg, personal communication), thus enabling study of the relative contributions of each mechanism under different circumstances.

Exogenous and mutant proteins are conventionally introduced into cells by transfection techniques or microinjection or either the DNA or protein. Primary migrating fibroblasts present a problem in this respect, as microinjection is technically very difficult in these cells and conventional transfection techniques are ineffective. With many reagents the efficiency of transfection is too low to be of use, while lipid-based transfection reagents cause a loss of cell polarity and migration. Alternative methods of gene transfer into the cells are therefore required. One system that has been of great use in difficult to transfect primary cells uses viral infection as a method of getting the DNA into the cell nucleus. Either retroviruses or adenoviruses can be used. Adenoviruses have the advantage of infecting both dividing and non-dividing cells and simplified methods for the generation of recombinant adenoviruses are available (He *et al.* 1998; Minamide *et al.* 2003). These rely on homologous recombination of a viral backbone plasmid and a shuttle vector containing the gene of interest in bacterial cells, thus eliminating the time-consuming step of generating recombinants in mammalian cells. In addition, the reagents needed to make these viruses are commercially available. This adenovirus system is used extensively in Chapters IV and V as a means of studying the effects of mutant AC proteins on the initiation and maintenance of cell polarity.

1.12 Cell polarity

As discussed above, cell migration requires a cell to become morphologically polarised and this part of the introduction examines some of the known molecular aspects of the polarisation process. The molecular regulation of cell polarisation and polarity has been extensively studied in recent years. Despite this, the picture remains far from clear. In addition to the predicted role for AC in polarised protrusion, the main hypothesis of this thesis (Chapters IV and V) there are other cytoskeletal proteins that have a known role in morphological cell polarity, including microtubules and myosin. This thesis expands knowledge of the role of these two proteins in the initiation of morphological polarity in fibroblasts (Chapter V). The next sections attempt to provide an overview of some aspects of cell polarity research that have emerged over the last few years, focussing on cell polarity during migration.

Morphological cell polarity

Polarised cells have a domain that is morphologically distinct from the rest of the cell. Formation of this domain is the process of polarity initiation and it can be sub-divided into distinct stages (fig. 1.8). Normally (but not necessarily – see below) an external spatial cue is required to signal the need for polarisation. A series of intracellular events then take place that result in a distinct protein or lipid population directed towards the site of polarisation. Next, the polarised region forms from a series of rearrangements of both the actin and microtubule cytoskeletons. The resulting cellular morphology varies according to the cell type and the function of the polarisation: yeast grow into a polarised shape during bud formation, a migrating cell extends a single lamellipodium and retracts its rear to form a front and a back, and epithelial cells develop distinct apical and basal regions separated by cell junctions. Once polarity has been established it must be maintained; however the system must be sufficiently flexible to take account of new polarity cues, for example as required by migrating cells during turning events. In this case, polarity is briefly lost and the cell protrudes multiple lamellipodia until the new direction is decided upon,

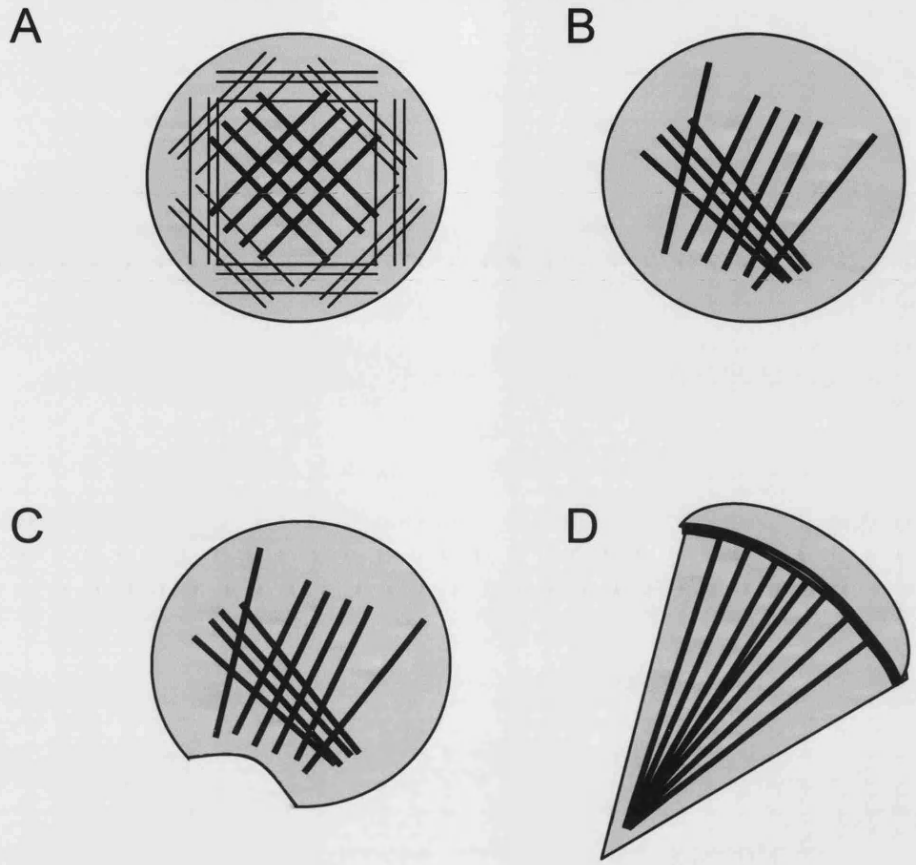


Figure 1.8: Diagrammatic representation of the sub-steps of cell polarisation in a migrating cell. (A) The cell is initially symmetrical or orthogonal. (B) Upon receipt of an external or internal cue, biochemical symmetry is lost and re-arrangements of the cytoskeleton occur. It is unclear whether the cytoskeletal rearrangements occur at this stage or the next. (C) Morphological symmetry is lost. This diagram shows retraction at the rear leading to tail formation, although it is unclear whether the tail or the leading edge forms first. (D) Further morphological rearrangements mediated by the cytoskeleton result in a polarised cell with a single, stable lamellipodium. Once attained, the polarised shape must be maintained throughout migration. (A) to (D) Black lines represent the actin cytoskeleton. For the sake of simplification the microtubules are not shown but are likely to be involved in most cell types.

whereupon a new polarised protrusion is extended and maintained. Chapter V focuses on morphological polarity in migrating fibroblasts, and discovers distinct morphological sub-steps and actin organisation changes during polarity initiation. The molecular details of some of these sub-steps are then examined in more detail, uncovering important, previously unrecognised roles for AC, myosin II and microtubules.

Spontaneous versus cue-dependent cell polarity

In the majority of biological situations cell polarisation happens in a directed fashion in response to a spatial cue such as a chemoattractant. Examples of this kind of polarity response during cell migration include guidance of neuronal growth cones and chemotaxis of macrophages and neutrophils. Much of the work on the molecular mechanism of polarisation has stressed the importance of asymmetrical stimuli and how these translate into an asymmetric response from the cell. Non-chemical stimuli for polarity also exist, for example application of an external mechanical stimulus (Anderson *et al.* 1996; Verkhovsky *et al.* 1999), however how this produces an internal biochemical and spatial asymmetry remains unclear. In other cases, cells have the ability to polarise spontaneously, even if in a random direction, in the absence of an external cue. These cells gain asymmetry in a less understood fashion that may rely only on tiny regional amplifications of their internal biochemistry (Wedlich-Soldner and Li 2003). One of the known examples of spontaneous cell polarisation is the random polarisation of chemotactic cells in a uniform gradient (Wedlich-Soldner and Li 2003). Recent work on the self-polarisation of neutrophils has begun to unravel some of the molecular mechanisms that aid in the spontaneous gain of asymmetry. In the absence of chemoattractant these cells polarise and become motile in response to a positive feedback loop involving the lipid PtdIns(3,4,5)P_3 and the Rho GTPases (Niggli 2000; Weiner *et al.* 2002). Localised concentration of PtdIns(3,4,5)P_3 at the leading edge activates Rac, Cdc42 and/or Rho, which in turn stimulate the increase of PtdIns(3,4,5)P_3 (Weiner *et al.* 2002). Interestingly this same loop in conjunction with F-actin also appears to be vital for maintaining neutrophil polarity and migration (Wang

et al. 2002). A second amplification loop has been found in yeast, which can exhibit spontaneous polarisation during bud-site selection. These cells depend on formin-mediated nucleation of F-actin cables for polarity, and the nucleation in turn requires Cdc42 for activation (Pruyne *et al.* 2002; Sagot *et al.* 2002). The Cdc42 is localised by myosin V-based transport of the protein on the actin cables (Wedlich-Soldner *et al.* 2003). A mathematical model has therefore been postulated in which polarity could result from a positive feedback loop between the Cdc42-dependent cable formation and cable/myosin V-based Cdc42 transport (Wedlich-Soldner *et al.* 2003).

De-adhesion, microtubules and cell polarity

The formation and remodelling of the cytoskeleton during polarisation requires changes in cellular adhesions. Cell adhesions are large structural and signalling complexes that are formed from the linking of the actin cytoskeleton to the integrin receptors that mediate signalling to and from the extracellular matrix. They form as "focal complexes" in association with lamellipodia and filopodia and aid protrusion of the lamellipodium. Focal complexes can either be disassembled or become the much larger and longer-lived "focal adhesions" that are found further into the cell body and tail (Small *et al.* 2002a). During polarity initiation prior to migration, disassembly of adhesion sites must occur on one side of the cell to break the symmetrical shape. The mechanism underlying this process is not understood although in some cells mechanical force is sufficient, pointing to a role for myosin (Verkhovsky *et al.* 1999).

For maintenance of polarity during migration, an adhesion-deadhesion cycle is set up with the small focal contacts forming at the front of the cell and the focal adhesions disassembling towards the rear. Fewer focal adhesions make the cell less "sticky" with respect to the substratum and correlate with increased migration speed (Small *et al.* 2002a). The contractile force exerted by myosin actually increases the strength of the adhesion rather than helping to rip it off the sub-stratum (Riveline *et al.* 2001). Presumably once the cell is migrating, deadhesion occurs during tail retraction. Disruption of contacts seems to require

the microtubule cytoskeleton. During cell migration microtubules align along the longitudinal axis of the cell, with the plus ends facing towards the leading edge (Gundersen and Cook 1999; Waterman-Storer and Salmon 1999) and in many cell types, disassembly of the microtubule network induces a loss of cell polarity and cell migration (Waterman-Storer and Salmon 1999). On the other hand, microtubules are not required for migration of keratocytes or leukocytes such as neutrophils and lymphocytes (Wittmann and Waterman-Storer 2001) and lamellipodium formation and protrusion can be microtubule-independent even in cells that otherwise require microtubules to migrate (Etienne-Manneville and Hall 2001). Microtubules appear to specifically and repeatedly target adhesion sites and this promotes their disassembly (Kaverina *et al.* 1999). Retraction of the rear of the cell then helps to promote protrusive behaviour at the front (Dunn *et al.* 1997; Small and Kaverina 2003).

An alternative suggestion has been proposed whereby microtubules exert a more overall control over polarised motility. Microtubules, in addition to actin, can be regulated by the Rho GTPases (Etienne-Manneville and Hall 2001; Palazzo *et al.* 2001) and this enables some complex cytoskeletal cross-talk to occur. In this scenario, growth of "pioneer" microtubules into newly formed regions of the leading edge is more persistent than that of other cellular microtubules (Waterman-Storer and Salmon 1997; Wadsworth 1999). Microtubule growth into these regions activates Rac, which promotes lamellipodium protrusion and the whole is maintained by a positive feed-back loop where active Rac promotes further microtubule growth (Wittmann *et al.* 2003).

The precise role of microtubules in the initiation of morphological polarisation remains unclear in most cell types – the exception being astrocytes (Etienne-Manneville and Hall 2001, 2003). Chapter V provides information that clearly shows that microtubules are not required for any of the sub-steps in morphological polarisation, but instead are needed to stabilise the newly formed polar shape and enable migration to occur.

Actomyosin and cell polarity

Asymmetries in myosin contractility can be sufficient to set up directional movement from initially symmetric cells. In disc-shaped keratocyte lamella fragments, which lack nuclei and microtubules (Verkhovsky *et al.* 1999) and consist mainly of the actin cytoskeleton and cytoplasm, local application of force to one side of the disc results in polarisation of the fragment by lamellipodial retraction at the site of force application. Motility ensues as the cellular asymmetry that has been set up is perpetuated (Verkhovsky *et al.* 1999). In fibroblasts, local inhibition of myosin is sufficient to dissolve adhesions on that side of the cell. The edge retracts, and protrusion on the other side of the cell allows directional motility (Kaverina *et al.* 2000). It should be noted, however, that actomyosin contraction of particular cellular regions might not control the acquisition of cell polarity in all cell types. Astrocytes induced to migrate in wound healing assays require microtubules, not actin, to become polarised, although actin-based protrusion is required for cell migration (Etienne-Manneville and Hall 2001, 2003).

It seems likely that most cells require the combined actions of both actin and microtubules to polarise and migrate, with the degree to which each is needed varying between cell types. One suggestion that has been put forward to explain the differing requirements for microtubules versus actomyosin concerns the strength of the cell-substratum attachments (Kaverina *et al.* 1999, 2002; Small *et al.* 2002a; Small and Kaverina 2003). In cells such as leukocytes and keratocytes, which form weak contacts, the force generated by protrusion of the lamellipodium may be sufficient to drag the cell forward. Cells that form strong attachments to the substratum, such as fibroblasts, require microtubules to mediate de-adhesion as the protrusive force is not enough to drive total cell translocation. Much more work is required to understand the complex relationship between the actin and microtubule cytoskeletons that triggers and maintains polarity and it is clear that not all the molecular components have yet been either defined or characterised.

1.13 Thesis aims

The major aim of this thesis is to understand the role of actin filament disassembly in initiating and maintaining morphological cell polarity. Using primary fibroblasts, the next chapters address several questions. These include:

- Why is actin filament disassembly required for lamellipodium protrusion during cell migration?

Where does the supply of actin monomer come from during polarised migration?

- How does regulating AC activity contribute to maintaining fibroblast polarity?
- What morphological events occur during acquisition of fibroblast polarity?
- Is there a role for AC in the acquisition of fibroblast polarity?
- What is the role of myosin in polarity acquisition?
- What role do microtubules play in the acquisition of fibroblast polarity?

As discussed above, proteins of the AC family play a key role in the regulation of actin dynamics by disassembling and severing actin filaments, ensuring that the cell is provided with actin monomer for assembly into a variety of actin structures. Based on the observation that actin filament disassembly is necessary for polarised lamellipodium protrusion, this thesis hypothesises that AC-mediated actin filament disassembly is crucial for a cell to initiate its polarity and subsequently maintain a single polarised protrusion throughout migration.

In Chapter III, the question of why migrating cells require continuous actin filament disassembly is addressed by examining the supply of actin monomer to the lamellipodium. Specific cell staining of cellular G-actin levels is used to investigate the explanation behind the previous observation that migrating cells, as distinct from non-migrating cells, require ongoing actin filament disassembly.

Maintenance of previously established polarity is the subject of Chapter IV. An explant-based culture system that distinguishes between polarised migrating and non-polarised non-migrating cells is described. Building on observations of the

levels on phosphorylated and non-phosphorylated AC within the lamellipodium of each cell phenotype, adenoviral-mediated gene transfer is used to perturb AC activity in migrating cells and the effects on polarity and migration are assessed.

Chapter V explores the acquisition of cell polarity in dissociated primary fibroblasts. The morphological and cytoskeletal sub-steps that take place during polarisation are described and the regulation of some of these is investigated in turn. The role of AC activity on the actin re-arrangements that occur during an early stage of polarisation is examined using several approaches: the pharmacological inhibitor of actin filament disassembly jasplakinolide is used alongside constitutively active LIM kinase and constitutively active and dominant negative AC. A role for ROCK and myosin in the stabilisation of the developing tail is evaluated using the drugs Y27632 (to inhibit ROCK) and blebbistatin (to inhibit myosin II), and finally the microtubule-specific drugs nocodazole and taxol are used to study the part played by microtubules in stabilising the polar shape once formed.

Chapter II: Materials and methods

2.1 Materials

All reagents were from Sigma unless otherwise indicated. All tissue culture media, penicillin-streptomycin and foetal bovine serum (FBS) were from Gibco; chicken serum was from Sigma. Tissue culture-ware was from Nunc. Antibody sources are detailed below.

Antibody reagents

The following specific antibodies (previously well-characterised) were used in all studies: mouse anti-actin antibody (clone C4, ICN), rabbit anti-myosin II (Sigma), mouse anti-tubulin (clone DM1α, Sigma), affinity-purified rabbit anti-human cofilin peptide antibody (Cytoskeleton Inc), which recognises both the non-phosphorylated and phosphorylated (inactive) forms of cofilin; affinity purified rabbit anti-chick ADF, which recognises both the non-phosphorylated and phosphorylated forms of ADF in chick cells (Morgan *et al.* 1993); rabbit anti-pAC phosphopeptide antibody, which recognises only the phosphorylated form of both ADF and cofilin (Meberg *et al.* 1998) (both gifts from J. Bamburg); rabbit anti-tropomyosin rabbit antibody raised to the αTm_f exon 9d peptide (Schevzov *et al.* 1997) (gift from R. Weinberger), affinity purified rabbit anti-XAC1 antibody (Rosenblatt *et al.* 1997)(gift from J. Rosenblatt), and rat anti-chick LIM kinase I antibody (gift from O. Bernard). All antibodies recognised a single band of the expected molecular weight on Western blots of cell extracts, except the tropomyosin antibody which recognised at least 5 isoforms as expected from other studies (Lin *et al.* 1988). Monoclonal antibody to adenoviral E2 protein was obtained from B6-8 hybridoma cells (gift from J. Bamburg). Fluorescently-conjugated secondary antibodies (fluorescent-conjugate AffiniPure F(ab')2 fragment goat anti-mouse, anti-rat or anti-rabbit IgG) were from Jackson Laboratories. Alkaline phosphatase-conjugated goat anti-mouse IgG (whole molecule) was from Sigma.

Adenoviral constructs

The AdEasy system (Stratagene) was used for all the adenoviral constructs used in this work. All constructs were made by A. Shaw, L. Minamide or J. Sneider and were generous gifts of J. Bamburg.

The following constructs are based upon the AdTrack CMV plasmid, which expresses a green fluorescent protein (GFP) reporter gene driven off a separate promoter from the gene of interest (He *et al.* 1998):
AdTrack alone for expression of GFP
LIMK1 EE508 for the expression of constitutively active LIM kinase I
XAC A3 KK95,96QQ for expression of constitutively active XAC that can disassemble but not sever actin filaments

The following constructs are based on the pShuttle CMV plasmid, which does not express a reporter gene (He *et al.* 1998):
XAC A3 for expression of constitutively active XAC (disassembles and severs actin filaments)
XAC E3 for expression of pseudophosphorylated, barely active, XAC

2.2 Methods

Cell culture and preparation of B6-8 antibody

Human embryonic kidney (HEK) 293 cells were grown at 37°C/5% CO_2 in 75cm^2 tissue culture flasks (Nunc) in Dulbecco's Modified Eagles Medium (DMEM) with 10% FBS and 1% penicillin-streptomycin. Cells were passaged at confluency by exposure to 2ml trypsin-EDTA at 37°C until the cells had detached from the flask. The trypsin was inactivated by the addition of an equal volume of tissue culture medium and the cell suspension centrifuged at 1000rpm for 3 minutes. Cells were re-suspended in medium and seeded at 1:10. B6-8 hybridoma cells were cultured at 37°C/5% CO_2 in 10cm tissue culture dishes in RPMI1640, 10%FBS and 1% penicillin-streptomycin and passaged every three days by scraping, and centrifugation and seeding as above. For

antibody collection, the cells were grown in 10cm plates for 7 days to become over-confluent. The cells were spun down and discarded and the supernatant containing the antibody reserved. 1M Tris-HCl pH 8.0 was added to 5% of the total volume and 1M sodium azide added to 0.5% of the total volume. The stabilised medium was stored at 4°C until required.

Preparation of migrating and non-migrating primary chick embryo fibroblasts from heart explants

Fertile eggs were incubated at 37°C in a humidified still-air Biohatch automated incubator (Brinsea) for 7-8 days. The resulting E7-8 embryos were removed from the egg, sacrificed by decapitation and the hearts extracted by the aorta. The aorta and the membrane surrounding the heart were removed and the tissue chopped/torn into small explants. The explants were plated onto coverslips (Scientific Laboratory Supplies) coated first with poly-L-lysine (Sigma) and second with matrigel (Becton Dickson), in CEF medium (DMEM +sodium pyruvate + pyroxidine + 1000mg/l glucose, 10% FBS, 1% penicillin-streptomycin, 10% chicken serum) and left to adhere. Separate populations of migrating and non-migrating fibroblasts were prepared by varying the length of time in culture as previously reported (Cramer 1999). Cells were used at 24-36 hours post-plating for a migrating population and at 8 days for a non-migrating population.

Preparation of dissociated primary chick embryo heart fibroblasts

CEF explants were prepared as described above and kept overnight in suspension culture in CEF medium using non-adherent tissue culture-ware (Sarstedt). Explants from 3-12 hearts were dissociated in 2.5mg/ml type 2 collagenase (Worthington Biochemical Corporation) in trypsin-EDTA for 40-45 minutes at 37°C with occasional trituration. The cell suspension was washed twice in 1-2 ml phosphate buffered saline (PBS)/10% FBS, centrifuged at 1000rpm for 2 minutes, the supernatant removed, the cells transferred to an appropriate volume of CEF medium and plated onto matrigel-coated coverslips

as above. For polarisation experiments cells were used at 10 minutes to 2 hours after plating.

Cell staining

For the majority of experiments, cells were fixed in 4% electron microscopy grade methanol-free formaldehyde (Taab) in cytoskeleton buffer (10mM MES pH 6.1, 3mM $MgCl_2$, 138mM KCl, 2mM EGTA) with 0.32M sucrose for 20 minutes to optimally preserve the cytoskeleton (Cramer and Mitchison 1993). For tubulin staining, cells were fixed in 4% electron microscopy grade methanol-free formaldehyde in cytoskeleton buffer for 20 minutes at 37°C to prevent microtubule disassembly. For myosin staining, cells were fixed in cold (-20°C) methanol for 45 seconds. Fixed cells were washed in PBS with 0.1% Triton X-100, permeablised in PBS/0.5% Triton X-100 for 10 minutes and blocked in antibody diluting solution (PBS, 2% bovine serum albumin, 0.1% Triton X-100 and 0.1% sodium azide). Antibodies and cytoskeletal probes were diluted in antibody diluting solution. Cells were stained with 0.1μg/ml Alexa594 phalloidin (Molecular Probes) alone for 30 minutes, or were co-stained simultaneously with 3μg/ml Alexa488 DNase (Molecular Probes) and 0.1μg/ml Alexa594 phalloidin for 30 minutes. For indirect immunofluorescence, cells were fixed, permeabilised and blocked as above, incubated with primary antibody (see list below for dilutions) for 1 hour, washed in PBS/0.1% Triton X-100, and incubated simultaneously with fluorescently conjugated secondary antibody (1:100) and 0.1μg/ml Alexa594-phalloidin for 45 minutes.

Antibody dilutions used for cell staining

Anti-actin	1:200
Anti-myosin	1:10
Anti-tubulin	1:200
Anti-cofilin	1:100
Anti-ADF	1:200

Anti-pAC	1:200
Anti-XAC	1:100
Anti-LIM kinase I	1:20

Preparation of cells for time-lapse microscopy

Non-infected CEF (explants or dissociated cells) were grown on 22mm poly-L-lysine and matrigel-coated glass coverslips. On the day of the experiment, coverslips were transferred to a custom-made aluminium chamber heated by a circulating water-bath set to 36°C, and covered with pre-warmed chamber medium (1:1 DMEM/Ham's F12 medium with 15mM HEPES buffer, L-glutamine and pyridoxine-HCl, without pyridoxal-HCl or phenol red plus 1% penicillin-streptomycin, 10% FBS and 10% chicken serum) with a film of poly(dimethylsiloxane) 200 oil viscosity 10 cSt (Aldrich) to prevent evaporation. For experiments using virally infected CEF, cells (explants or dissociated cells) in chamber medium were plated onto 35mm plastic dishes containing a matrigel (but not poly-L-lysine)-coated 12mm glass-base insert (Willco Wells). The medium was coated in oil and the dish was parafilmed tightly to ensure a sealed system before transferring to a HeatWave30 temperature controlled stage for 35mm dishes (Bioscience Tools).

Image acquisition

High-resolution images of fixed and stained cells were digitally acquired using a 12-bit cooled charge-coupled device camera (KAF 1400, Roper Scientific) on a Nikon microscope using a 100 x, 1.4 NA oil objective controlled by Metamorph software (Universal Imaging). Images were digitally processed in Metamorph followed by Photoshop (Adobe). High-resolution time-lapse data was recorded using a cooled charge-coupled device camera (KAF 1400, Princeton Instruments) camera on an Axiovert microscope (Zeiss) using a 63x, 1.4 NA oil objective controlled by Metamorph software. Images were digitally processed in Metamorph and saved first as stacks and second as movie files

(.AVI). Cell migration and lamellipodium protrusion rates were determined using Metamorph.

Fluorescence quantification

Cells were fixed and stained with phalloidin and DNase as described above. In each individual cell Metamorph software was used to measure total fluorescence (sum of all grey levels) in a 10-pixel wide line-scan across the lamellipodium perpendicular to the cell margin. Individual images were enlarged 200-400% on screen to enable accurate measurement of the lamellipodium. Staining conditions and camera exposures were constant. Background fluorescence was subtracted from all measurements but other than this, images for analysis were left unprocessed. In order that lamellipodia of different sizes could be compared, the total fluorescence was normalised to lamellipodium area. G-actin fluorescence was divided by F-actin fluorescence in each lamellipodium to give the G-/F-actin ratio, and the average of individual G-/F/actin ratios calculated for the population. Ratio images were constructed using Metamorph software. For actin filament disassembly analysis, the lamellipodium was identified by phalloidin staining and the actin monomer measured in the DNase image by line-scan analysis. Line-scans in individual cells were acquired across the lamellipodium and the population average determined.

Treatment of cells with jasplakinolide

Separate populations of migrating and non-migrating CEF were grown on coated coverslips as described above. Jasplakinolide (1μM, a gift from M. Sanders) was added to live cells in pre-warmed media from a frozen 500x DMSO stock. Treatment of cells with DMSO alone had no effect on cell morphology or the actin cytoskeleton. For analysis of G-actin pool usage, cells in explant culture were treated for 0, 5 and 15 minutes, fixed and stained with Alexa488 DNase and Alexa594 phalloidin as detailed above. The phalloidin image was used to identify the lamellipodium and the G-actin fluorescence

levels quantified. For analysis of the role of actin filament disassembly in the initiation of cell polarity, cells were dissociated, plated onto coated coverslips, and left to adhere for 10 minutes. Drug was added and cells were treated for 5-50 minutes, fixed and stained with Alexa594 phalloidin.

Treatment of cells with Y-27632

Dissociated primary chick embryo fibroblasts were plated as described and left to adhere for 10 minutes. Y-27632 (10μM, Tocris) was added to live cells in pre-warmed CEF medium (for time-course assays) or chamber medium (for time-lapse imaging) from a 1000x water stock. Cells were treated for 5 minutes to 2 hours. For time-lapse experiments, a short pre-treatment sequence was recorded and drug was added to cells directly on the microscope without stopping the time-lapse acquisition.

Treatment of cells with methyl-blebbistatin

Dissociated primary chick embryo fibroblasts were plated as described and left to adhere for 10 minutes. Methyl-blebbistatin (100μM, a gift from T. Mitchison) was added to live cells in pre-warmed CEF medium (for time-course assays) or chamber medium (for time-lapse imaging) from a 1000x DMSO stock. Cells were treated for 5 minutes to 2 hours and those for time-course assays were fixed and stained with Alexa594 phalloidin. Treatment of cells with 0.1% DMSO alone had no effect on cell morphology or the actin cytoskeleton. During time-lapse recordings the drug was added to cells directly on the microscope as above.

Treatment of cells with nocodazole

Dissociated primary chick embryo fibroblasts were plated as described and left to adhere for 10 minutes. Nocodazole (5ug/ml) was added to live cells in pre-warmed CEF medium (for time-course assays) or chamber medium (for time-lapse imaging) from a 2000x DMSO stock. Cells were treated for 5 minutes to 2

hours and those for time-course assays were fixed and stained with Alexa594 phalloidin. Treatment of cells with DMSO alone had no effect on cell morphology or the actin cytoskeleton. During time-lapse recordings the drug was added to cells directly on the microscope as above.

Treatment of cells with taxol

Dissociated primary chick embryo fibroblasts were plated as described and left to adhere for 10 minutes. Taxol (10uM, a gift from A. Hall) was added to live cells in pre-warmed CEF medium (for time-course assays) or chamber medium (for time-lapse imaging) from a 1000x DMSO stock. Cells were treated for 5 minutes to 2 hours and those for time-course assays were fixed and stained with Alexa594 phalloidin. Treatment of cells with 0.1% DMSO alone had no effect on cell morphology or the actin cytoskeleton. During time-lapse recordings the drug was added to cells directly on the microscope as above.

Expansion of adenoviruses

HEK293 cells were grown to 80% confluency and the medium changed. The flask was inoculated with 100_l virus and incubated for 2 days. The cells were harvested using, a cell scraper, transferred to a 50ml tube (Corning Costar), pelleted by centrifugation and resuspended in 5ml PBS. The cells were lysed by three cycles of freezing in dry ice/ethanol, quick thawing at 37°C and vortexing gently. The sample was centrifuged and the supernatant containing the virus aliquotted into 0.5ml lots for storage.

Adenoviral titering

HEK 293 cells were plated at $6x10^5$ cells per well of a 6-well plate (Nunc) and left overnight to become 70-95% confluent. The virus to be titered was diluted in serum-free DMEM at both $1:10^4$ and $1:10^5$ virus solutions. The medium was removed from the confluent cells and 0.5ml diluted virus solution added per well. The plate was incubated at 37°C for 45 minutes with occasional gentle

rocking. Following this, the virus was removed and 2ml DMEM plus 2% FBS was added to each well; the plate was returned to the incubator and left for 16 hours. Cells were fixed in 4% methanol-free formaldehyde in PBS for 20 minutes, permeabilised in 90% methanol in PBS for 2 minutes, and washed once with PBS alone and once with PBS plus 1% bovine serum albumin (BSA) (fraction V). B6-8 (anti-E2) antibody (1:5) was added to the cells in PBS/1% BSA for 45 minutes, washed off in PBS/1% BSA and alkaline phosphatase-conjugated secondary antibody (1:1000) added for 45 minutes. Cells were washed and transferred to high pH buffer (50mM Tris pH 9.5, 100mM NaCl, 1mM $MgCl_2$) before incubating with 1-2ml 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (Sigma) for 45 minutes-1 hour until the nuclei of infected cells appeared dark brown. The number of infected cells in 10 fields of view was counted using a 20x objective on a Xenophot microscope (Nikon) and the viral titre calculated using the following equation:

Average positive cells per field x (*) x 2 x dilution = titre in focus forming units (ffu)/ml

(*) is the conversion factor for comparison between the area of the field of view on the microscope and the area of the 6-well plate.

Adenoviral infection of primary chick embryo heart fibroblasts

For experiments using both explants and dissociated cells, cells were initially held in suspension culture in the presence of the virus according to the following method. CEF explants were prepared as described above and transferred in a minimal volume (less than 100µl) to a 8mm glass cloning ring (Bellco) adhered to a 5cm suspension culture dish with silicone vacuum grease (Beckman). CEF medium was added to the dish surrounding the cloning ring to prevent evaporation of the medium containing the explants. An appropriate amount of virus (as determined by titre assay, see below for amounts) was added to the cloning ring and the dish transferred to 37°C. For experiments using dissociated CEF, explants were held in suspension culture in the cloning

ring in the presence of the virus for 48 hours, after which time they were dissociated and plated as described above. For experiments using CEF explants, the explants were held in suspension in the presence of the virus for 24 hours. The entire contents of the cloning ring were then removed, added to coated dishes/plates and incubated in the continued presence of the virus for a further 24 hours before use.

Adenovirus titres and volumes added to each cloning ring

GFP	2.4x109 ffu/ml	30µl
LIMK EE508	8.8x109 ffu/ml	13µl
XAC A3	3x108 ffu/ml	80µl
XAC E3	1.5x108 ffu/ml	80µl
XAC A3 KKQQ	1.4x109 ffu/ml	80µl

The above amounts are for experiments based on CEF explants. Where two constructs were infected simultaneously the amount of each virus was halved. For experiments using dissociated cells, the viral amounts given above were halved, and where two viruses were added together the amount was further halved (i.e. 25% of the volume given above was added).

Statistical analysis

All graphs are given as mean +/- standard error mean (SEM) calculated within Excel (Microsoft). The student's 2-tailed t-test was calculated using Minitab software.

Chapter III: Distribution and availability of actin monomer during polarised migration

Chapter III: Distribution and availability of actin monomer during polarised migration

3.1: Introduction.

Previous published work from our lab has demonstrated that continuous actin filament disassembly is necessary for lamellipodium protrusion in migrating cells, but not for non-polarised protrusion in non-migrating cells. To begin to test why migrating cells have this requirement, information on how the cycle of actin is used during migration is needed. To date there is little information on either the distribution or the availability of actin monomer in cells. This chapter attempts to find an explanation for the requirement for actin filament disassembly by assessing the concentration and availability of the actin monomer pool both in and immediately behind the lamellipodium of polarised migrating, and non-polarised non-migrating CEF. The data presented indicate that very little G-actin is present in the lamellipodium of polarised migrating cells relative to the amount of F-actin. The ratio between G-actin and F-actin was lower in the lamellipodium of migrating cells compared to non-migrating cells. Furthermore, this G-actin was not available (at least to the resolution of detection of our camera and sensitivity of the assay) to fuel lamellipodium protrusion in migrating cells, but could be used in non-migrating cells.

3.2: Results.

Optimising the preparation of migrating and non-migrating chick embryo heart fibroblasts

In order to assess a role for AC proteins in polarised cell migration, it was first necessary to establish an amenable experimental system. Chick embryo heart fibroblasts (CEF) are a well-studied model system for cell migration studies and have the advantage of changing migration capacity according to the length of time they spend in culture (Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b). This enables the study of two opposing cell behaviours: polarised protrusion in migrating cells and non-polarised protrusion in non-migrating cells. Differences between the two can then be used to gain an insight into what might be important in polarised migration.

Optimal conditions for obtaining migrating and non-migrating cells by varying the length of time spent in culture (24-36 hours for migrating cells, 4-6 days for non-migrating cells) have previously been reported ((Couchman and Rees 1979; Cramer *et al.* 1997; Cramer 1999b), see Introduction). These studies were repeated to determine the viability of the culture system under current lab conditions and for the investigations in this and the next chapter. CEF explants were prepared and plated according to the scheme shown in figure 3.1 and fibroblasts grown out for varying lengths of time. Fibroblasts that reached the boundary of the explant and were not in contact with other cells (e.g. fig. 3.2, arrows) were analysed for cell morphology and appearance of the actin cytoskeleton. At 24 hours post-plating cells had a clearly polarised morphology (fig. 3.3A, A'), being crescent (fig. 3.3A) or kite-shaped (fig. 3.3A') with a single well-spread lamellipodium identified as the heavily phalloidin-stained region at the front of the cell (fig. 3.3A, A', arrow) and actin bundles oriented in the direction of movement. When these cells were time-lapsed, they were observed to be rapidly migrating at around 1-1.5μm/minute (data not shown and (Cramer 1999b)). By two days the cells, while in the main still polar, had a more elongated appearance (fig. 3.3B) and were slower migrating (data not shown). After this time, as it has long been recognised

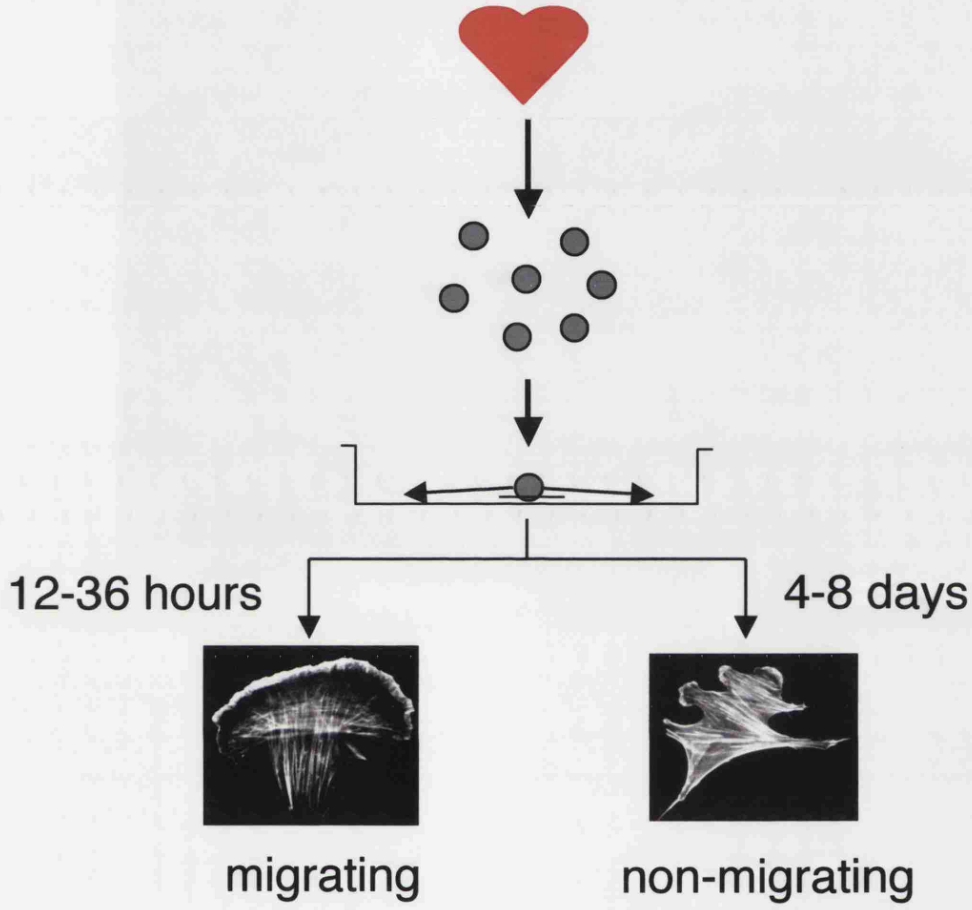


Figure 3.1: Preparation of migrating and non-migrating chick embryo fibroblasts.

Embryonic day 7 chick heart explants are plated onto coverslips and left to adhere. Cells migrate out from the explants for two days before losing polarity and becoming non-migrating. Varying the length of time in culture allows the preparation of separate populations of migrating and non-migrating cells.

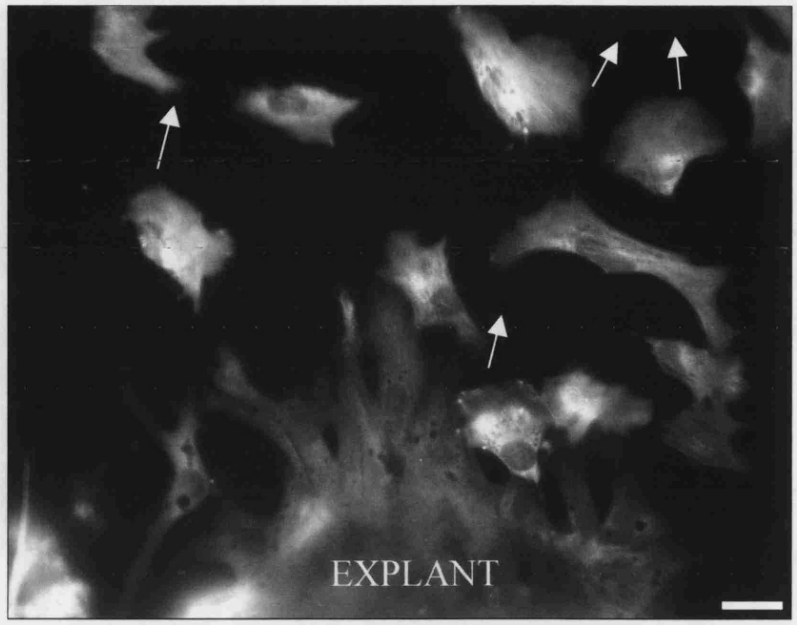


Figure 3.2: Cells migrating out from a chick heart explant.

The explant adheres to a coverslip and fibroblasts grow out from the tissue mass over time. Single migrating cells at the explant boundary (arrows) are used for experiments. The morphology and migration status of these cells changes over time in culture.

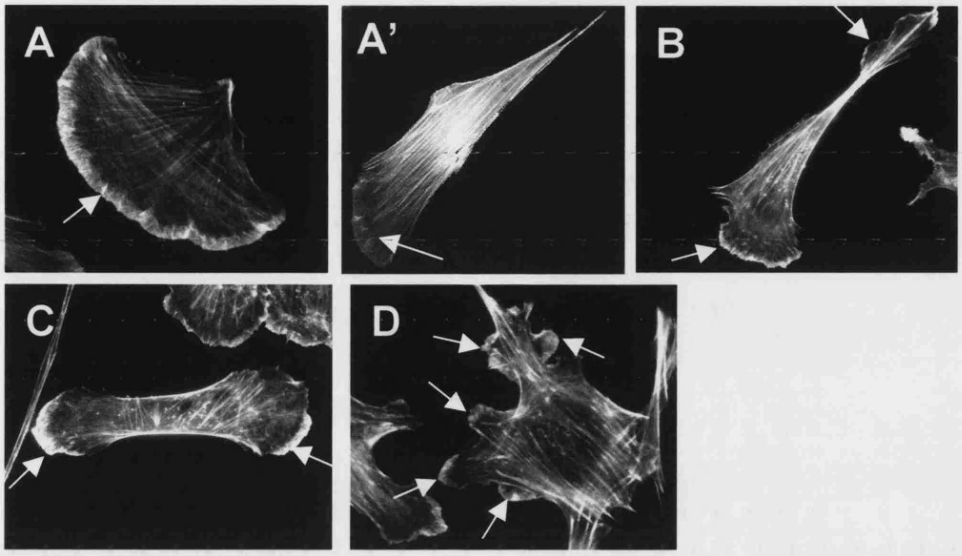


Figure 3.3: Chick fibroblasts change their polarity over time in culture. Chick heart explants were prepared and left to adhere. Cells were fixed and stained for F-actin with phalloidin at 1 day (A, A'), 3 days (B), 4 days (C) and 8 days (D) post-plating. Note the increasingly depolarised morphologies as defined by increase in lamellipodium number (arrows) over time in culture. By using cells at 1 day (A and A') and 8 days post-plating (D), separate populations of polarised migrating cells and non-polarised non-migrating cells can be obtained.

(Couchman and Rees 1979), the cells began to lose their polarised morphology, becoming bi-polar (fig. 3.3C) and eventually losing all morphological polarity by day 7 to 8 post-plating (fig. 3.3D). These cells were non-migrating by time-lapse microscopy (data not shown), and had multiple lamellipodia protruding randomly from around the cell margin (fig. 3.3D, arrows). Thus, longer periods in culture were required than had been found in previous work to provide a fully non-migrating fibroblast culture. In order to maximise the numbers of rapidly migrating and non-migrating cells while minimising the numbers of cells whose behaviour could not be easily quantified by morphology, all experiments were therefore carried out on 24-36 hour and 8 day cultures, respectively.

Concentration of actin monomer in the lamellipodium of migrating and non-migrating CEF

The actin monomer concentration was assessed in migrating and non-migrating cells by co-staining cells with fluorescent phalloidin to probe F-actin and fluorescently conjugated DNase I to probe G-actin. Under specific fixation conditions (methanol-free formaldehyde followed by permeabilisation) DNase I specifically stains G-actin and not F-actin and accurately reflects G-actin concentration (Cramer *et al.* 2002). DNase staining appeared homogenous throughout the cell, including the lamellipodium (fig. 3.4A). The lamellipodium was identified from the F-actin image and the relative DNase I fluorescence intensity normalised to lamellipodium area measured in the lamellipodium of both migrating (fig. 3.4B) and non-migrating (fig. 3.4B) cells. No significant difference was seen in G-actin concentration within this region between migrating and non-migrating cells (fig. 3.4B compare the two bars, n=121 cells over 4 experiments).

As there was no apparent difference in G-actin concentration in the lamellipodium of migrating and non-migrating cells, the amount of F-actin found within the same region was analysed. Fluorescence intensity line-scans of both the DNase and phalloidin images were taken across the lamellipodium from the front to the back in both migrating (fig. 3.5A) and non-migrating (fig. 3.5B) cells. While the DNase fluorescence (red diamonds) was similar for both

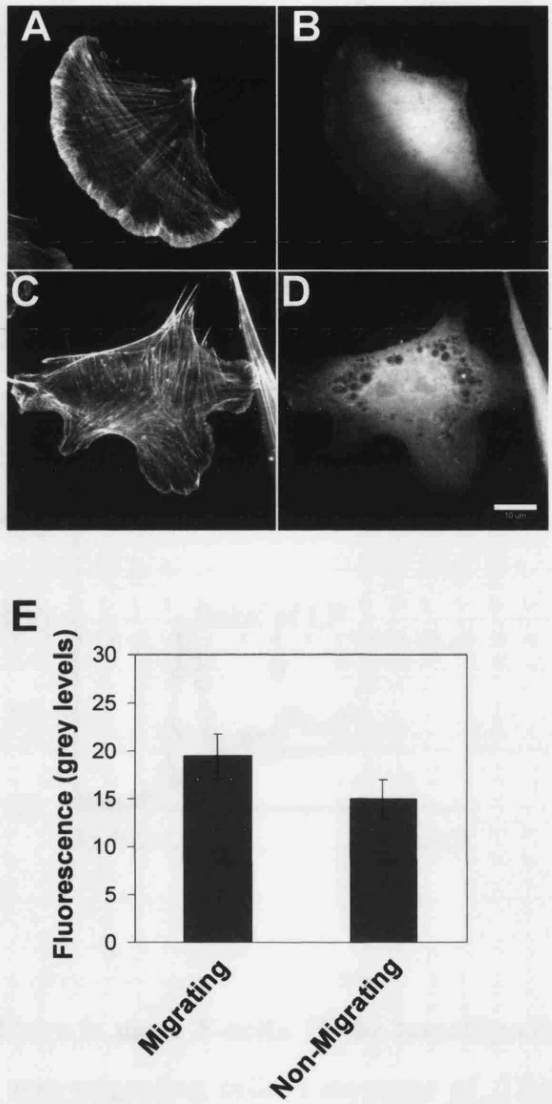


Figure 3.4: G-actin levels are similar in both migrating and non-migrating fibroblasts. Migrating (A) and non-migrating (B) fibroblasts were fixed in methanol-free formaldehyde and co-stained with DNase I for G-actin (A, B) and phalloidin for F-actin (C, D). The lamellipodium was identified from the F-actin image and the fluorescence intensity (total grey levels normalised to the area of the lamellipodium) of the DNase stain measured for this region. The population average (n=121) and standard error mean were calculated for both migrating and non-migrating cells (E). The scale bar in (D) represents 10μm.

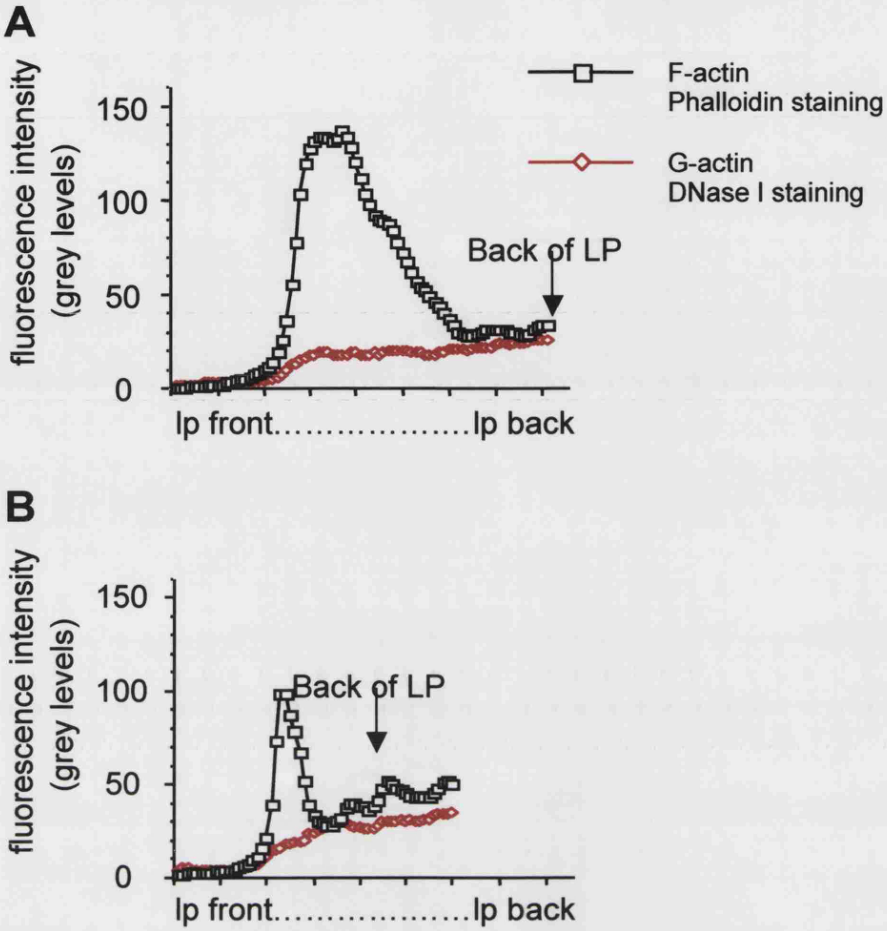


Figure 3.5: There is more F-actin in the lamellipodium of migrating cells than in non-migrating cells. Line-scans of DNase (G-actin) and phalloidin (F-actin) fluorescence intensity were taken from the front to the back of the lamellipodium of the cells shown in the previous figure. (A) shows the migrating cell, (B) the non-migrating cell. Each of the line-scans shown here shows a single cell that is representative of at least 20 separate cells. Note that while the DNase fluorescence (red diamonds) is similar for both migrating and non-migrating cells, the phalloidin intensity (black squares) within the lamellipodium is approximately 50% greater in the migrating cell compared to the non-migrating cell.

migrating and non-migrating cells, the phalloidin staining intensity (black squares) within the lamellipodium was approximately 50% greater in the migrating cell compared to the non-migrating cell. Therefore, it appears that the ratio of G-actin to F-actin is lower in migrating cells.

To test this directly, the amount of G-actin relative to the amount of F-actin was compared in individual migrating and non-migrating cells. Cells were co-stained with DNase (fig. 3.6A and B) and phalloidin (fig. 3.6C and D) under equivalent conditions and imaging conditions were kept constant. The G-actin to F-actin ratio was obtained by dividing the G-actin image by the F-actin image (fig. 3.6E and F, ratio imaging carried out by L. Cramer). A low G-actin to F-actin (G/F) ratio is coloured blue, an intermediate G/F ratio is green and high G/F ratio is red. The G/F ratio was clearly lower in migrating cells (fig. 3.6E white arrow, blue colour) than in non-migrating cells (fig. 3.6F white arrow, green colour). This difference in G/F ratio was quantified by calculating the G/F ratio in individual migrating and non-migrating lamellipodia and determining the population average. The average G/F ratio in the lamellipodium of migrating cells (0.22 ± 0.01, n=121 cells) was 1.7-fold lower than in the lamellipodium of non-migrating cells (0.37 ± 0.01, n=121 cells), a highly significant difference when tested in a Student's 2-tailed t-test (P = 0.003). The G/F ratio was lower not only in the lamellipodium of migrating cells compared to non-migrating cells, but also in the front region of the lamella situated immediately behind the lamellipodium (compare fig. 3.6E black arrow, green colour to fig. 3.6F, black arrow, red colour). Therefore relatively speaking there is less G-actin available within the lamellipodium of migrating cells to maintain assembly of the amount of F-actin observed in this region.

Availability of the actin monomer pool in the lamellipodium of migrating and non-migrating CEF.

To distinguish consumption of G-actin from the cellular pool and monomer derived from filament disassembly during protrusion of the cell margin, actin filament disassembly was specifically and rapidly blocked in live cells with jasplakinolide (Cramer 1999b). Jasplakinolide-treated cells were then fixed and

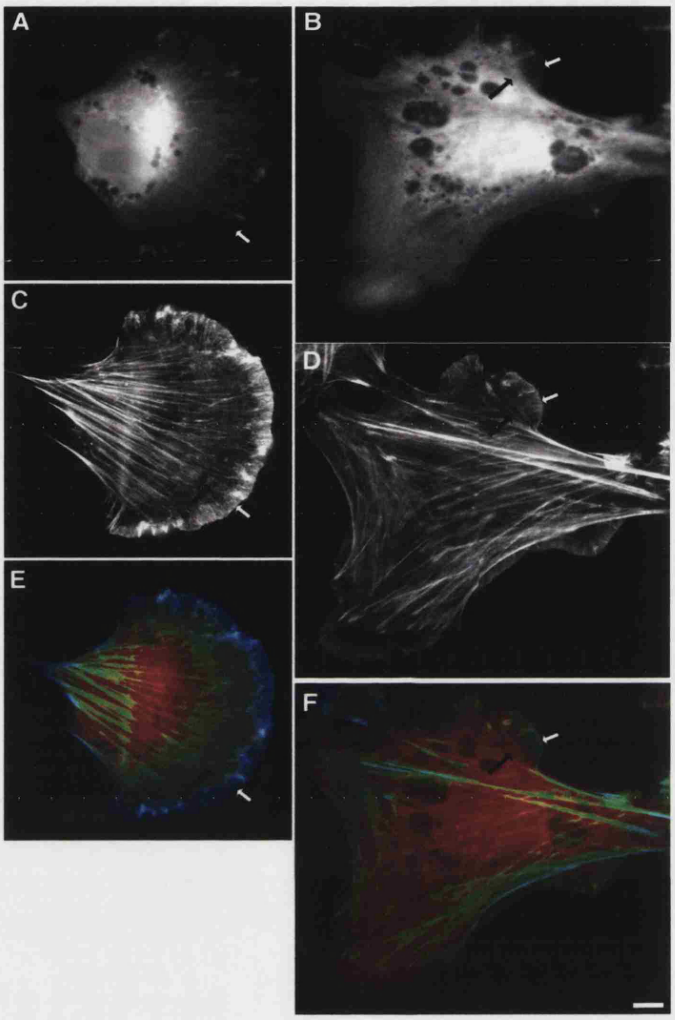


Figure 3.6: The ratio of G-actin to F-actin is lower in migrating compared to non-migrating fibroblasts. Migrating (A, C, E) fibroblasts were fixed in methanol-free formaldehyde and co-stained with DNase I for G-actin (A, B) and phalloidin for F-actin (C, D). The ratio image (E, F, provided by L. Cramer) was determined by dividing the G-actin image by the F-actin image. The ratio varies from low (blue) through intermediate (green) to high (red). White arrows denote the lamellipodium, black arrows a part of the lamella immediately behind the lamellipodium. Note that in migrating cells the G-/F-actin ratio in both the lamellipodium and lamella is lower compared to non-migrating cells. The scale bar in (F) represents 5μm.

DNase I was used to measure any remaining G-actin in the lamellipodium. Migrating cells were treated with 1μM jasplakinolide for 5 minutes and non-migrating cells for 15 minutes, times by which it is known that protrusion of the lamellipodium in all cells of these two respective populations had been on-going and then blocked (Cramer 1999b). In the lamellipodium of migrating fibroblasts treated with jasplakinolide, no obvious decrease in DNase I staining intensity was detected (n=18, fig. 3.7 compare C & D between arrowheads and compare line-scan intensity in I). In contrast, in non-migrating cells there was a decrease in G-actin staining in the lamellipodium (fig. 3.7 compare G & H between arrowheads and compare line-scan intensity in J) accompanied by an increase in lamellipodium length. As expected, in non-migrating cells when filament disassembly is blocked but protrusion on-going, by five minutes of jasplakinolide treatment G-actin levels within the first 0.3μm of the lamellipodium had decreased to virtually below the level of detection (n=19, fig. 3.7J, diamonds), and by 15 minutes, DNase I staining had decreased by approximately 50% throughout the entire lamellipodium (n=22, fig. 3.7J, compare squares to circles). This difference in reduction in DNase I staining intensity in migrating and non-migrating cells is not due to any potential difference in the cellular volume through which DNase I fluorescence staining is detected: confocal Z-sections show that the lamellipodium height in migrating and non-migrating cells is similar, ranging from 1-1.5μm (data not shown).

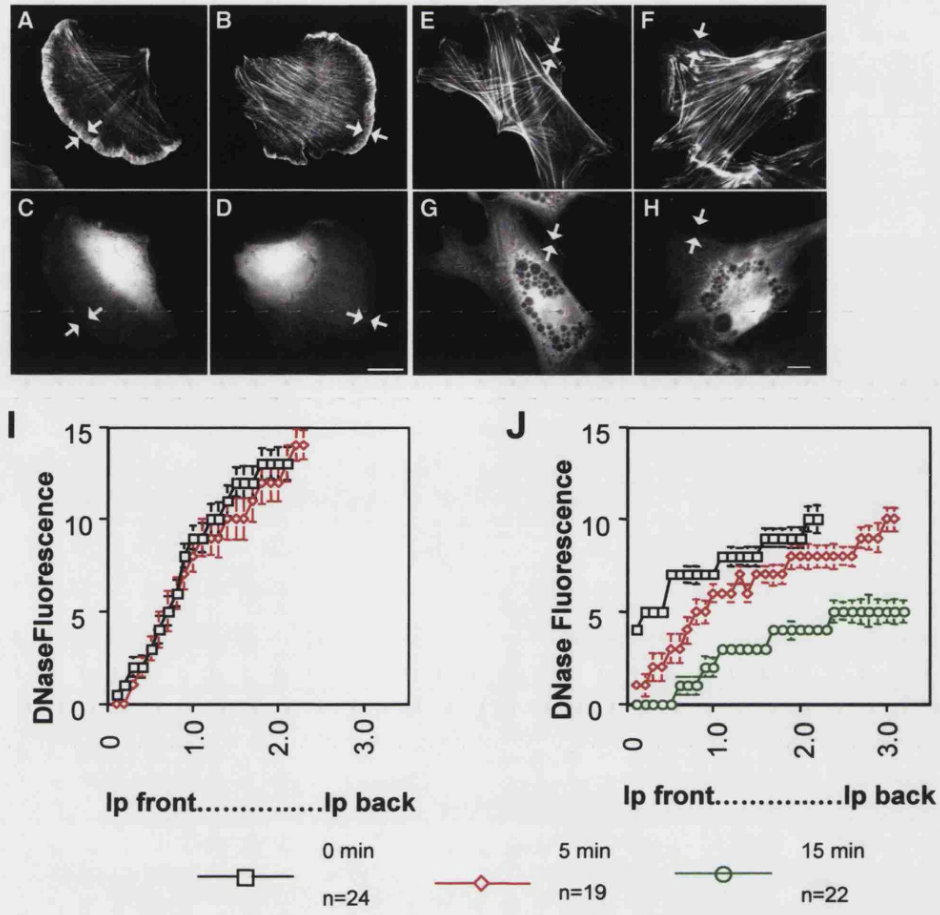


Figure 3.7: Non-migrating fibroblasts consume the G-actin pool during lamellipodium protrusion. Migrating (A-D, I) and non-migrating (E-H, J) were treated with jasplakinolide (B, D, F, H), formaldehyde fixed and co-stained with co-stained with DNase I for G-actin (C, D, G, H) and phalloidin for F-actin (A, B, E, F). Migrating cells were treated with 1μM jasplakinolide for 5 minutes and non-migrating cells for 5 and 15 minutes, times by which we know that protrusion of the lamellipodium in all cells of these two respective populations had been on-going and then blocked (Cramer, 1999). The lamellipodium (arrows) was identified from the phalloidin stain and fluorescence intensity line-scans of the level of DNase I staining (I, J) were taken across the lamellipodium for untreated cells (black squares), cells treated for 5 minutes (red diamonds) and cells treated for 15 minutes (green circles). Note the increase in lamellipodium length (arrows are further apart in (H)) and reduction in DNase I staining in treated, non-migrating cells (compare G and H between arrows and green circles compared to black squares in J). As phalloidin and jasplakinolide compete for F-actin binding, the phalloidin stain here is only used as a qualitative marker for the lamellipodium. The scale bars in (D) and (H) denote 10μm.

3.3: Discussion.

Actin monomer supply during polarised cell migration

The comparatively low G-actin to F-actin ratio within and behind the lamellipodium of migrating cells compared to non-migrating cells implies that only a limited amount of actin monomer is available for assembly in the lamellipodium during protrusion. In this situation therefore, it may be expected that monomer availability is tightly linked to the ability of the lamellipodium to protrude. In contrast, the higher G-actin to F-actin ratio in non-migrating cells in these two regions implies that the monomer pool here is relatively more available for assembly. This study also revealed differences between migrating and non-migrating cells in the use of the actin monomer pool during lamellipodium protrusion. No detectable decrease in monomer levels in the lamellipodium of migrating cells during protrusion was observed. While the possibility that monomer is diffusing into the lamellipodium of migrating cells at the same rate at which it is being used (and therefore not stored for any length of time that the assay can detect) cannot be excluded, this can only be for a maximum of 5 minutes before protrusion is blocked. This strongly suggests that actin monomer is highly limiting in the lamellipodium of migrating cells. While non-migrating cells can access the cellular monomer pool, it is evident that they cannot do this indefinitely, as inhibiting filament disassembly with jasplakinolide does eventually block lamellipodium protrusion (Cramer 1999b). Whether this is because only the lamellipodium pool of actin monomer is assembly-competent, or because monomer diffusion is insufficiently fast remains to be seen. Recent evidence from rat fibroblasts has shown that the rates of actin monomer transport during lamellipodium protrusion are too rapid to be accounted for by diffusion (Zicha *et al.* 2003). Therefore, some form of active transport may be required to deliver actin monomer to the leading edge. In any case, these data support the requirement for direct recycling of newly released actin monomer for lamellipodium protrusion in migrating, but not non-migrating, cells and the idea that recycling of the actin is the rate-limiting step for membrane protrusion in these cells.

Delivery of actin monomer to sites of actin filament assembly

How does the cell distinguish between the cellular actin monomer pool and monomer derived from recent actin filament disassembly? The actin monomer pool is in the main prevented from spontaneous assembly by being bound to thymosin beta-4 (Safer *et al.* 1990). One possibility is that a proportion or all newly disassembled actin monomer within the lamellipodium does not bind to the monomer sequestering protein thymosin but is instead "marked" for immediate assembly by another actin binding protein. Speculating highly, two potential candidates are profilin and twinfilin. Profilin is known to be responsible for catalysing the regeneration of ATP-actin from the ADP-actin monomer resulting from filament disassembly (Pollard *et al.* 2000), needed if the monomer is to undergo assembly has been shown to localise to regions of dynamic actin (Buss *et al.* 1992). Twinfilin is a recently identified ADF-homology-containing protein that lacks the ability to disassemble actin, but instead is a G-actin binding protein that has been shown to interact genetically with AC (Goode *et al.* 1998). As twinfilin is also localised to sites of rapid actin dynamics (Vartiainen *et al.* 2000), it has been speculated that it can act as a "molecular mailman" (Palmgren *et al.* 2002) delivering actin monomer to sites of assembly.

Why is continuous actin filament disassembly required for polarised, but not non-polarised protrusion?

Presumably, limiting the concentration of the actin pool and forcing the cell to use actin monomer derived from recent disassembly is used to set up a gradient of actin monomer availability with highest availability at the leading edge. Provided that actin filament disassembly was restricted to one spatial location, i.e. the lamellipodium, this would promote protrusion in that one spatial location. A higher availability of actin monomer might allow deregulated protrusion, as observed in non-polarised, non-migrating cells. How then, could disassembly be restricted to one spatial location? Since actin filament disassembly needs to be catalysed in cells by AC proteins, one possibility is that

AC activity is spatially directed towards the leading cell margin. This is addressed in the next chapter.

Chapter IV: The role of ADF/cofilin in the maintenance of cell polarity during fibroblast migration

Chapter IV: The role of ADF/cofilin in the maintenance of cell polarity during fibroblast migration

4.1 Introduction

Polarised cell morphology is required for normal cell migration. Morphological polarity of migrating cells can be divided into two main sections: acquisition of the polarised shape, and its subsequent maintenance throughout migration. The previous chapter established that sustained polarised migration requires continuous actin filament disassembly because actin monomer within the lamellipodium of migrating cells is highly limiting. In this chapter the role of AC proteins in the maintenance of polarised cell migration is discussed. The AC family of proteins is essential for filament disassembly/severing in cells (Moon and Drubin 1995; Bamburg 1999). It may be hypothesised, therefore, that controlling AC activity within the lamellipodium would regulate polarised lamellipodium protrusion during migration. A role for AC in cell polarity during migration has not previously been investigated. One study (Zebda *et al.* 2000) showed that AC is necessary for protrusion in EGF-stimulated adenocarcinoma cells, but it is difficult to assess a role for AC in cell polarity in this study as cells were analysed at a very early step. Cell polarity is also difficult to assess, as at this early stage lamellipodia appeared delocalised around the entire cell periphery. AC activity in cells has been modified in cells by over-expressing constitutively active or dominant negative LIM kinase (LIMK) (Arber *et al.* 1998; Yang *et al.* 1998) but these studies focussed on non-polarised, non-migrating cell types. Here, the localisation of ADF, cofilin, and inactive, phosphorylated, AC (pAC) within the lamellipodium of constitutively migrating chick embryo heart fibroblasts is examined. Using mutant AC proteins and constitutively active LIMK 1, for which AC are the only known substrates, this chapter shows that maintaining non-pAC within the lamellipodium is necessary for maintaining cell polarity during fibroblast migration

4.2: Results

Phosphorylated ADF/cofilin is depleted from the lamellipodium of polarised migrating CEF

As a first clue to identify candidate mechanisms for how AC might regulate polarised lamellipodium protrusion during migration, the distribution of AC and pAC in separate populations of fixed migrating and non-migrating chick embryo fibroblasts were investigated. A specific antibody that recognises both ADF and pADF in chick cells (Morgan *et al.* 1993) was used to show ADF was localised fairly homogenously throughout the cell, including the lamellipodium, in both migrating (fig. 4.1C) and non-migrating (fig. 4.1D) fibroblasts. The position of the lamellipodium was easily identified as an F-actin rich band as probed with phalloidin in stained cells (fig. 4.1A, B, between arrows). A similar distribution was observed for cofilin when cells were stained with a specific antibody that recognises both cofilin and phosphorylated cofilin (ACFL02, Cytoskeleton Inc; fig. 4.2). In contrast, pAC was differentially distributed in cells stained with a specific antibody that recognises only the phosphorylated form of both ADF and cofilin (Meberg *et al.* 1998). In migrating cells, it was clear from superimposing pAC and phalloidin staining in co-stained cells that pAC was localised to the cell body but depleted from the lamellipodium itself (fig. 4.3F, absence of fluorescence between the arrows). Longer fixation time (45 minutes) did not alter the cellular distribution of pAC (data not shown) thus it may be concluded that the observed distribution of pAC is not due to any poor fixation within this region. The same differential distribution of AC and pAC was observed in individual cells that we knew were migrating (by time-lapse microscopy) prior to cell fixation and staining in situ on the time-lapse microscope (data not shown). In contrast, in non-migrating fibroblasts, pAC (fig 4.3H) was clearly detectable in both the cell body and the lamellipodium in a generally even distribution (fig. 4.3, compare G and H between arrows). A similar staining pattern was observed in other cell types. In migrating J774 macrophages as in migrating fibroblasts, pAC was absent from the lamellipodium while in PtK2 cells, a non-migrating epithelial cell line like non-

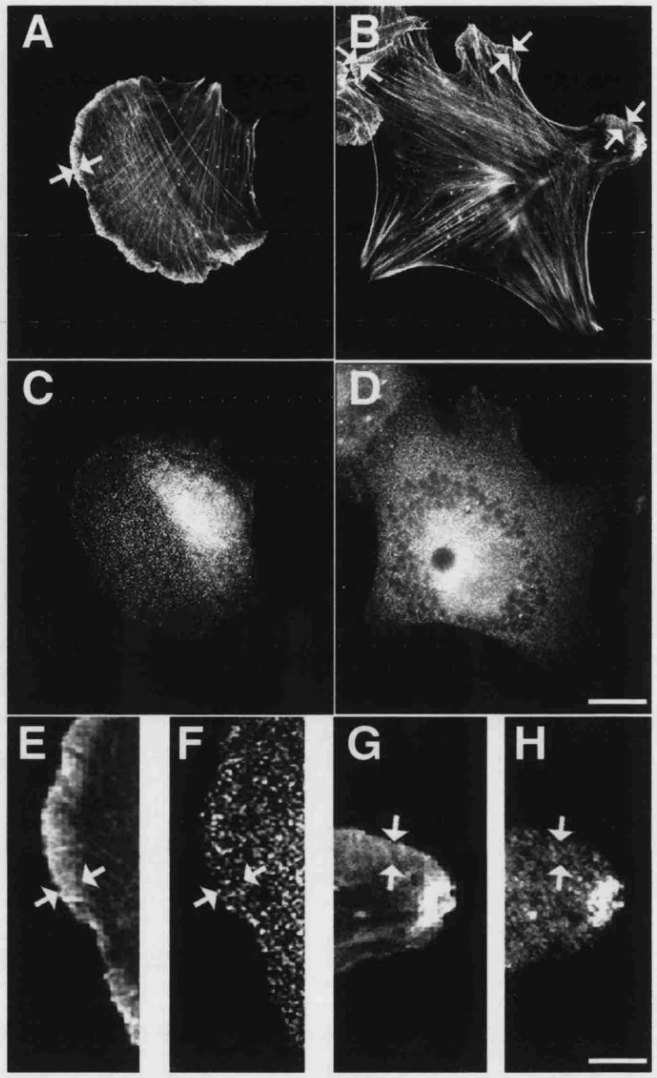


Figure 4.1: ADF is localised to the lamellipodium in migrating and non-migrating fibroblasts. (A-H) Migrating (A, C, E and F) and non-migrating (B, D, G and H) fibroblasts were fixed and co-stained with (A, B, E and G) phalloidin and (C, D, F and H) anti-ADF, which recognises ADF and pADF only. (E-H) Enlargements of the lamellipodium in (A-D), respectively. The lamellipodium is denoted between the arrows. The scale bar in (D) represents 10μm, the scale bar in (H) represents 5μm.

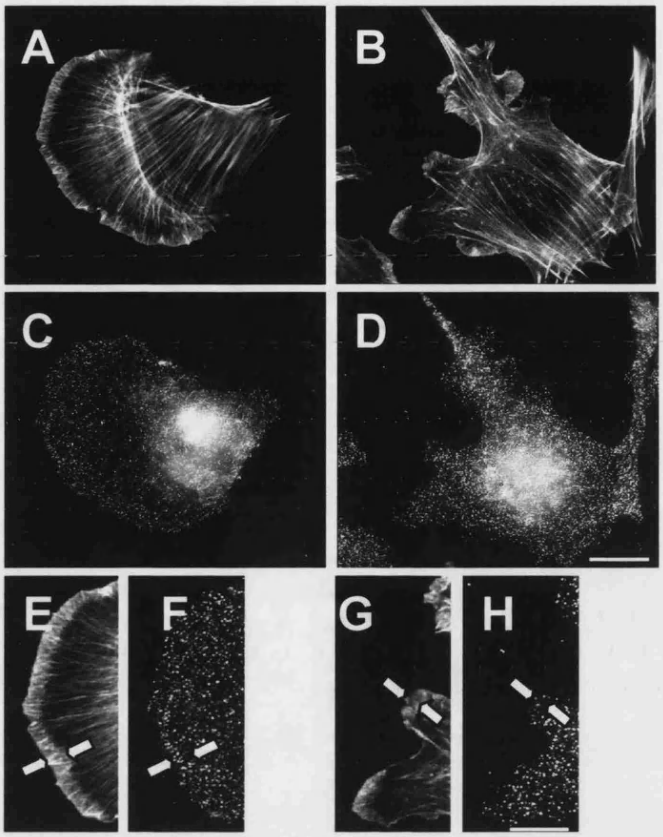


Figure 4.2: Cofilin is localised to the lamellipodium in migrating and non-migrating fibroblasts. (A-H) Migrating (A, C, E and F) and non-migrating (B, D, G and H) fibroblasts were fixed and co-stained with (A, B, E and G) phalloidin and (C, D, F and H) anti-cofilin, which recognises cofilin and phospho-cofilin only. (E-H) Enlargements of the lamellipodium in (A-D), respectively. The lamellipodium is denoted between the arrows. The scale bar in (D) represents 10μm, the scale bar in (H) represents 5μm.

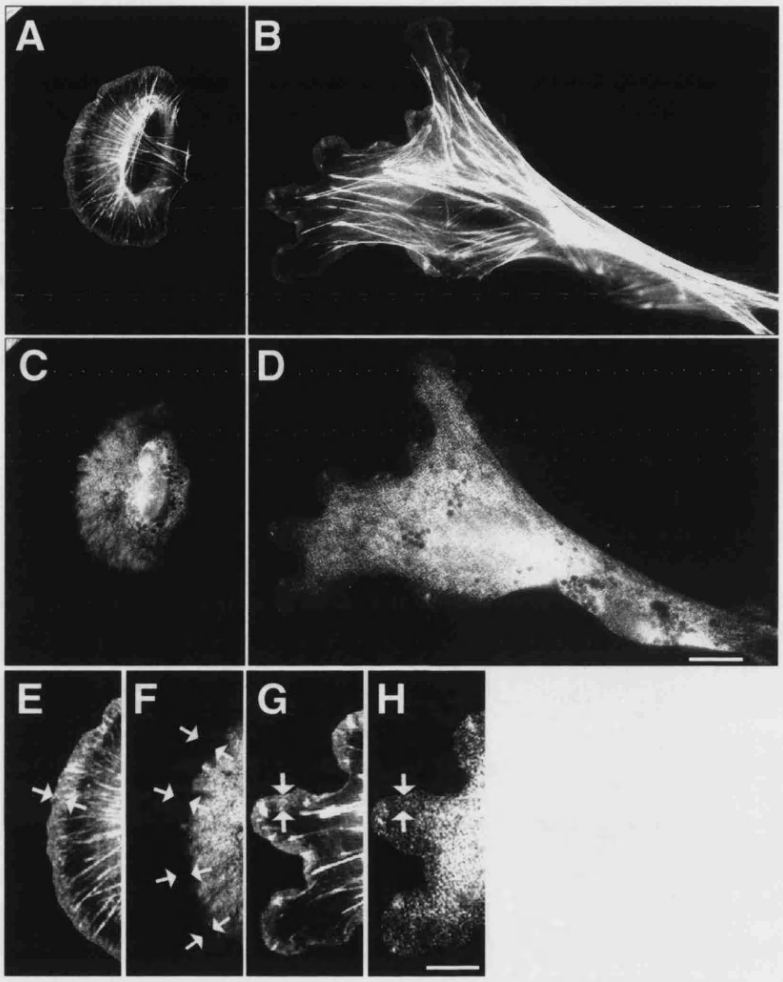


Figure 4.3: pAC is depleted from the lamellipodium in migrating fibroblasts. (A-H) Migrating (A, C, E and F) and non-migrating fibroblasts (B, D, G and H) were fixed and co-stained with (A, B, E and F) phalloidin and (C, D, G and H) anti-pAC, which is specific for the phosphorylated forms of both ADF and cofilin. (E-H) Enlargements of the lamellipodium (denoted between the arrows) in (A-D), respectively. The arrows in (F) denote the position of the lamellipodium in the migrating fibroblast as defined by the position of the F-actin rich band stained with phalloidin. Note the lack of pAC staining in the lamellipodium in the migrating cell (F, between arrows). The scale bar in (D) represents 10μm, the scale bar in (H) represents 5μm.

migrating fibroblasts, pAC was present throughout the lamellipodium (data not shown).

Adenovirus efficiently infects primary fibroblasts without affecting polarised cell migration.

To investigate whether the observed distribution of AC and pAC in cells is important for controlling cell polarity during cell migration it was necessary to perturb AC activity in cells. As with other primary cell types, however, gene transfer into migrating heart fibroblasts is very inefficient using conventional transfection protocols and certain lipid-based transfection reagents such as Lipofectamine have a deleterious effect on cell polarity and migration (data not shown). An adenoviral approach was therefore adopted as this has previously been found to work well in other difficult-to-transfect primary cell types such as hippocampal neurones (Minamide *et al.* 2003). It was found that cells infect and express exogenous gene products efficiently using the AdEasy adenoviral system (Stratagene). Viral infection with the empty AdTrack vector containing only GFP had no effect on cell polarity or cell migration speed and the appearance of infected cells was indistinguishable from non-infected controls (fig. 4.4 compare A to C). There was a time lag before good gene expression was attained and optimal protein expression of infected cells occurred after 48h of infection (fig. 4.5A), the time that fibroblasts grown on coverslips in primary culture begin to lose polarity and the capacity to migrate (fig. 4.5B). To circumvent this problem, the experimental protocol was therefore modified. In addition to simultaneously infecting and growing out cells for 24 hours from explants plated on matrigel-coated coverslips, explants were infected in suspension culture for a further 24h prior to plating the explants (fig. 4.6). While suspension culture of individual cells resulted in extensive cell death within 8 hours, explants could be successfully held in suspension without loss of viability for up to 5 days (data not shown). The period in suspension culture had no effect on the ability of fibroblasts to adhere to coverslips and migrate out from the explants with expected morphology (fig. 4.7A) and viral infection of

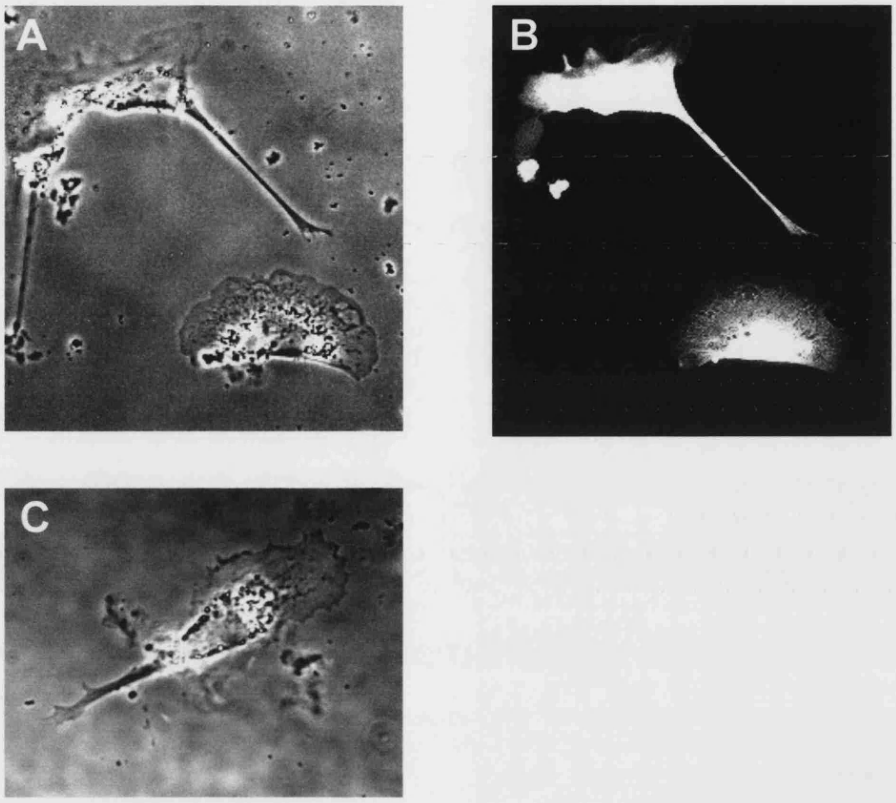


Figure 4.4: Morphology of GFP-infected fibroblasts. Explants were plated in the presence of GFP-adenovirus for 48 hours, fixed, and their morphology examined. (A) and (B) show an infected cell as determined by GFP expression (B). (C) shows an uninfected cell.

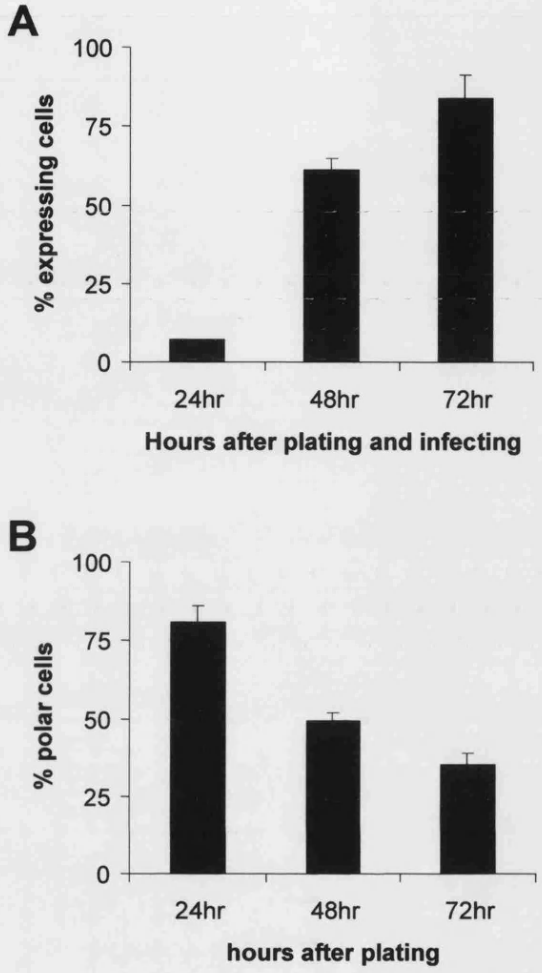


Figure 4.5: Time-course analyses of GFP expression and cell polarity. (A) Explants were plated in the presence of GFP-adenovirus for 24 hours, 48 hours and 72 hours, fixed, and the percentage of GFP-expressing cells in the outermost band of cells grown out from the explant quantified. (B) Explants were plated for 24 hours, 48 hours and 72 hours, fixed, stained with phalloidin, and the percentage of polar cells quantified. Graphs are expressed as the mean percentage of three independent experiments (n=200 cells per experiment) ± SEM. Note that good GFP-expression requires at least 48 hours in the presence of the virus, while by this time the number of polar cells has decreased.

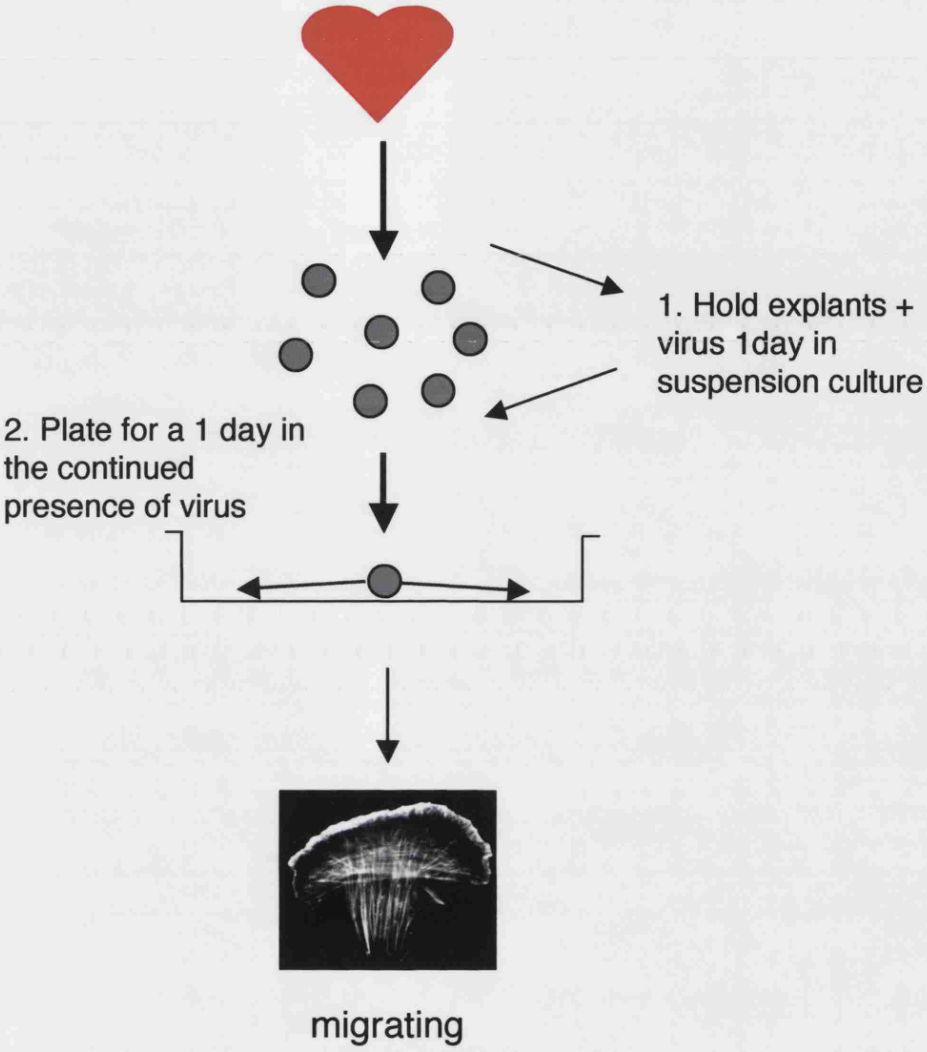


Figure 4.6: Modification of the CEF preparation protocol to accommodate viral infection. Embryonic day 7 chick heart explants are prepared and held in suspension culture in the presence of adenovirus for 24 hours, plated onto coverslips for a further 24 hours, fixed and stained.

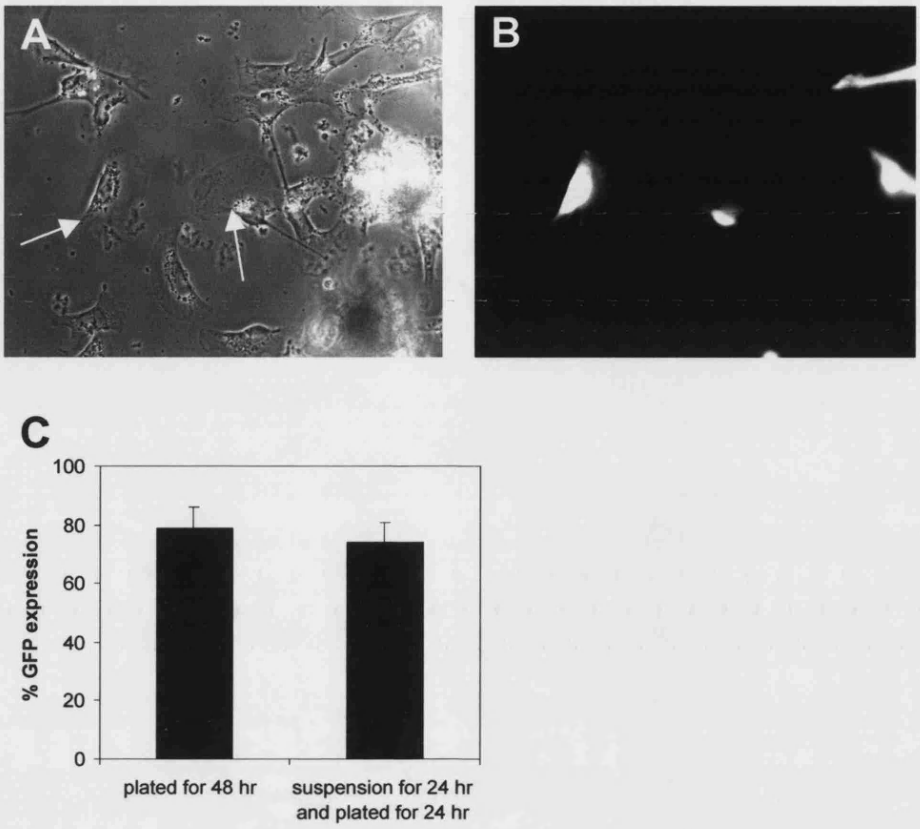


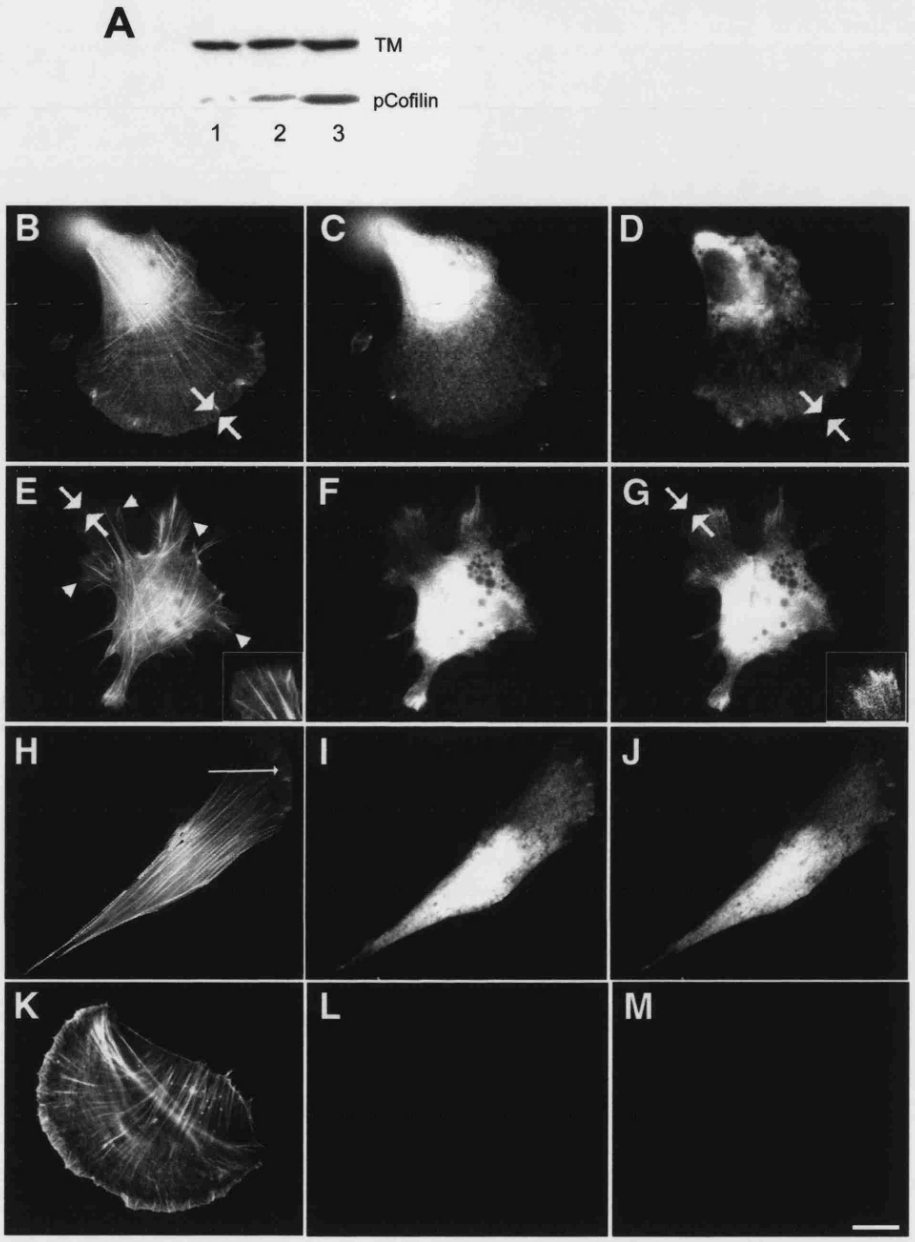
Figure 4.7: Adenovirus infects CEF efficiently in suspension. CEF explants were prepared and either plated in the presence of virus for 48 hours (C, left bar) or held in suspension culture in the presence of adenovirus for the expression of GFP for 24 hours and subsequently plated in the continued presence of the virus for a further 24 hours (A, B, C right bar).(A and B) Phase contrast (A) and fluorescence (B) images of infected cells. Arrows show the infected cells as determined from the GFP expression shown in (B). (C) Comparison of GFP expression in cells grown out from explants plated immediately and cells grown out from explants initially held in suspension for 24 hours prior to plating.

explants occurred as readily in suspension culture as when explants had been plated (fig. 4.7B).

Non-phosphorylated AC is required within the lamellipodium to maintain cell polarity during fibroblast migration

Using this adenoviral infection method of transferring genes into cells, the proportion of inactive, pAC was increased in migrating cells by expressing EE508 (thr508 replaced by glu and insertion of an additional glu), a constitutively active form of LIM kinase 1 (Edwards and Gill 1999). The activity of the EE508 construct was confirmed in cultured cells by L. Minamide. In LLPCK A4.8 cells infection with adenovirus expressing LIMK EE508 caused a dose-dependent increase in the amount of phosphorylated AC (fig. 4.8A, compare lanes 2 (low dose) and 3 (high dose) with the uninfected control in lane 1).

Infected cells expressing EE508 were identified by GFP expression driven off a second promoter from the same adenovirus, as previously described (He *et al.* 1998). In fibroblasts expressing EE508, cell polarity and protrusion of a single polarised lamellipodium was abolished. Instead these cells adopted a non-migrating appearance, characterised by the presence of multiple, non-polarised lamellipodia (fig. 4.8E, multiple lamellipodia in non-polar cells denoted by arrowheads). In contrast to non-infected migrating cells (fig. 4.8A, C), pAC was localised to these non-polarised lamellipodia (see fig. 4.8E and G, between arrows, and inserts in fig. 4.8E and G). In control cells, infected with adenovirus for expressing only GFP (vector alone), cell polarity was unaffected; cells protruded a single polarised lamellipodium (fig. 4.8B-D), indistinguishable from the uninfected cell (fig. 4.8K-M) and as expected, pAC, as with uninfected cells, remained virtually undetectable within the lamellipodium (fig. 4.8D, between arrows). 87% of cells expressing CA LIMK did not exhibit polarised morphology as characterised by protrusion of a single polarised lamellipodium


A
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L
M

Figure 4.8: Constitutively active LIM kinase induces loss of morphological cell polarity that is rescued by an active, nonphosphorylatable *Xenopus* XAC. (A) Western blot of extracts of cells infected with the LIMK EE508 construct (carried out by L. Minamide). Extracts (10μg total protein) from (lane 1) uninfected LLPCK A4.8 cells and extracts from cells infected for 24 hours with (lane 2) 25μl aliquot or (lane 3) 150μl aliquot of adenovirus for CA LIMK. Blots were developed first with the anti-pAC antibody and then re-probed with a mouse monoclonal antibody to exon 1b of tropomyosin 5 as a loading control. (B-M) The effect of LIMK EE508 and XAC A3 on cell polarity in migrating fibroblasts. In each experiment 1.5-2 x 10^6 viral focus forming units were added per heart explant. Cells were fixed, permeabilised and co-stained with (B, E, H & K) phalloidin and either (D and G) anti-pAC or (J and M) anti-XAC. Migrating fibroblasts were infected with (B-D) GFP only, or with (E-G) LIMK EE508 only or were co-infected (H-J) with LIMK EE508 and XAC A3 (rescue). Non-infected controls are shown in (K-M). The single lamellipodium in polar cells is denoted by a thin arrow, multiple lamellipodia in non-polar cells are denoted by arrowheads. Note the loss of cell polarity and protrusion of multiple lamellipodia in the cell expressing LIMK EE508 (E-G) and rescue of polarity in LIMK EE508 and XAC A3 co-expressing cells (H-J). The scale bar in (M) represents 10μm.

compared to 27-30% of non-infected cells and cells infected with vector alone (fig. 4.9, n=400 cells over 3 experiments).

The AC-specificity of the CA LIMK effect on polarised lamellipodium protrusion was tested using two independent approaches. First, the active, nonphosphorylatable *Xenopus* AC mutant, XAC A3 (ser3 replaced by ala, (Meberg and Bamburg 2000)) was expressed together with CA LIMK in co-infected cells. Co-expressing cells were identified by GFP expression to identify CA LIMK (see above) and by indirect immunofluorescence using a XAC1-specific antibody (Rosenblatt *et al.* 1997) that exhibited minimal cross-reactivity with chick AC, being barely detectable by fluorescence in non-infected cells when compared with cells expressing exogenous XAC (compare fig. 4.8J with 4.8M). Co-infection completely rescued cell polarity (fig. 4.9, n=270 cells over 3 experiments) and protrusion of a single polarised lamellipodium was restored (fig. 4.8H, single lamellipodium in polar cells denoted by thin arrow).

In the second approach, a pseudo-phosphorylated (less active) XAC mutant (E3) (ser3 replaced by glu, (Meberg and Bamburg 2000)) was expressed by itself in migrating cells. Cells expressing E3 lost their capacity to protrude a single polarised lamellipodium (fig. 4.10 compare C and D with the control cell in A and B, fig. 4.10G n=260 cells over 3 experiments) and, as with cells expressing only CA LIMK, cells protruded multiple lamellipodia (fig. 4.10C, arrowheads).

To investigate the effect that the loss of polarity induced by CA LIMK expression has on cell behaviour and movement, the behaviour of cells infected with CA LIMK was analysed by time-lapse microscopy. As expected, for cells with multiple protrusions persistent, directed cell movement was abolished. However, net movement was not zero and cells retained some capacity for movement, albeit in a slower and abnormal fashion (fig. 4.11). Overall cell speed was reduced (fig. 4.11C n=10 cells ± SEM) due to frequent changes in the direction of movement (fig. 4.11D). In other words, the persistence of cell movement in a given direction was greatly reduced. To test if this decrease in

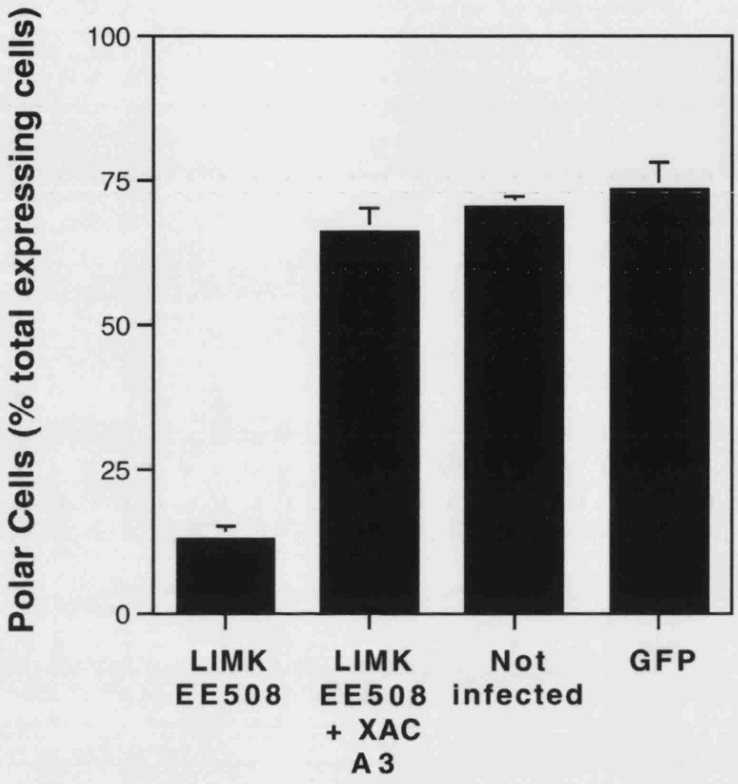


Figure 4.9: Quantification of cell polarity in virus infected cells
Graph shows % polar cells ± SEM of the data shown in the previous figure. Note that for uninfected cells, the graph is expressed as polar cells (% total cells) ± SEM .

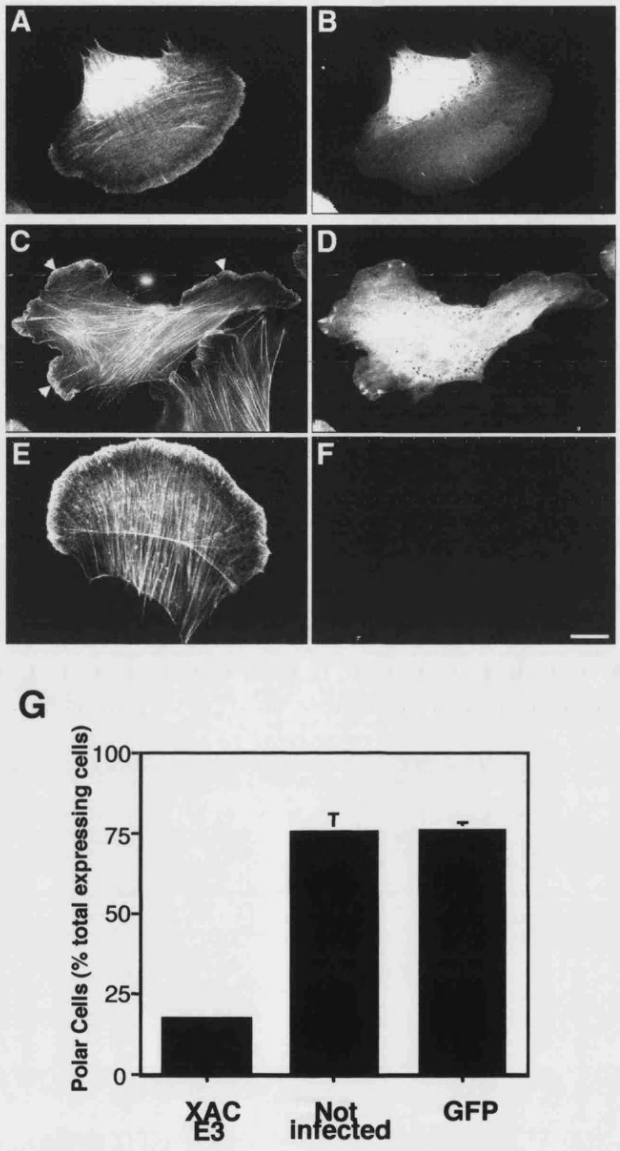


Figure 4.10: Expression of pseudophosphorylated XAC induces loss of cell polarity. (A-F) Migrating fibroblasts were infected either with (A and B) GFP or (C and D) XAC E3 or (E and F) were not infected, fixed and stained with (A, C and E) phalloidin or (D and F) co-stained with phalloidin and anti-XAC. The scale bar in (F) represents 10μm. (G) Quantification of staining. A graph of polar cells is shown (% total expressing cells ± SEM). Note the loss of cell polarity and protrusion of multiple lamellipodia in the cell expressing XAC E3 (arrowheads).

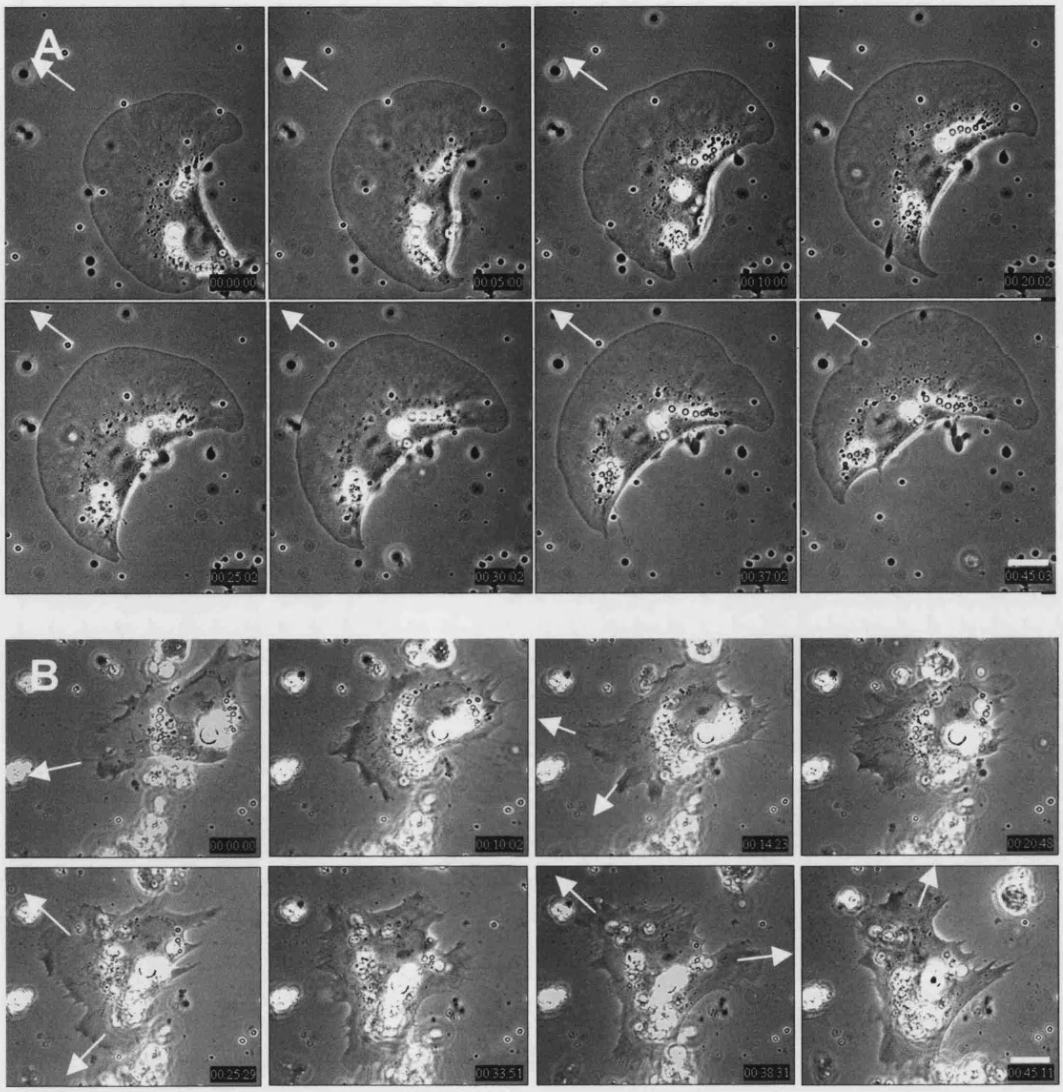

A
B
C
Migration rate (um/minute)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
LIMK EE508 infected
D
Frequency cell changes direction of movement in 20 minutes
7
6
5
4
3
2
1
0
Control
EE508 infected

Figure 4.11: Cell movement is less persistent and slower in cells infected with constitutively active LIM kinase Stills from phase-contrast movies of (A) a non-infected cell and (B) a cell expressing LIMK EE508. LIMK EE508-infected cells can move, but abnormally; cell speed is 3-fold slower (C) and the cell changes direction more frequently (D). Direction of arrows in (A) and (B) illustrate the net direction of movement of the cell. Time is in (hrs:mins:secs). The scale bars in (A) and (B) represent 10μm. Each movie is representative of at least 10 cells.

cell speed and in persistence of directed movement could be explained by lamellipodium protrusion behaviour, the history of lamellipodium behaviour was evaluated in both non-infected and CA LIMK infected CEF (fig. 4.12A and B). Interestingly, the rate of instantaneous protrusion was increased in CA LIMK-infected cells, but protrusion persistence was 3-4-fold lower with net reduced overall protrusion rate, explaining the large decrease in cell speed. Overall, a single cell had multiple protrusions, with one or two that were dominant protruding at any given time in a given direction. However, this protrusion was transient and underwent catastrophe after 1-2 minutes (fig 4.12B, traces show 2 lamellipodia in 2 representative cells; note the multiple troughs in each trace that represent retraction events). Following this event, either a new protrusion was initiated (at the same location or elsewhere around the cell periphery), or an existing protrusion became dominant. This was in contrast to control cells, where protrusion was persistent over at least 10 minutes (fig. 4.12A, slope of graph constantly increases).

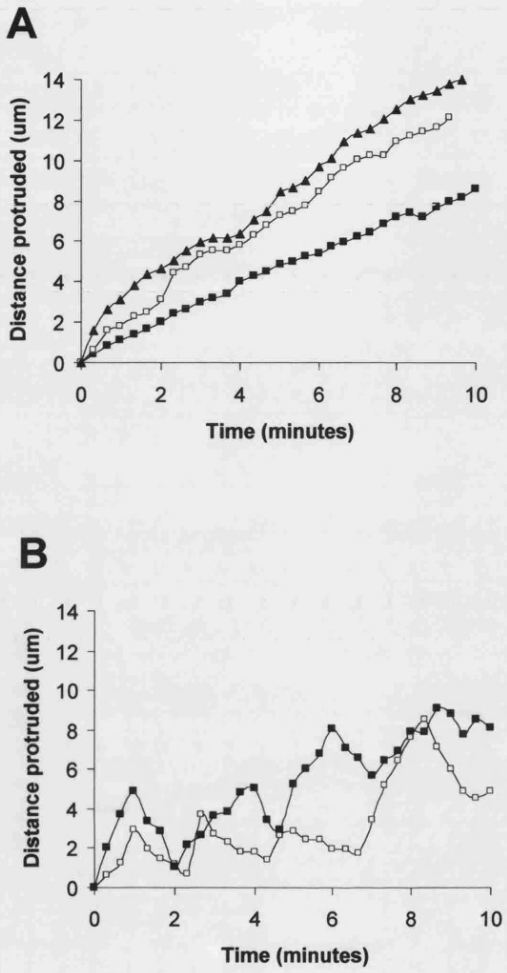


Figure 4.12: Protrusion is less persistent in cells expressing constitutively active LIM kinase. (A, B) History of lamellipodium protrusion over 10 minutes. Non-infected cells and cells expressing LIMK EE508 were imaged by time-lapse microscopy and the protrusion rate of the dominant lamellipodium determined. (A) graph shows 3 individual lamellipodia in 3 separate non-infected cells. (B) graph shows 2 individual lamellipodia in 2 separate LIMK EE508-infected cells. Note that the instantaneous protrusion rate is faster in (B) but less persistent, resulting in a decrease in overall protrusion rate.

4.3: Discussion.

The importance of pAC localisation in maintaining cell polarity during fibroblast migration

These data indicate that regulating the amount of phosphorylation of AC proteins in specific cellular regions is important for maintaining a single polarised protrusion during cell migration. These data support the idea that locally restricting required actin filament disassembly to one spatial location can control the maintenance of a polarised protrusion. Locally maintaining AC in the active, non-phosphorylated state within the lamellipodium ensures a continual supply of actin monomer derived from disassembly for re-assembly and protrusion, directed in one spatial location. This stabilises and maintains polarised protrusion and net migration. Conversely, when pAC is increased in the lamellipodium, the local monomer supply is depleted without being replenished by AC-catalysed filament disassembly. As monomer is abundant in the rest of the cell body (Cramer *et al.* 2002), it would be expected that a new protrusion would randomly form where actin monomer was available for assembly, resulting in a loss of net cell polarity.

In migrating cells, the presence of one protrusion can apparently suppress other protrusions from forming in the same cell, probably because there is insufficient actin monomer at other spatial locations to fuel production of further lamellipodia. Inhibiting AC function and polarised protrusion results in multiple, albeit depolarised, lamellipodia. This could be physiologically relevant, for example when a migrating cell reaches its destination there must be a way of halting its movement. Equally, during cell turning events, the cell must pause and become transiently depolarised to sense its environment before deciding on a new direction. It is tempting to speculate that locally regulating the phosphorylation state of AC controls switching between the two mechanisms.

How does spatially and temporally regulating AC activity maintain a single polarised protrusion?

The lamellipodium of a polarised migrating cell is composed of a meshwork of short, stiff and highly branched actin filaments, needed if actin polymerisation is to provide the force to drive the plasma membrane forward during protrusion (Mogilner and Oster 1996; Svitkina *et al.* 1997; Svitkina and Borisy 1999; Pollard *et al.* 2000; Mogilner and Oster 2003). In order to maintain this situation, the level of actin polymerisation must not out-strip the level of depolymerisation. A high level of AC activity in the lamellipodium may therefore be important not only for providing an on-going source of actin monomer, but also as a counterweight to actin filament assembly. In this scenario, AC is vital for maintaining the structure of the dentritic brush in the lamellipodium. High levels of AC activity in the cell body would almost certainly be inappropriate in a migrating fibroblast as the cell body predominantly consists of long graded polarity bundles (Cramer *et al.* 1997), therefore a large pool of pAC in conjunction with filament bundling and stabilising proteins is likely to maintain these actin structures. Blocking AC activity may promote the formation of longer, less stiff actin filaments. These would be less effective at pushing up against the plasma membrane and membrane tension would therefore result in frequent retraction events, as seen when AC was blocked with CA LIMK. In addition, the rapidity of instantaneous protrusion seen in cells expressing CA LIMK is not balanced by replenishment of the actin monomer. This bias towards filament assembly can only be maintained so long as actin monomer is available. Exhaustion of the local monomer pool may cause destabilisation of the protrusion until more actin monomer is provided either from newly disassembled actin filaments, or from the stored actin monomer pool within the cell body. These two situations may explain the transience of protrusion persistence and halting movement of LIMK EE508-expressing cells.

Interestingly, similar behaviour can be seen in cells with high levels of Ena/VASP activity at the cell margin. These are a family of actin binding

proteins that play key roles in cell motility (Machesky 2000; Reinhard *et al.* 2001; Cramer 2002; Kwiatkowski *et al.* 2003). Ena/VASP concentration within the lamellipodium correlates with increased instantaneous protrusion rate (Rottner *et al.* 1999), however Ena/VASP decreases the rate of cell migration (Bear *et al.* 2000). Findings by Bear and colleagues (2002 (Bear *et al.* 2002), reviewed by (Cramer 2002)) can explain this paradox in terms of a shift from short actin filaments to long filaments when Ena/VASP is in excess in the lamellipodium. Lamellipodia containing the long filaments are more prone to retraction than those containing the short filaments, leading to transient, depolarised lamellipodium protrusion around the cell. As a consequence, in spite of the increased protrusion rate, the migration rate drops. Whether Ena/VASP and AC act in concert – low Ena/VASP activity and high AC activity for polarised protrusion, and vice versa for depolarised protrusion - to regulate actin filament length and hence cell behaviour remains an interesting point for future study.

Spatial regulation of AC activity in polarised migrating cells

Locally maintaining AC proteins in the non-phosphorylated, active state within the lamellipodium is necessary to maintain polarised lamellipodium protrusion during cell migration. It is not yet known how this is regulated in cells. One open possibility is that LIM kinase localisation may be restricted to outside the lamellipodium in migrating cells. Another scenario is that the rate of AC dephosphorylation, acting via the recently identified specific AC phosphatase Slingshot (Niwa *et al.* 2002) is higher in the lamellipodium in migrating cells compared to the rest of the cell. Conversely, 14-3-3zeta binding may stabilise phosphorylated-AC specifically outside the lamellipodium as this protein has newly been shown to be a pAC-binding protein that protects pAC from dephosphorylation (Gohla and Bokoch 2002). Preliminary data suggests that a combination of both decreased kinase levels and increased phosphatase levels in the lamellipodium of migrating compared to non-migrating cells may be responsible for maintaining AC in the non-phosphorylated state, but further work is required to establish if this is the case.

Since there is very little pAC within a polarised lamellipodium, how could the extent of actin filament disassembly be controlled in migrating cells? One possibility is the net degree of polymerisation in the lamellipodium may be greater than the net degree of depolymerisation. Recent data has shown that, while the basal levels of both actin assembly and disassembly are largely even throughout the lamellipodium and occurring constantly, actin assembly is also promoted at the extreme lamellipodium tip (first 1μm) (Watanabe and Mitchison 2002). This would have the effect of using actin monomer as soon as it became available, preventing the formation of a non-limiting supply of actin monomer and ensuring that protrusion continues to be directed in one location. The amount of tropomyosin bound to actin within the lamellipodium could help to restrict the location of AC-mediated actin filament disassembly. Unpublished data from our laboratory shows that tropomyosin staining is very weak and diffuse at the extreme leading edge of migrating CEF, inferring the existence of a relatively tropomyosin-free compartment at this location. This is in agreement with the recent observation by DesMarais et al. (DesMarais *et al.* 2002) that tropomyosin is absent from the extreme leading edge and suggests that specialised sub-compartments within the cell may be maintained by differential distribution of tropomyosin isoforms (Gunning *et al.* 1998). A gradient of hydrolysis of ATP-actin to ADP-actin, the preferred state for AC binding, is undoubtedly a factor. This ensures that only older actin filaments are disassembled and prevents wasteful disassembly of newer actin. pH may also play a part in regulating ADF activity within the lamellipodium: large pH changes are likely to occur close to the membrane at the leading edge of cells, where high levels of ion exchange activity by transporters such as the Na^+/H^+ pump occur. Interestingly, this exchanger has been implicated in the control of cell polarity during fibroblast migration (Denker and Barber 2002) and one speculative mechanism for this may be to control actin dynamics via AC regulation.

Remaining questions

While some of the upstream signalling processes that act to regulate polarised protrusion are becoming clearer, less well understood is how this information is refined by the cell in order to produce an appropriate response. One example of this kind of problem is the well-characterised control of lamellipodium protrusion behaviour by the small GTPase Rac. Rac acts to promote lamellipodium protrusive behaviour during cell migration (Nobes and Hall 1999), however one of its downstream targets is to activate LIM kinase (Gungabissoon and Bamburg 2003), which inactivates AC and, as shown in this chapter, thus prevents polarised migration. How can this contradiction be rationalised? One possibility is that the signal downstream of Rac bifurcates: one branch going to activate LIM kinase, the second to activate the AC phosphatase Slingshot. The balance between kinase and phosphatase activity would then regulate AC phosphorylation. Such cellular fine-tuning could ensure that protrusion of the cell margin remains highly responsive to changes in extracellular cues. Elucidation of the as yet unknown signalling pathways upstream of Slingshot should help to answer this question.

This chapter has provided the first indication of a pAC analogue (E3) acting as a dominant negative. It remains for forthcoming studies to determine why E3 can function in this role, however this observation should prove a useful tool to aid in future work on the role of these essential proteins in cell motility. In addition, we do not yet know whether it is ADF, cofilin, or both that regulate cell polarity during cell migration, however both ADF and cofilin are apparently present in these cells as we obtained positive staining with specific antibodies. Other than AC, lamellipodium protrusion during migration is also maintained by microtubules (Wadsworth 1999; Wittmann and Waterman-Storer 2001; Small *et al.* 2002a). The ways in which the microtubule and actin cytoskeletons interact are beginning to be elucidated, however more work is required to understand under what conditions microtubules take the lead, when AC activity is most important, and whether the two systems are regulated separately or together. Aside from lamellipodium protrusion, migrating cells need to move the cell body forward and retract the tail, stages of migration for

which adhesion and myosin are likely to play vital roles. Studies for the future will need to address the spatial and temporal coordination of all of these processes for the maintenance of polarised cell migration.

Finally, this chapter has defined a role for localised AC activity in maintaining morphological polarity during cell migration. As yet unanswered, however, are the questions of how a fibroblast acquires its polarised morphology and the role, if any, that AC-mediated actin filament disassembly/severing plays in this process. These will be addressed in the next chapter.

Chapter V: Acquisition of morphological polarity in primary fibroblasts

5.1 Introduction

Initiation of cell migration requires a cell to become morphologically polarised and continuously protrude a single lamellipodium oriented in the direction of migration. This acquisition of the polarised cell morphology in CEF can be subdivided into three major stages: formation of a polarised protrusion, organisation of actin into oriented cables perpendicular to the protrusion and tail formation. The role of microtubules in maintaining directed migration is well established (Wadsworth 1999; Wittmann and Waterman-Storer 2001; Small *et al.* 2002a; Small and Kaverina 2003), however in many cells assembly of the actin cytoskeleton is the driving force in the formation of a polarised shape and reorganisation of the actin cytoskeleton is crucial if a cell is to migrate. To date however, our understanding of the mechanism of acquisition of a polarised protrusion remains limited. As described in Chapter I, initiation of morphological polarity is a multi-stage process. This chapter examines the stages of fibroblast polarisation. Three of these stages are analysed in turn to investigate the roles that the actin and microtubule cytoskeletons play in forming a polarised cell shape. Molecular, pharmacological and genetic approaches have been used to identify many of the upstream components that are necessary for directed cell polarity. In addition to actin and tubulin, two families implicated in this process are the AC proteins and myosins (Drees *et al.* 2001).

The precise role that AC proteins play in the acquisition of cell polarity has not previously been investigated. The previous chapter showed that maintaining a pool of active AC at the leading edge of a migrating cell is required to maintain polarity during cell migration, but the initiation of cell polarity was not studied. A second study showed that AC is necessary for lamellipodium protrusion in EGF-stimulated adenocarcinoma cells (Chan *et al.* 2000; Zebda *et al.* 2000) but it remains unclear which actin filaments AC was affecting and whether this activity was necessary for the cell to polarise.

Myosin II activity is likely to play an important role in cell body translocation and tail retraction (Mitchison and Cramer 1996) and, although various studies have implicated myosin in cell polarisation and polarity (Verkhovsky *et al.* 1999; Kaverina *et al.* 2000) the mechanism underlying this is unknown. Since asymmetries in actomyosin contractility across an otherwise uniform cell are sufficient to induce polarisation in fibroblasts by initiating tail formation (Kaverina *et al.* 2000), myosin may be involved in tail formation during polarisation. Alternatively, myosin-based forces could be responsible for the required re-orientation of the actin cytoskeleton.

This chapter shows that fibroblasts polarise in a series of stages, with the tail forming before the definition of the final leading edge. Perturbation of actin filament disassembly using jasplakinolide, mutant AC proteins and constitutively active LIM kinase showed that AC was required for vital early actin rearrangements that were needed for successful completion of all subsequent stages. Both the severing and depolymerising activities of AC were required for optimal numbers of polar cells. Blocking myosin function with the ROCK inhibitor Y27632 (Uehata *et al.* 1997) and the myosin II inhibitor blebbistatin (Straight *et al.* 2003) revealed two separable roles for myosin: myosin stabilised the newly defined cell rear through regulation of cell body movement and maintained a polarised shape through tail retraction. Interestingly, microtubules were not required to form a polarised shape, however in the absence of microtubule dynamics polarisation appeared transient, the leading edge was unstable, and the cells failed to migrate in a directed fashion.

5.2 Results

Fibroblasts polarise in distinct morphological stages

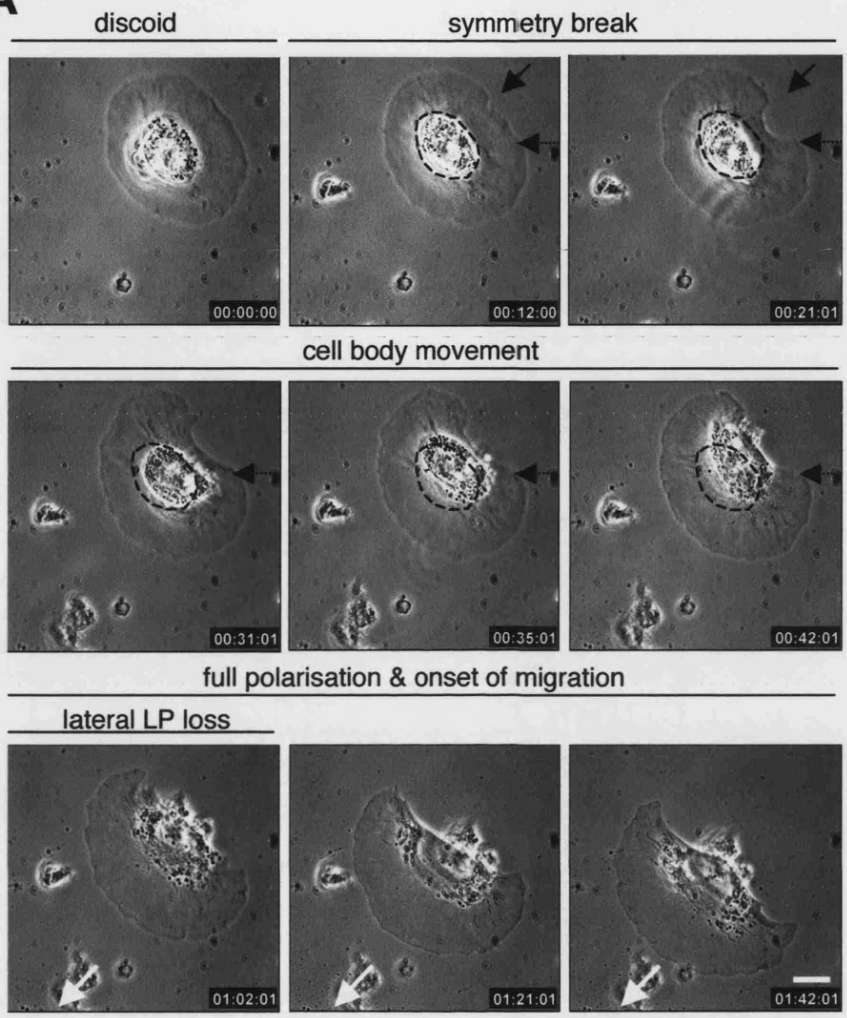
In order to evaluate the roles played by individual proteins in the initiation of cell polarity, the morphological changes that occur during the polarisation process had first to be defined. The explant culture system for primary chick embryo fibroblasts was inappropriate for use in these studies as the fibroblasts at the edge of the explant that were used in the previous chapter were already clearly polarised and migrating. An alternative assay system was needed that would enable the study of polarisation from the earliest stages to eventual migration. CEF can polarise and migrate in media containing serum without any additional external polarity cue; therefore a dissociated culture system allows the study of polarisation without the need to apply an exogenous stimulus. Dissociation proved easier and yielded more cells if the explants were held overnight in suspension culture in growth media prior to dissociation (data not shown). This is probably because residual cardiac membrane is very sticky and hinders the separation of individual cells. The adhesiveness of the residual membrane was reduced by overnight incubation in suspension culture. Following dissociation, and upon plating, the fibroblasts polarised and subsequently migrated at the same rate as in explant culture (data not shown) except for a difference in time-scale. In contrast to the explant culture system, there was no lag period before the cells began to migrate, presumably because the lag period is due to the time taken for the explant to adhere to the coverslip. Migration accordingly ceased sooner in dissociated culture; by 12 hours after plating fewer than 20% of the cells remained polarised and migrating (data not shown). The majority of the cells polarised in the first 2 hours (data not shown). Thus to study polarisation cells were used at 0-2 hours after plating as this maximised the number of cells with similar morphology at a given time.

Time-lapse phase-contrast microscopy of live cells and fluorescence microscopy of fixed and stained cells showed that fibroblasts polarise in distinct stages as defined by morphology and actin organisation. Cells adhered to the

coverslip within 5 minutes of plating. The fibroblast was initially fairly symmetrical and discoid in shape (fig. 5.1A left panel of top row, n=14 movies). The first sign of a break in the symmetry was an inwards movement or loss of the plasma membrane (fig. 5.1A middle panel of top row, black arrow). This enlarged over the course of approximately 10 minutes until a distinct crescent-shaped portion had been taken out of one side of the cell (fig. 5.1A top right panel). This break in symmetry ultimately formed the rear of the cell, or cell tail. Throughout this process the cell body was a tight phase-bright ball, which moved rearwards across the cell towards the break in symmetry (fig. 5.1A compare the initial position of the cell body as marked by the dashed circle to its position over time). Once the cell body was located in this region, further inwards retraction of the hole stopped (fig. 5.1A right panel of middle row). However, retraction did continue to enlarge the hole, but did so laterally by lateral loss of lamellipodium flanking the hole, and this correlated with cell spreading until the characteristic crescent or kite shape of the migrating fibroblast had been attained (fig. 5.1A bottom row). On completion of the formation of the polarised shape, the cell began to migrate (fig. 5.1A direction of migration shown by white arrows). Formation of the cell tail thus yielded a single polarised lamellipodium in one spatial location by default. From the earliest sign of lamellipodium loss to cell migration took an average of (75 minutes +/- 45 minutes) In individual cell the biggest variation was in the time taken to achieve the break in symmetry, however once this had been attained cells proceeded through to a completely polarised morphology at the same rate (fig. 5.1B). However, the cells were not synchronised and therefore, at any given time point, there was a mixed population of morphologies with individual cells showing different respective stages of polarisation. Due to this fact, the percentage of fully polarised migrating cells was never more than 45-50 % at any one time.

Having made this initial characterisation of the stages of morphological polarisation in live cells, the relationship of the actin cytoskeleton to each of these morphological stages was examined. Each stage occurred sequentially in live cells at approximately the same time after the break in symmetry had been attained; this consistency enabled the use of cell morphology together with

A



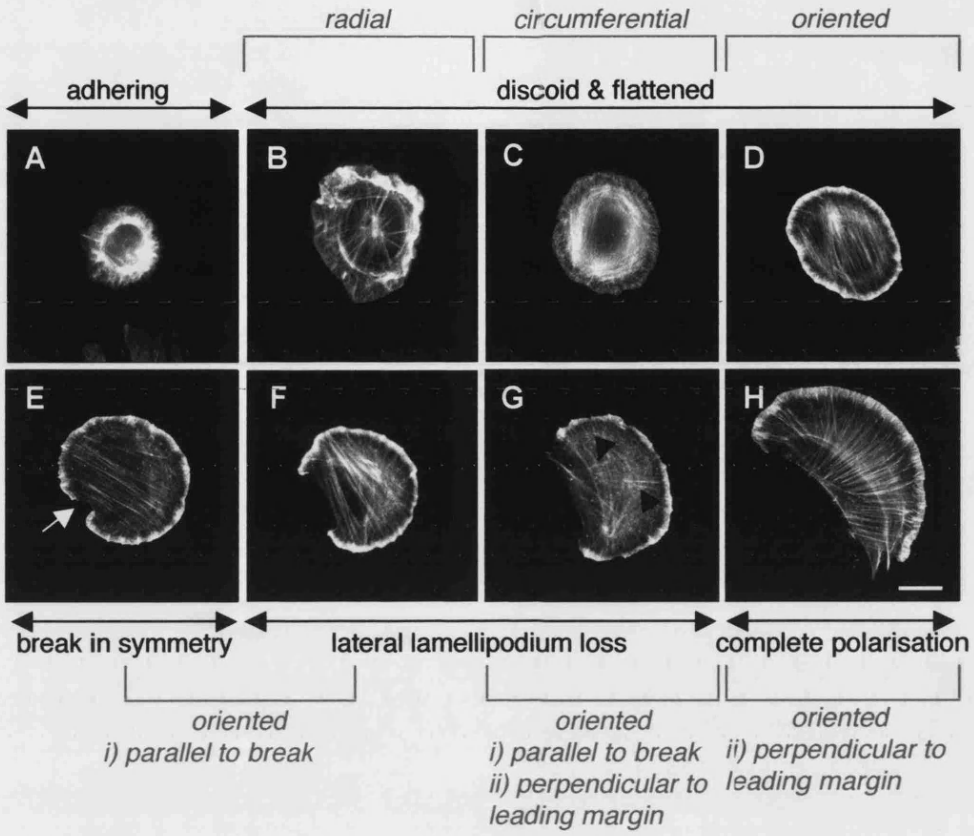
B

Shape	Time after plating in cell population	
Flattened & discoid	0-50 minutes	
Break in symmetry	10-60 minutes	
Rearward cell body movement	25-75 minutes	Initial tail formation
Lateral loss of lamellipodium	45-85 minutes	Complete tail formation
Complete polarisation & onset of migration	55 minutes onwards	

Figure 5.1: Polarisation of primary chick embryo fibroblasts. (A) Still images extracted from a phase contrast movie of newly plated dissociated CEF. The black arrow (12 and 21 minute time-points) indicates the first break in morphological symmetry. Note the rearward movement of the cell body in the middle panels (moving to the right, away from the dashed circle) compared to its location when the break in symmetry occurs (12 min time-point, dashed circle). The rear of the cell remains still (dotted arrows). White arrows show the direction of migration (onset at 60 minutes). Note that the location of the break in symmetry becomes the rear of the cell. This movie is representative of 14 cells. The scale bar represents 10μm, and the time is in hrs: mins: secs. (B) Table to summarise the relationship between cell shape and time after plating. Times are given as a range across the cell population.

known time after plating as markers of polarisation stage for fixed time-point assays. Dissociated fibroblasts were plated for 5-60 minutes, fixed and stained with phalloidin. A number of actin rearrangements occurred while the cell shape remained discoid and flattened and before any morphological sign of polarisation could be seen. After 5 minutes the cells were mostly flattened out on the coverslip (fig. 5.2 compare A to B). Within the cell body, actin was distributed in a radial arrangement outwards from the centre (fig. 5.2 B). This distribution persisted for less than 5 minutes before the actin re-oriented into a circumferential array close to the plasma membrane (fig. 5.2 C). A further rapid series of actin rearrangements occurred and by 15 minutes after plating the majority of cells were very slightly ovoid (rather than totally discoid) in shape with actin arranged in an oriented fashion across the longest length (fig. 5.2 D). Only after this had taken place did the break symmetry occur. This always occurred at a position perpendicular to the actin orientation (fig. 5.2 E). Actin remained in this orientation throughout formation of the tail and subsequent (though at reduced abundance, see below) full polarisation of the cell. During the stabilisation of the tail, the first actin bundles oriented perpendicular to the leading margin appeared (fig. 5.2 G, arrow). These increased in number, accompanied by a decrease in the number of actin bundles oriented parallel to the cell margin of the break in symmetry, until by the time that the cell was fully polarised, the majority of the actin was oriented perpendicular to the leading margin (fig. 5.2 H). The relationship of each type of actin bundle organisation to the morphology of the cell is summarised in figure 5.2I and a graph of the variations in the 4 main morphological stages over time is shown in figure 5.2J.

The above section has shown that polarisation occurs by a series of sub-steps, each defined by a shape change. Each shape change is accompanied by a distinct actin organisation. The remainder of this chapter examines the regulation of 3 of these sub-steps in turn: the regulation of the transition from circumferential to oriented actin organisation, the rearward movement of the cell body during the initial stages of tail formation, and the maintenance of the fully polarised shape.



I

Shape	Actin organisation
Adhering	
Flattened & discoid	radial circumferential oriented
Break in symmetry	oriented i) parallel to break
Lateral loss of lamellipodium	oriented i) parallel to break ii) perpendicular to leading margin
Complete polarisation	oriented iI) perpendicular to leading margin

J

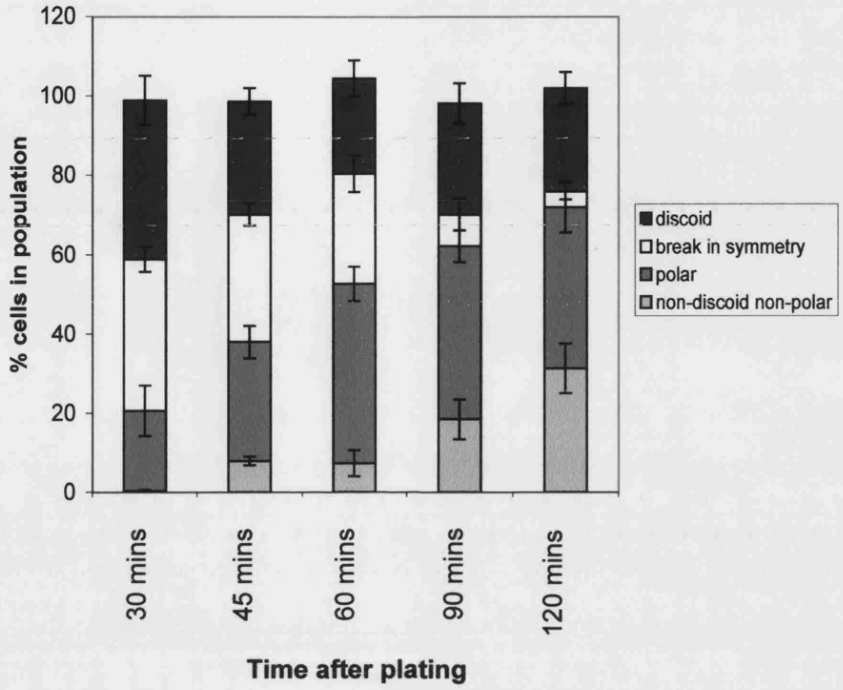


Figure 5.2: Actin changes during morphological polarisation. (A) to (H) dissociated CEF were plated for (A) 1, (B) 5, (C) 10, (D) 15, (E) 25, (F) 40, (G) 50 and (H) 60 minutes, respectively, fixed and stained with phalloidin. Note that the break in symmetry occurs at a position perpendicular to the orientation of the actin filaments (E, arrow). The arrowheads in (G) mark the appearance of the first front-back oriented actin bundles. (I) Table to summarise the relationship between cell shape and actin organisation. (J) Histogram showing the variation in the four main polarisation stages with time after plating

AC regulates changes in actin organisation during polarity initiation

As the section above has shown, a series of changes in actin organisation occur while the cell is discoid and flattened. These happen in a defined order in the vast majority of polarising cells, suggesting the importance of changes in actin organisation not only for each shape change that occurs during polarisation, but also to the overall driving mechanism of polarisation. One key change, that is the focus of this part of the chapter, is the formation of oriented actin bundles. Two obvious possibilities for controlling the formation of these bundles are re-orientation of the existing radial or circumferential actin bundles to form a new higher order actin structure, or disassembly of the radial and circumferential bundles and re-assembly of the released monomer into the newly required structure. As AC has been implicated in the initiation of cell polarity in yeast (Drees *et al.* 2001), formation of oriented bundles seemed a likely point for AC to be involved in polarisation.

Requirement for AC activity in formation of oriented actin bundles

To test a requirement for AC activity in the formation of oriented actin bundles, we increased the proportion of phosphorylated (inactive) AC by virally expressing the constitutively active LIMK construct EE508 (thr508 replaced by glu and insertion of a second glu) that was used in chapter IV. As shown in chapter IV, efficient infection of CEF required 48 hours in the presence of the virus, 24 of which took place in suspension culture, and 24 during plating of the explants. As the dissociated cells were to be used immediately upon plating, this method would not work. To give the virus time to infect the CEF explants, viral infection of explants therefore took place over 48 hours in suspension culture. The infected cells were then dissociated (see methods). In contrast to the explants used in chapter IV, virally-infected dissociated CEF proved fragile. At least 50% of cells were lost during dissociation; on plating these did not adhere and were non-viable by trypan blue staining (data not shown). Despite this, the

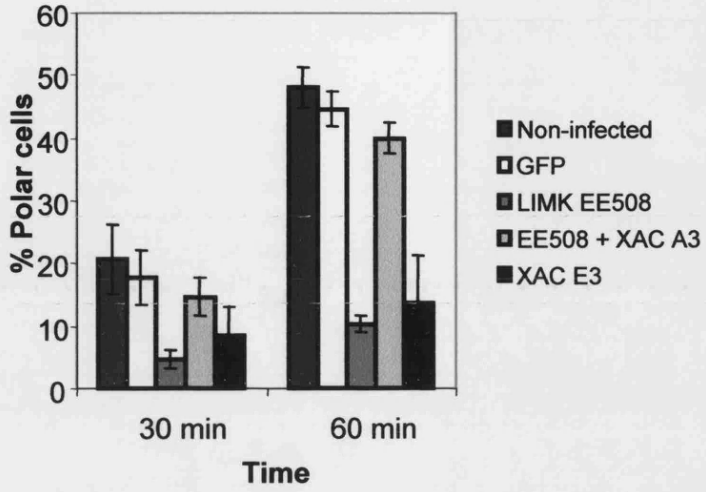
cells that survived appeared healthy and adhered, polarised and migrated with no further problems (data not shown).

Polarity was assessed at fixed time-points as judged to best reflect each stage in polarisation as determined from figures 5.1 and 5.2. Fluorescently labelled phalloidin was used to show cell morphology, the GFP reporter gene to demonstrate LIMK EE508 infection, and indirect immunofluorescence with anti-XAC to detect XAC A3 or E3. Cells expressing LIMK EE508 failed to polarise; by 60 minutes after plating only 10% of infected cells were polar (fig. 5.3 A, mid grey bar) compared to 45-50% of non-infected (fig. 5.3 A, dark grey bar) and control (vector containing GFP marker only) infected cells (fig. 5.3 A, white bar). The AC specificity of this effect was tested. Co-infection with LIMK EE508 and the constitutively active XAC A3 restored acquisition of polarity to wild-type levels (fig. 5.3 A, light grey bar). Infection with the dominant negative XAC E3 prevented polarisation and only 13% of cells had polarised by 60 minutes after plating (fig. 5.3 A, black bar).

Hypothesising that AC activity was required to provide actin monomer for formation of oriented actin bundles, oriented bundle formation during the first 60 minutes after plating was assessed by phalloidin staining (fig. 5.3 B n= at least 150 cells over 3 experiments per construct per time-point). Cells in which AC activity was impaired by infection with either LIMK EE508 (fig. 5.3 B black circles) or XAC E3 (fig. 5.3 B open triangles) exhibited a 4-fold reduction in oriented bundle formation compared to non-infected (fig. 5.3 B black squares) and GFP-infected (fig. 5.3 B black triangles) cells. This failure to form oriented bundles could be prevented by co-infection with LIMK EE508 and XAC A3 (fig. 5.3 B open squares). The time-course of oriented bundle formation in cells expressing both constructs was indistinguishable from control cells; thus AC activity is needed for oriented bundle formation.

To test whether AC was required to disassemble radial actin, circumferential actin or both, the precise nature of the actin changes under the control of AC was analysed. Since oriented bundle formation and this occurs before the break in symmetry, the majority of cells that have attained a break in symmetry have

A



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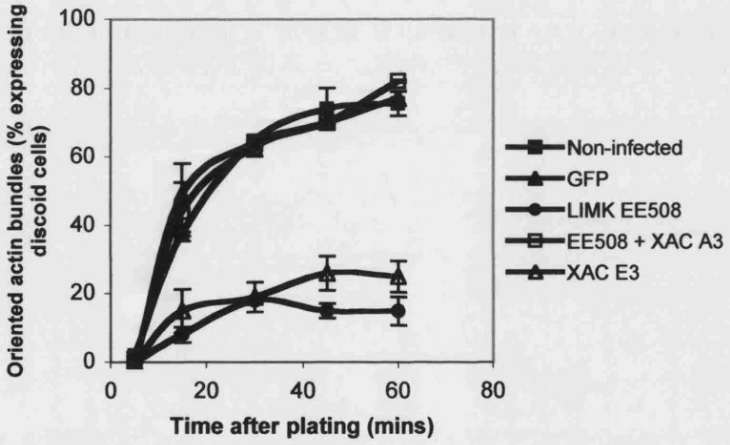


C

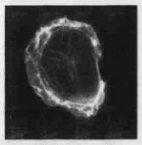
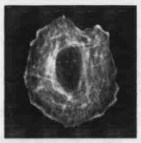
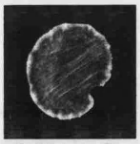
	radial	circumferential	oriented	disorganised
				
GFP	3% ± 0	11% ± 2	78% ± 2	8% ± 1
LIMK EE508	4% ± 1	72% ±4	18% ±2	6% ± 3
LIMK EE508 + XAC A3	4% ± 1	17% ± 2	78% ± 4	4% ± 0

Figure 5.3: AC is required for circumferential actin to oriented actin transition. (A) Quantification of the effect of LIMK EE508 and mutant AC proteins on cell polarity. (B) Time-course of phalloidin stained cells showing the effect of LIMK EE508 and mutant AC proteins on oriented bundle formation in discoid cells. (C) The appearance of actin in CEF infected with GFP, LIMK EE508, or co-infected with LIMK EE508 and XAC A3. Cells were counted at 1 hour after plating and only cells that had attained a break in symmetry were quantified. Graphs are expressed as percentages of total expressing cells ± SEM. Note that data in (C) are expressed as % of expressing cells with a break in symmetry ± SEM and not as a % of the whole population of expressing cells. At least 300 cells were counted over 3 experiments for each of the viral constructs used

parallel bundles. Blocking AC activity should result in cells with actin organised in the orientation found prior to that for which AC is required. If AC mediates the radial to circumferential transition or both this and the circumferential to oriented transition, cells in which AC activity is perturbed should have radially-oriented actin. On the other hand, if AC activity is required for circumferential to oriented transition, the cell body actin of cells with disrupted AC should appear circumferential. LIMK EE508 infected cells retained the ability to initiate symmetry breaks at the periphery, enabling the comparison of like cells infected with GFP, LIMK EE508 or co-infected. Cells were analysed by phalloidin staining at 1 hour after plating and the appearance of the cell body actin quantified (fig. 5.3 C, n= at least 200 cells over 3 experiments for each construct). As before, LIMK EE508 infection caused a 4-fold reduction in oriented bundle formation. Instead, actin was circumferentially oriented in 72% of LIMK EE508-infected cells compared to in 11% and 17% of GFP infected and co-infected cells, respectively. No difference was seen between GFP-infected, LIMK-EE508 infected or co-infected cells in the percentage of cells with radial actin structures in the cell body. Therefore, AC severing and/or depolymerising activity is required during the transition from circumferential to oriented actin.

An explanation was sought for the failure of cells to polarise when oriented bundle formation was aberrant. Cells infected with LIMK EE508 were dissociated and imaged by phase-contrast time-lapse microscopy (fig. 5.4, n=5 movies). The symmetry of the discoid cell was broken by lamellipodium loss at the cell periphery (fig. 5.4 top row middle panel, arrow), however it appeared deregulated. Rather than proceeding with enlargement and subsequent stabilisation of this region to form a polar cell, further loss of lamellipodium occurred randomly at points around the cell periphery (fig. 5.4, arrows mark each point of lamellipodium loss, compare to fig. 5.1, middle row onwards). Consequently the tail did not form. These regions enlarged to the point where the cell was completely non-polarised and polygonal in shape (fig. 5.4 bottom row). This suggests that actin organisation into an oriented array is necessary for restricting lamellipodium loss to one spatial location, allowing the tail to form and hence enabling full acquisition of a polarised cell shape.

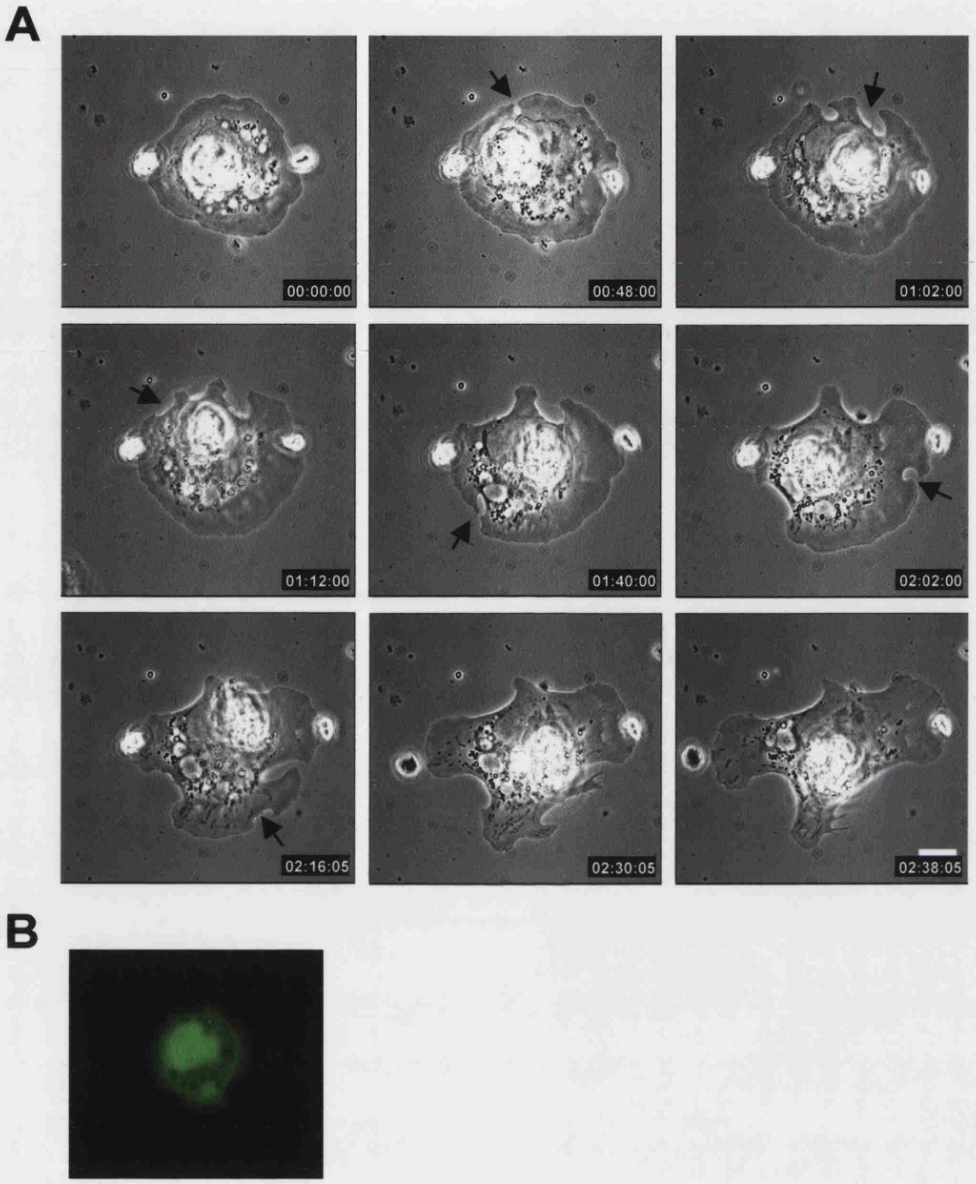


Figure 5.4: A block in AC induces multiple retraction events. (A) Still images extracted from a phase contrast movie of newly plated CEF infected with LIMK EE508. (B) Infection was determined using GFP expression. Black arrows indicate the appearance of each instance of lamellipodium loss around the cell periphery. This movie is representative of 8 cells. The scale bar represents 10μm, and the time is in hrs: mins: secs.

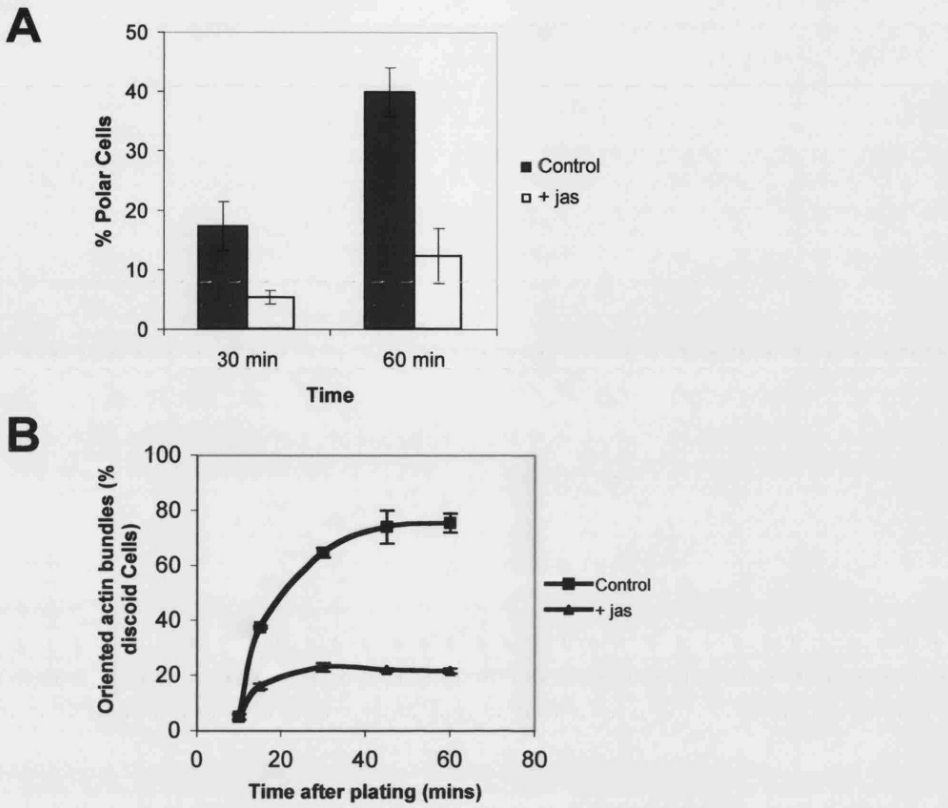
Jasplakinolide blocks polarity acquisition and the circumferential to oriented actin bundle transition.

Having established a requirement for AC activity in the formation of oriented actin bundles, the need for actin filament disassembly during this stage was tested. At low doses, jasplakinolide specifically inhibits disassembly of actin filaments in cells within a minute of its addition (Cramer 1999b). This rapid action makes it possible to look at cells during the transition from circumferential to oriented actin, which takes only 5 minutes.

0.5 μM jasplakinolide was added to dissociated CEF 10 minutes after plating, when the majority of the cells exhibit a circumferentially arrayed actin cytoskeleton and the appearance of the actin within the cell body assessed over time by phalloidin staining. Jasplakinolide competes with phalloidin for binding to actin bundles (Bubb *et al.* 1994), making it difficult to assess actin structures in cells stained with phalloidin, however doubling the amount of phalloidin revealed sufficient detail. The ability of the cells to polarise was severely impaired by drug treatment. In a fixed time-point assay, only 12% of drug-treated cells exhibited a polar morphology by 1 hour after plating, compared to 40% of control cells (fig. 5.5 A, n=600 cells over 3 experiments).

When this was examined in more detail it was clear that as observed when AC was blocked, lamellipodium loss occurred randomly around the periphery of the cell (data not shown). Quantification of oriented bundle formation showed that cells treated with jasplakinolide showed up to a four-fold reduction in the formation of oriented bundles over time compared to control cells (fig. 5.5 B, n=at least 300 cells over 3 experiments per time-point per treatment).

To corroborate that parallel bundle formation required prior disassembly of circumferentially arrayed actin and that failure to form these oriented bundles was not due to aberrant formation of other actin organisations or a delay in the process, a detailed analysis was made of actin appearance in cells with a single break in symmetry at 45 minutes after plating (35 minutes after jasplakinolide



C

	radial	circumferential	oriented	disorganised
Control	3% ± 3	18% ± 3	76% ± 4	3% ± 2
Jas	3% ± 2	69% ±2	22% ±1	6% ± 1

Figure 5.5: Actin filament disassembly is required for circumferential actin to oriented actin transition. Dissociated CEF were plated for 10 minutes and then treated with 0.5μM jasplakinolide for 5-50 minutes. (A) Quantification of the effect of jasplakinolide on cell polarity. (B) Time-course of phalloidin stained cells showing the effect of jasplakinolide on oriented bundle formation. (C) The appearance of actin in CEF treated with jasplakinolide. Cells were counted at 1 hour after plating and only those that had attained a single break in symmetry were quantified. Graphs are expressed as percentages of total cells ± SEM. Note that data in (C) are expressed as % of cells with a break in symmetry ± SEM and not as a % of the whole cell population.

addition); cells with more than one retraction event were not included. Actin appearance was normal (lacking abnormal actin aggregates caused by drug treatment) in over 90% of cells (data not shown). The actin of cells in which filament disassembly was blocked remained oriented in a cortical array in 69% of cells compared to 18% of non-treated cells. No difference in the percentage of cells with radial cell body actin could be seen between treated and non-treated cells. Thus, actin filament disassembly is required for the circumferential to oriented actin transition.

Requirement for AC severing activity

As described in the introduction, AC has two activities in cells: actin filament severing and end-wise depolymerisation of actin monomer from the pointed end (Carlier *et al.* 1997; Maciver 1998). It is not clear which activity takes precedence at any given time in cells. Here, the two activities of AC were separated to determine which or both are required for polarity.

The KK95,96QQ mutation (Replacement of the two lysines at positions 95 and 96 with glutamates) within AC abrogates severing activity without affecting depolymerisation from the pointed end (J. Bamburg, personal communication). Adenovirus for the expression of the constitutively active form of human ADF with the KKQQ mutation (A3KKQQ) was co-infected into cells alongside LIMK EE508 and its ability to rescue polarity assessed. A requirement for severing as well as end-wise depolymerisation should result in no polarity rescue or a partial rescue, while a requirement for end-wise depolymerisation but not severing should lead to a rescue. Both constructs are in the AdTrack vector (He *et al.* 1998), thus it is not possible to use the GFP reporter gene as a marker for expression of either protein in cells. Instead, expression of the A3KKQQ protein was identified with the anti-XAC antibody, and expression of the LIMK EE508 identified using a rat monoclonal antibody to LIM kinase 1. As each cell was to be labelled with 3 fluorescent colours, it was not possible to assess actin structures in infected cells without using four-colour fluorescence, which was beyond our technical capabilities; current laboratory microscope

filters allowed too much bleed-through to use 4 colours. Polarity was therefore scored by cell morphology. Thus, only conclusions relating to any role in polarity overall may be drawn, and not those relating to which population of actin is affected.

Co-infection with LIMK EE508 and A3KKQQ partially restored acquisition of polarity (fig. 5.6). Comparison of the situation at 30 minutes after plating versus 60 minutes after plating revealed a difference in the amount of polar cells. At 30 minutes after plating co-infection with LIMK EE508 and A3KKQQ restored polarity to wild-type levels (fig. 5.6 compare the white bar to the dark grey bar (non-infected) and the light grey bar (LIMK EE508 + XAC A3, able to disassemble and sever). At 60 minutes after plating 29% of cells could be classed as polar (fig. 5.6 white bar) compared to 40% of control (non-infected) cells (fig. 5.6 dark grey bar) and 40% of cells co-infected with LIMK EE508 and XAC A3 (fig. 5.6 light grey bar). Analysis of later time-points did not reveal any differential requirement for severing over end-wise disassembly over time (data not shown). Thus both the severing and disassembling activities of AC are required for polarisation.

Myosin stabilises the newly formed tail

These data have identified a regulatory mechanism for the earliest stages of polarisation. In summary, AC is required for the transition between circumferential to oriented actin organisation. This transition is required for full acquisition of polarity. Without AC activity, the cell tail does not form as the location of the break in symmetry becomes deregulated. The rest of this chapter puts AC to one side to examine the parts played by myosin and microtubules in the polarisation process. Myosin has previously been implicated in tail formation. Localised myosin relaxation has been shown to aid the loss of lamellipodium that leads to formation of the cell rear (Kaverina *et al.* 2000). Here, the role of myosin in the formation of the cell tail was therefore investigated.

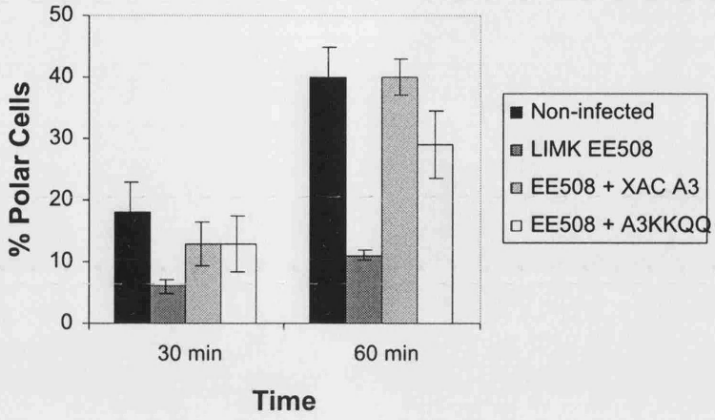


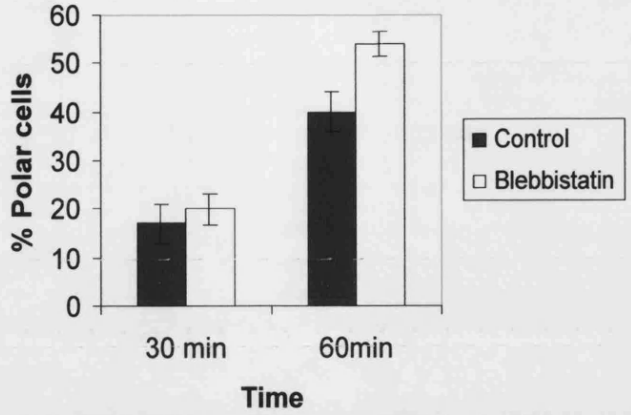
Figure 5.6: The severing and depolymerising activities of AC are required for polarity initiation. Quantification of the ability of the non-severing, but still disassembling, AC mutant A3KKQQ to rescue the failure to polarise induced by LIMK EE508. Graph is expressed as percentage of total expressing cells ± SEM. At least 300 cells were counted over 3 experiments for each of the viral constructs used.

The role of myosin was tested using myosin-targeted drugs: the myosin II inhibitor blebbistatin (Straight *et al.* 2003) and subsequently the Rho kinase (ROCK) inhibitor Y-27632. Y27632 indirectly inhibits myosin and, until the discovery of blebbistatin, was one of the best myosin inhibitors available for use in the laboratory. The caveat to its use, however, is that multiple signalling cascades are affected by inhibiting ROCK including the LIM kinase, AC pathway. Despite this, the fact that blebbistatin and Y-27632 give the same results here means it is possible to be confident that, at least in this system, Y-27632 is targeting myosin.

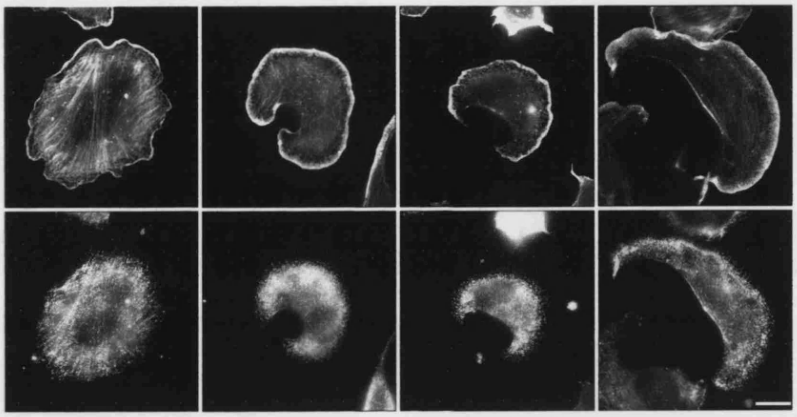
Myosin II inhibition causes aberrant tail formation

The selective myosin inhibitor methyl-blebbistatin, which blocks activity of myosin II without affecting myosin Ib, Va or X (Straight *et al.* 2003), was used to assess the role of myosin in tail formation. Cells were plated for 10 minutes prior to the addition of 100μM blebbistatin, incubated in drug for 20 to 50 minutes, fixed and cell morphology analysed using phalloidin and indirect immunofluorescence with anti-myosin antibody (Sigma). Treatment with blebbistatin did not inhibit the cell's ability to polarise (fig. 5.7 A). On the contrary, the number of cells with clearly identifiable morphological polarity was actually slightly increased by blebbistatin treatment at 60 minutes after plating (fig. 5.7 A). It was clear, however, that the morphology of the polarised cells was not normal. Blebbistatin-treated cells had an abnormally large tail region, often resulting in a highly exaggerated crescent shaped cell (compare fig. 5.7 B, far right panel to fig. 5.7 C). The development of this abnormal tail was analysed in more detail. Actin re-orientation into a parallel array occurred normally, as did the break in symmetry (fig. 5.7 B, far left and centre left panels), thus polarisation proceeded normally in the first 15 minutes after plating (5 minutes in drug). After this point, the loss of the cell margin began to encroach into the cell body (fig. 5.7 B, centre right panel) and this became more pronounced over time, so that by 60 minutes after plating (50 minutes in the presence of the drug), the majority of the cells had an atypical crescent morphology (fig. 5.7 B far right panel). The percentage of polar cells with

A


60
50
40
30
20
10
0
% Polar cells
30 min
60min
Time
Control
Blebbistatin

B



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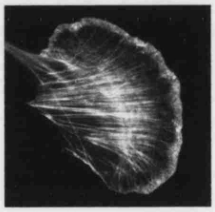

D

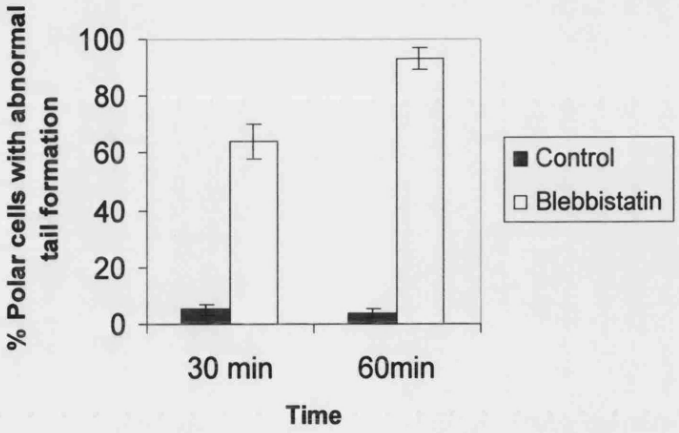
100
80
60
40
20
0
% Polar cells with abnormal tail formation
30 min
60min
Time
Control
Blebbistatin

Figure 5.7: Myosin II is responsible for tail stabilisation. Dissociated CEF were plated for 10 minutes and then treated with 100μM methyl-blebbistatin for 5-50 minutes. (A) Quantification of the effect of blocking myosin II on cell polarity. (B) The effect of myosin II inhibition on cell morphology. Cells treated with blebbistatin for (left to right) 5, 20, 35 and 50 minutes were fixed and co-stained with anti-actin (top row) and anti-myosin (bottom row). (C) Control cell (untreated) fixed at 60 minutes after plating and stained with phalloidin. Note the enlarged tail region and increasingly crescent-shaped cells in (B) compared with the control cell in (C). (D) Quantification of abnormal tail presence. Graphs are expressed as mean percentage of total cells ± SEM. The scale bar in (B) represents 10μm.

abnormal tail formation (fig. 5.7 B middle right and far right morphologies as assessed against control cells such as the one shown in fig. 5.7C) was quantified. In 93 % of cells the tail had encroached into the cell body by 60 minutes after plating compared to only 4% of untreated cells (fig. 5.7 D). Throughout this process myosin was predominantly localised within the cell body, with little staining apparent at the cell margin (fig. 5.7 B bottom panels). Interestingly, in many (but not all) cells formation of the front-back oriented graded polarity actin bundles appeared greatly reduced (fig. 5.7 B compare the appearance of the actin in the cell on the far right with the cell in fig. 5.7 C).

The reason for this effect on polar morphology was examined by time-lapse microscopy (fig. 5.8, n=4, carried out by T. Mseka). As with the fixed time-point assays, dissociated cells were plated for 10 minutes before treatment with 100μM blebbistatin. Two effects of the drug became clear, an early effect during acquisition of polarity involving the cell body and a late effect involving tail retraction during migration. Treatment did not block the initiation of tail formation (fig. 5.8, black arrow) but it did affect the appearance of the cell body. Rather than remaining as a tight ball, the cell body seemed to relax and spread out within the cell, and appeared less phase-bright (fig. 5.8 compare the appearance of the cell body in the top left panel with that of the rest of the images). The cell body failed to move rearwards across the cell to the position of the new tail (compare fig. 5.8, dashed circle indicates the original position of the cell body at tail initiation to the behaviour of the cell body in fig. 5.1), which continued to encroach gradually inwards and was not stabilised, forming an abnormal cell rear. This effect of blebbistatin is consistent with unpublished data on the effect of blebbistatin in cell body movement in migrating fibroblasts, which is blocked by blebbistatin treatment (L. P. Cramer, personal communication). Failure of the cell to form the tail correctly did not block protrusive behaviour, however, the cell was unable to detach the sides of the cell rear from the substratum and these trailed behind the rest of the cell (fig. 5.8, white arrows).

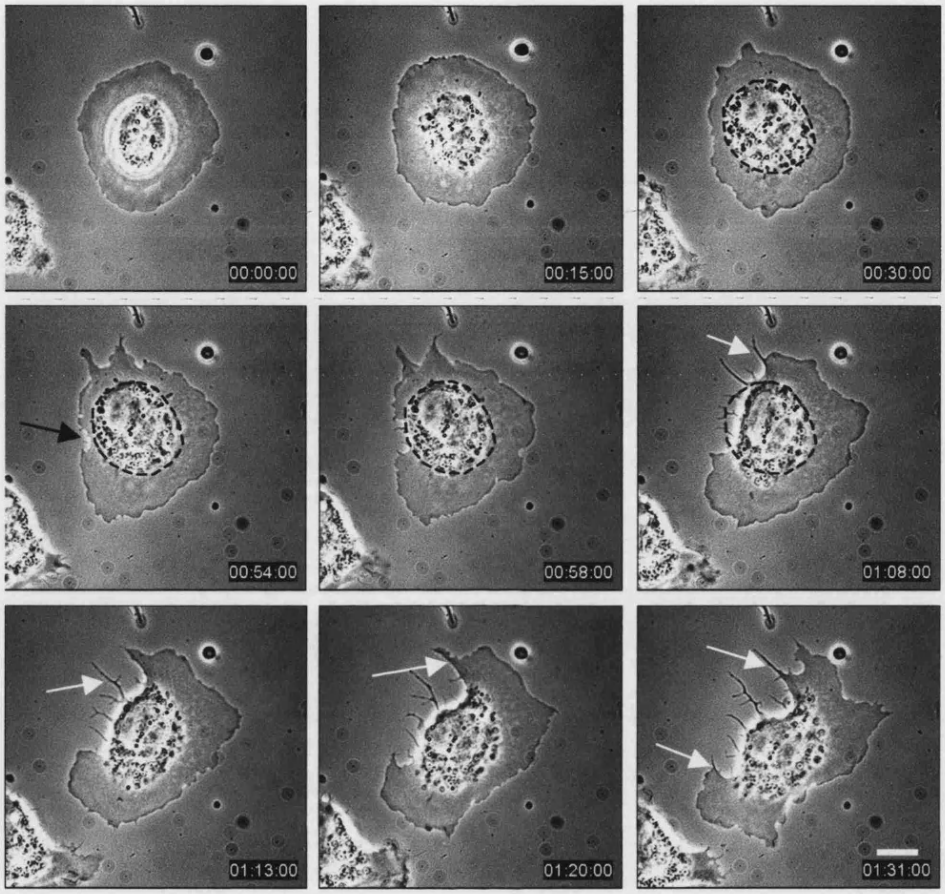

Figure 5.8 Cell body movement requires myosin activity. Still images extracted from a phase contrast movie of newly plated CEF treated with 100μM methyl-blebbistatin (see methods). The black arrow indicates the initiation of tail formation. The dashed circle shows the position of the cell body at the initiation of tail formation. Note the cell body does not move rearwards to meet the tail. White arrows show positions where tail retraction has been compromised. This movie is representative of 4 cells. The scale bar represents 10μm, and the time is in hrs: mins: secs. Movie taken by T. Mseka

The effect of Y-27632 treatment on tail formation

To strengthen the blebbistatin data, the ROCK inhibitor Y27632 was used to block myosin activity. 10μM Y27632 was added to cells 10 minutes after plating for 20-50 minutes and cell morphology assessed by phalloidin staining. Blocking ROCK did not affect the ability of the cells to polarise and the numbers of polar cells on drug-treated coverslips were similar to those on untreated coverslips (fig. 5.9 A), however Y27632-treated cells developed an abnormally large tail region and formation of front-back oriented actin bundles perpendicular to the leading cell margin was again reduced (compare fig. 5.9 B, far right panel to fig. 5.9 C far right panel). The percentage of polar cells with abnormal tail formation (fig. 5.9 B middle right and far right morphologies) was quantified. In 74 % of cells the tail had encroached into the cell body by 60 minutes after plating compared to only 4% of untreated cells (fig. 5.9 D).

Time-lapse microscopy (fig. 5.10, n=6) revealed the same defects to those observed in blebbistatin treated cells: the cell body became relaxed (fig. 5.10 compare the appearance of the cell body in the top left panel with that of the rest of the images) and failed to move across the cell to the location of the break in symmetry (fig. 5.10, dashed circle indicates the original position of the cell body at tail initiation). The break continued to move inwards (compare the appearance of the cell rear in fig. 5.10 bottom row with that shown in fig. 5.1A bottom row), however protrusion was not affected and the cell began to migrate (fig. 5.10 bottom row). Retraction of the trailing edges was compromised and these trailed behind the rest of the cell (fig. 5.8, white arrows). As the trailing edges grew progressively longer the migrating portion of the cell became thinner and thinner and developed the highly exaggerated crescent shape seen in fixed cells. Thus, myosin II is important for the regulation of tail stabilisation and for tail retraction during migration, but does not control the break in symmetry.

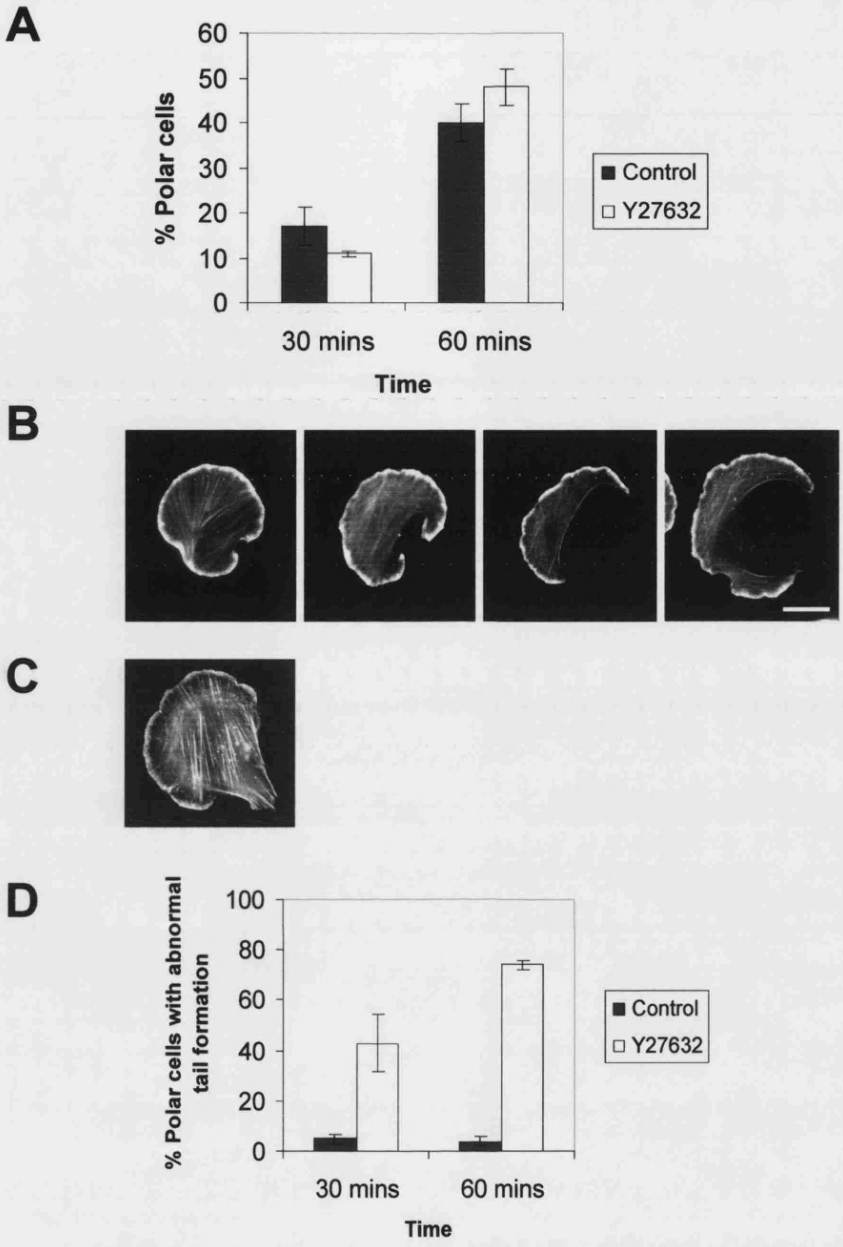

Figure 5.9: Myosin is required to stabilise the cell tail. Dissociated CEF were plated for 10 minutes and then treated with 10 μM Y-27632 for 5-50 minutes. (A) Quantification of the effect of ROCK inhibition on cell polarity. (B) The effect of ROCK inhibition on cell morphology. Phalloidin stained images of cells treated with Y-27632 for (left to right) 5, 20, 35 and 50 minutes. (C) Control (untreated) cell fixed at 1 hour after plating and stained with phalloidin. (D) Quantification of abnormal tail presence. Graphs are shown as mean percentage ± SEM. The scale bar in (B) represents 10μm.

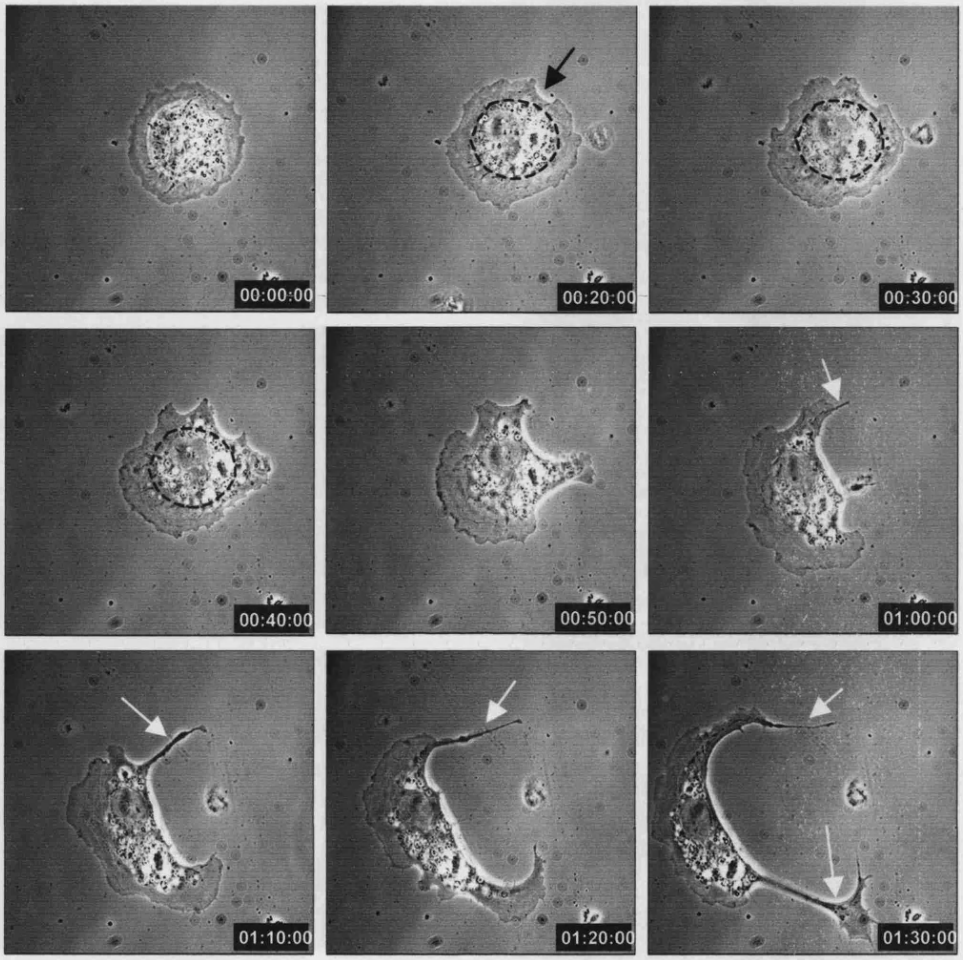

Figure 5.10: Tail stabilisation requires cell body movement. Still images extracted from a phase contrast movie of newly plated CEF treated with Y27632 (see methods). The black arrow indicates the initiation of tail formation. The dashed circle shows the position of the cell body at the initiation of tail formation. Note the position of the cell body does not change. White arrows mark positions where the tail has failed to retract. This movie is representative of 6 cells. The scale bar represents 10μm, and the time is in hrs: mins: secs.

Microtubules stabilise the polarised lamellipodium once it is formed

Microtubules are crucial for polarity in many, but not all, cell types. In astrocytes they play a critical role in setting up polarity by regulating a host of signalling molecules (Etienne-Manneville and Hall 2001, 2003). Microtubules also maintain polarity during cell migration, either by regulating adhesion turnover at the rear of the cell (Small *et al.* 2002a; Small and Kaverina 2003), or by promoting and stabilising growth of the leading edge (Wittmann and Waterman-Storer 2001). The part that microtubules play in the acquisition of particular cell shapes during gain of polarity remains less clear. In this section, the part played by microtubules in the stabilisation of the leading edge is assessed.

Microtubules are required for persistent cell polarity

As a first step to investigate the role of microtubules in polarised migration, the microtubule cytoskeleton was disrupted by addition of the microtubule destabilising drug nocodazole. 5μg/ml nocodazole was added to dissociated CEF and the polarisation of cells without microtubules was analysed by time-lapse microscopy (fig. 5.11, n=10, carried out by T. Mseka). The process of polarity acquisition was not compromised by nocodazole treatment and the cell developed a polarised morphology. Polarisation was not persistent, however, and the leading edge became destabilised and was retracted (fig. 5.11, asterisks). Further transient protrusion events occurred around the cell periphery over time (fig. 5.13, direction of each leading cell margin indicated by arrows). The results of this were that polarised morphology was short-lived, and the cell failed to migrate. The behaviour of nocodazole treated CEF was quantified. Depolymerisation of the microtubule cytoskeleton had no effect on the cell migration rate (fig. 5.12A), however the persistence of the polarised shape as a percentage of the total time-lapse length was reduced from 100% in control cells (which maintained a single leading cell margin in the same spatial location

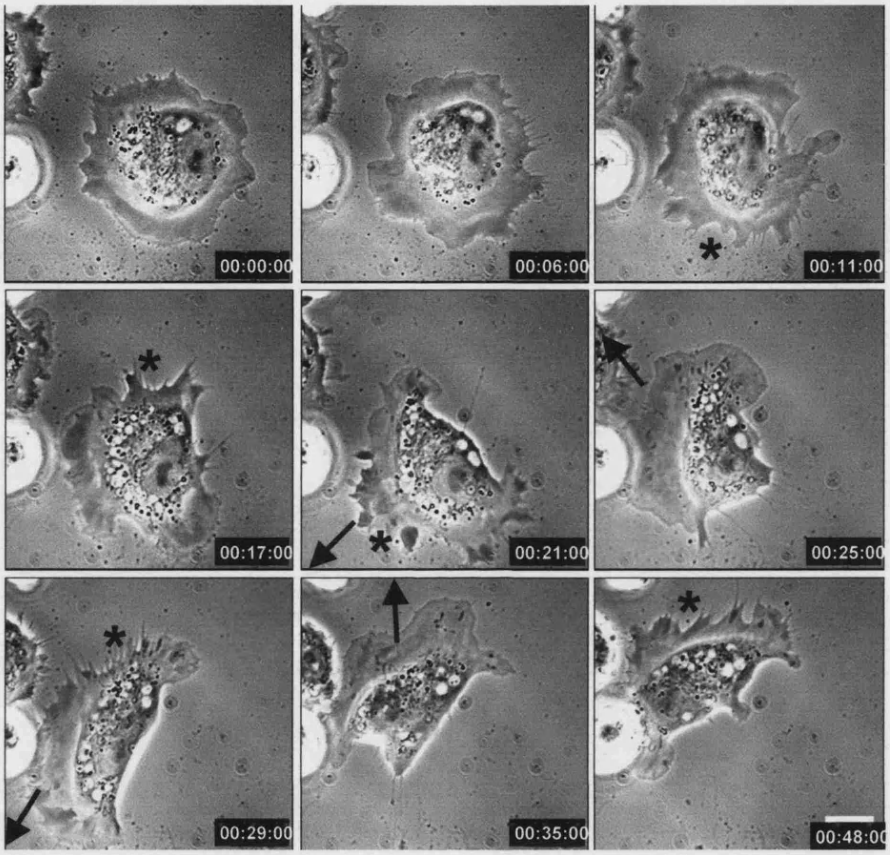

Figure 5.11: Microtubules are required to stabilise the leading edge. Still images extracted from a phase contrast movie of newly plated CEF treated with 5μg/ml nocodazole. This movie is representative of 10 cells. Arrows mark the direction of migration. Asterisks show regions of destabilised lamellipodium. Note the transience of polarisation in any given direction. The scale bar represents 10μm, and the time is in hrs: mins: secs. Movie taken by T. Mseka.

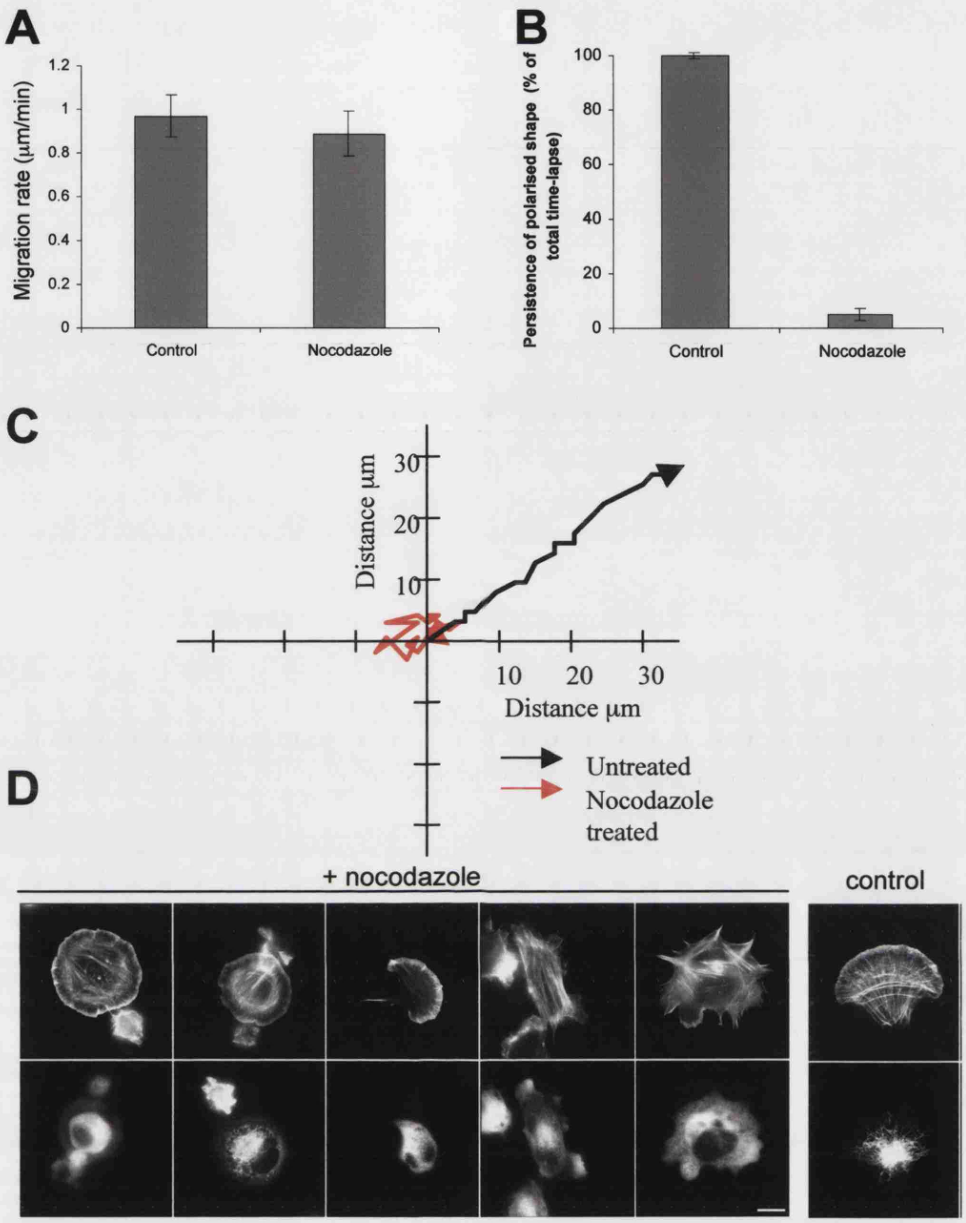

Figure 5.12: Once polarity is acquired, microtubules maintain polarity persistence. Dissociated CEF were plated for 10 minutes and then treated with 5µg/ml nocodazole for 50 minutes. (A) Quantification of the effect of depolymerising microtubules on cell migration rate. (B) The effect of nocodazole on the persistence of the polarised shape. (C) Schematic showing the path of migration of a single representative cell tracked by time-lapse microscopy for 1 hour after first attaining a polarised morphology. Graph (A) is expressed as mean percentage of total cells ± SEM. Graph (B) is expressed as mean percentage of the total time-lapse length ± SEM.(D) The effect of nocodazole treatment on cell morphology. Cells were untreated and left for 1 hour (far right) or were treated with nocodazole for (left to right) 5, 20, 35, 35, and 50 minutes, fixed and co-stained with phalloidin (top row) and anti-tubulin (bottom row). The scale bar in (D) represents 10µm

throughout the time-lapse) to 5% in nocodazole-treated cells (fig. 5.12B). When the path of migration of a single cell was tracked for one hour after the first acquisition of a completely polarised morphology, it was clear that, while control cells migrated in a single direction throughout the time-lapse, nocodazole-treated cells showed a dramatic decrease in migration persistence and were unable to move in a single direction. To test whether microtubules were required for any of the earlier steps in polarisation, or for actin organisation, fixed time-point assays of nocodazole-treated CEF stained with phalloidin and anti-tubulin were carried out. As expected from the time-lapse, microtubules were not required for any morphological or actin change during polarity acquisition, however it was very difficult to find cells with the truly polarised morphology shown in the middle panel of figure 5.12D. Rather than possessing a single smooth leading edge (fig. 5.12D third panel from left), most of the few cells that were polar had a very ragged appearing lamellipodium (fig. 5.12D fourth panel from left, arrow) in 39% of cases in comparison to 2% of control cells (n=150 cells over 4 experiments). The majority of cells lacked a polarised morphology and appeared spiky around the periphery with few clear regions of lamellipodium (fig. 5.12D right panel). This was in contrast to control cells, which polarised normally in the same time period (fig. 5.12D far right panel). Thus, stabilisation of the leading cell margin and directional migration both require the presence of microtubules.

Persistent polarisation and directional motility require microtubule dynamics

To investigate whether the presence of microtubules was sufficient for persistent lamellipodium protrusion or whether microtubule dynamics were required, dissociated CEF were treated with the microtubule stabilising agent taxol (10μM) and analysed by time-lapse microscopy (fig. 5.13 n=10, carried out by T. Mseka). The drug was added to a cell with disc-shaped morphology and its effect on polarisation examined. The behaviour of taxol treated cells was similar to that observed using nocodazole. Cells polarised normally (fig. 5.13 arrows show the direction of migration), however they appeared to lack a sense of direction for motility and had an unstable leading edge (fig. 5.13, asterisks).

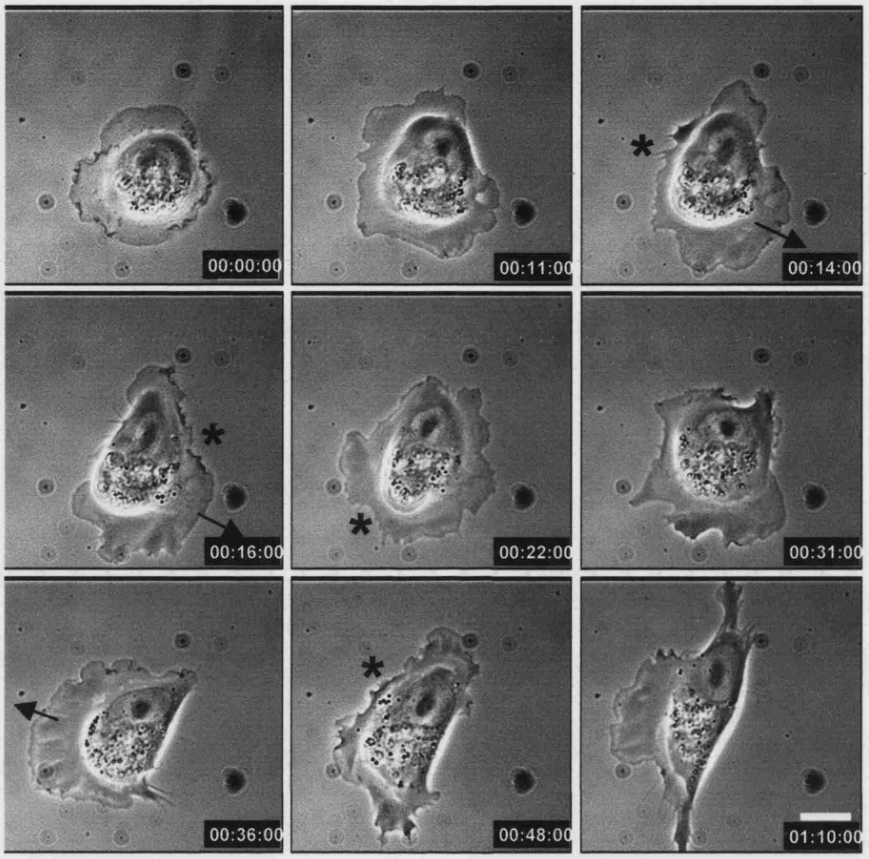

Figure 5.13: Blocking microtubule dynamics blocks leading edge stability. Still images extracted from a phase contrast movie of newly plated CEF treated with 10μM taxol. This movie is representative of 10 cells. Arrows mark the direction of migration. Asterisks show regions of destabilised lamellipodium. Note the transience of polarisation in any given direction and lack of cell migration. The scale bar represents 10μm, and the time is in hrs: mins: secs. Movie taken by T. Mseka.

This underwent frequent retractions and was re-protruded at alternative locations around the cell margin (fig. 5.13 note the changing position of the asterisk with each new lamellipodium), resulting in impaired locomotion and reduced productive migration. Quantification of these data showed that, like nocodazole-treated cells, taxol-treated cells exhibited the same migration rate as control cells (fig. 5.14A), however the polarised cell morphology persisted for only 10% of the time-lapse length (fig. 5.14B) and the cells did not migrate in a directional fashion (fig. 5.14C). Together these data indicate that microtubule dynamics, and not just an intact microtubule cytoskeleton, is required for the final stage of polarisation: maintaining a persistent protrusion.

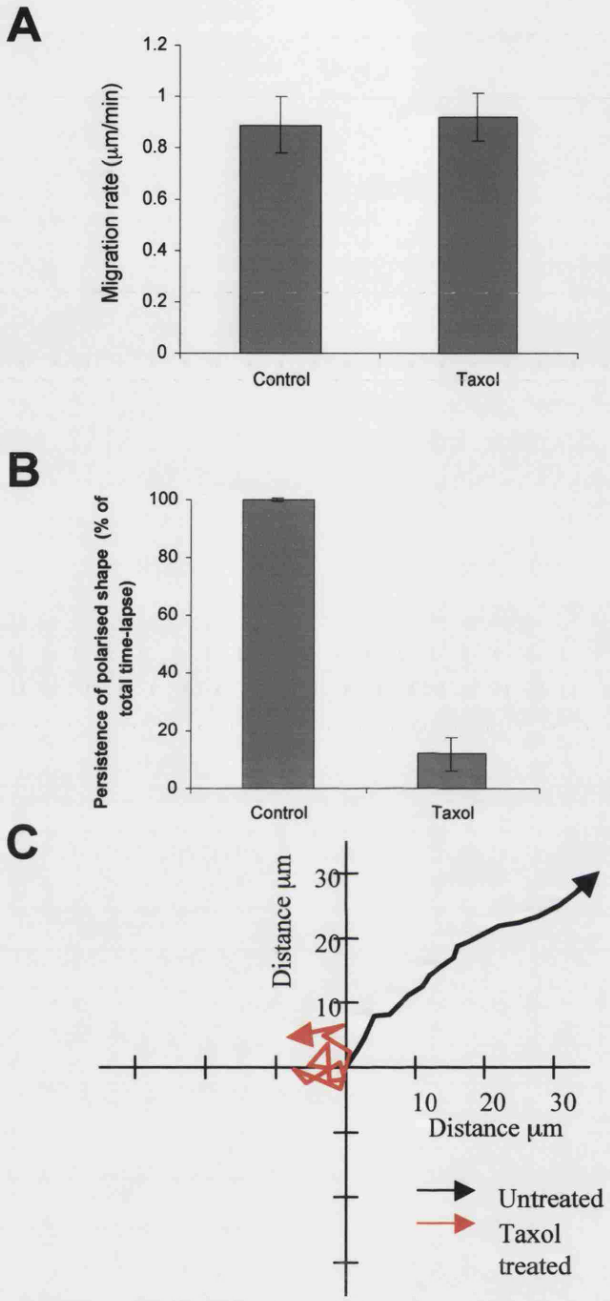

Figure 5.14: Blocking microtubule dynamics blocks polarity persistence. Dissociated CEF were plated for 10 minutes and then treated with 10µM taxol for 50 minutes. (A) Quantification of the effect of blocking microtubule dynamics on cell migration rate. (B) The effect of taxol treatment on the persistence of the polarised shape. (C) Schematic showing the path of migration of a representative cell tracked for 1 hour after first attaining a polarised morphology. Graph (A) is expressed as mean percentage of total cells ± SEM. Graph (B) is expressed as mean percentage of the total time-lapse length ± SEM.

5.3 Discussion

These data have demonstrated that fibroblasts polarise in a series of closely regulated morphological stages. Successful completion of each of these stages in turn is necessary if the cell is to polarise correctly and subsequently migrate. Fibroblasts form the tail first rather than protruding a dominant lamellipodium, and are not the only cell type to polarise in this fashion. Keratocyte cytoplasmic fragments and goldfish fibroblasts (Verkhovsky *et al.* 1999; Kaverina *et al.* 2000) also form the tail first however astrocytes that polarise in a wound-healing assay do not (Etienne-Manneville and Hall 2001). Instead, these cells form a large dominant protrusion in response to the wound, and migrate to close the gap. This study has shown that, at least in migrating fibroblasts, acquisition of polarity is dependent on a tightly regulated series of steps, hingeing on specification of the tail position by AC and tail stabilisation by myosin II.

Positioning of the tail appears to be tightly related to the actin orientation within the cell body, forming perpendicular to the orientation of the oriented actin bundle array, not in the same orientation as might have been pre-supposed. At first glance this would seem to make more work for the cell, which must go through another series of actin rearrangements to form the front-back oriented graded polarity bundles that are needed for migration to occur. Bundles oriented at right angles to the direction of movement must have some functional purpose for the cell, as these remain strongly evident as arcs in a polarised migrating cell (see numerous examples in the last 3 chapters). One possible role for these bundles would be to generate tension across the cell, thus helping to maintain the cell shape.

How does radial to circumferential transition occur?

AC mediates the circumferential to oriented transition, but how is the initial radial array of actin reorganised into the circumferential orientation? AC is not involved, suggesting that actin filament disassembly/severing is unlikely to play a role in this process. This chapter has not ruled out a part for myosin as at 10

minutes after plating, when all drugs were added, the majority of the cells already have cortically arrayed actin. Myosin activity was therefore blocked too late to affect the formation of these bundles. Blocking myosin function prior to plating the cells will be necessary to determine if this could be the case. Finally, actin bundling proteins such as a-actinin, fimbrin, or a variety of others could also be involved.

AC function during polarisation

The stored actin monomer pool is not used for assembly into the oriented array, as cells treated with jasplakinolide remain with their actin arranged circumferentially. Therefore the function of AC during polarity initiation is two-fold. First, it removes the unwanted actin filaments from the cell and second, in so doing it provides the actin monomer that is required for formation of the parallel array. How is AC regulated so that only the circumferential bundles and not the newly forming oriented bundles get disassembled? This remains an open question but is highly important that these bundles are stabilised in order for polarisation to succeed. Of all the mechanisms regulating AC activity, the most likely in this case is competition with tropomyosin. Preliminary observations using a pan-tropomyosin antibody may indicate that oriented bundles are more highly tropomyosin-decorated than radial or circumferential bundles, however this is by no means certain. Binding of some tropomyosin isoforms to F-actin is inhibitory to AC binding, while others promote the interaction (Bernstein and Bamburg 1982; Ono and Ono 2002; Bryce *et al.* 2003). Further experiments using antibodies to specific tropomyosin antibodies may help to distinguish which bundles are bound to isoforms that aid AC function, and which are bound to isoforms that compete with AC. It seems improbable that phosphorylation of AC plays a significant role in this process as there was no apparent difference in pAC localisation either across the cell, or during the various stages of polarisation (data not shown). Approximately 50% of discoid cells undergoing the actin rearrangements at the start of polarisation had clear pAC staining within the leading edge (data not shown). No clear relevance to polarisation could be seen, however it may be noteworthy that an average of 50% of

fibroblasts succeed in completing polarisation (data not shown). Whether these two observations are related remains an interesting possibility that requires further study. It may be expected that a cell that is destined to polarise would lose pAC from the lamellipodium at some point during the polarisation process, as migrating cells lack pAC in this region (Chapter IV). The timing of this event is unclear and ideally requires the tracking of pAC in live cells throughout the polarisation process and subsequent migration.

Why is it necessary to form oriented bundles/why does polarisation in LIMK cells go wrong?

Cells in which AC activity is abolished fail to form oriented bundles and fail to restrict lamellipodium loss at the cell periphery to one spatial location. What might the oriented bundles be doing? One idea is that oriented bundles somehow play a role in specifying the location of the tail. In this scenario, the failure to disassemble the circumferential actin array means that there are many "mini" arrays of oriented actin all around the cell periphery. The cell body cannot then move in a directional fashion to regulate tail formation as it does not know where to go. The cell may try to overcome the failure to stabilise the tail by initiating new tail locations elsewhere around the cell, leading to the polygonal non-polar cells observed when AC or actin disassembly was blocked. How the location of the tail is specified remains unclear in this case, however the oriented bundles are likely to play some part in this process as the tail is always formed at such a precise location perpendicular to the actin orientation.

Requirement for filament severing

The constitutively active but non-severing AC failed to completely restore the ability of the cell to polarise in the presence of LIMK EE508, suggesting that filament severing plays a more minor role than end-wise depolymerisation, but is still required for optimal polarisation. Actin severing by AC has been implicated in the generation of large numbers of free barbed ends at the leading edge on EGF-stimulation of adenocarcinoma cells (Chan *et al.* 2000). Within the cell body severing activity may produce the free barbed ends, which could

be oriented and elongated to rapidly form the new actin organisation, thus accelerating the polarisation process. Depolymerisation from the pointed end provides a plentiful supply of actin monomer for re-assembly wherever required, thus preventing the inappropriate assembly of actin that could occur if the monomer pool were used.

Mechanism of formation of the break in symmetry

Surprisingly, neither actin disassembly nor the microtubule cytoskeleton were required for the break in symmetry, nor it is not clear what is responsible for this event. It seems likely that a de-adhesion event is responsible, although in contrast to evidence from already polarised, migrating cells (Kaverina *et al.* 1999), microtubules do not mediate this process by targeting selected focal complexes for disassembly. Localised de-adhesion must however be regulated and it remains for future work to determine how this occurs. The integrin-blocking peptide RGD (Ruoslahti and Pierschbacher 1986) has been shown to cause release of cell contacts on one side of the cell and promote extension of the opposing cell edge (Kaverina *et al.* 2000). Focal complex and focal adhesion formation is triggered by tyrosine phosphorylation (Adams 2002). The most likely situation, therefore, is for the concerted action of protein tyrosine phosphatases in response to an internal biochemical cue that determines where the break in morphological cell symmetry should occur. Individual adhesions can be modulated in response to force without altering neighbouring cell-matrix contacts (Choquet *et al.* 1997), thus it is possible for such phosphatase action to be highly localised. A hint that this might be the case comes from a preliminary experiment using the supposed protein tyrosine phosphatase inhibitor sodium orthovanadate. Treatment with this compound at both 1mM and 5mM blocked the break in symmetry in dissociated CEF but did not affect any of the earlier stages (data not shown). It should be noted however, that orthovanadate appears to be highly non-selective. Therefore these experiments require repetition with more specific inhibitors. A second possibility mediating the break in symmetry is selective proteolysis by the calcium-dependent protease calpain, which has been implicated both in the control of cell migration by enhancing focal

adhesion turnover (Bhatt *et al.* 2002) at the cell rear (Huttenlocher *et al.* 1997) and in the regulation of cell migration (Dourdin *et al.* 2001; Denker and Barber 2002; Lokuta *et al.* 2003). A combination of the two is also possible, with the de-adhesion event occurring, followed by calpain-mediated degradation. Finally, tail formation could be initiated by regulated endocytosis of plasma membrane.

The requirement for myosin in stabilisation of the tail but not in forming the break in symmetry is in contrast to previous data which showed that locally inhibiting myosin promoted localised de-adhesion, tail formation from that point and subsequently cell polarisation (Kaverina *et al.* 2000). The authors concluded from this that localised myosin relaxation aided focal adhesion turnover at that point and promoted formation of a polarised cell (Kaverina *et al.* 2000). How can these results be rationalised in the context of this present study? Examining the morphology of the goldfish fibroblasts used in the study, it becomes apparent that, while polar, the cells have very large tail regions matching the appearance of the myosin-inhibited CEF used in the current work. In both cases, therefore, blocking myosin activity leads to deregulated tail formation and the generation of abnormally thin crescent-shaped cells. This suggests a similar role for myosin in regulating the extent of tail formation in both systems, even if in fish fibroblasts the break in symmetry can also be myosin-mediated. Maintenance of the cell tail and subsequent cell migration are likely to be dependent on targeted loss of adhesion complexes (Sheetz *et al.* 1998; Small and Kaverina 2003). Under normal polarisation conditions a balance must be attained whereby sufficient de-adhesion occurs to form the tail region, but not so much that exaggerated polarisation is the result. One possibility is that myosin provides the counterweight to balance de-adhesion, perhaps by increasing the strength of cell-matrix adhesions (Riveline *et al.* 2001). Maybe myosin II-mediated movement of the cell body stabilises the tail region by locally promoting adhesion stabilisation. These adhesions would then become available for disassembly following forward movement of the cell body during migration, thus enabling movement of the cell over the substratum whilst maintaining the cell shape.

The role of microtubules in fibroblast polarisation

Interestingly, microtubules were not required to form a polarised cell, although they were needed to maintain cell polarity and directed migration. This is in contrast to the situation in other cell types, where microtubules are an integral part of the polarisation process. Contrary to expectations, microtubule dynamics were not required for the break in symmetry to occur, suggesting that the role of microtubules in CEF may be other than to regulate de-adhesion during initiation of cell polarisation. Microtubules are thought to be crucial in regulating the maintenance of polarity and directional migration and the results presented here are entirely consistent with this idea. Exactly how microtubules act to maintain polarisation and directional motility in primary fibroblasts remains unclear. The two major hypotheses are that either microtubules act to aid cell body movement and tail retraction during migration by regulating the targeted disassembly of adhesion sites (Small *et al.* 2002a; Small and Kaverina 2003), or that growth of microtubules into the region of the leading edge promotes Rac-mediated protrusion, and hence polarised cell migration (Wittmann and Waterman-Storer 2001). The leading edge appears highly unstable in the absence of microtubule dynamics, but these data cannot distinguish between the two ideas as it is not clear whether the leading edge becomes unstable due to a failure to maintain the balance of adhesion and de-adhesion, or whether it becomes unstable due to a lack of Rac activation at the leading edge. Examination of microtubule and adhesion dynamics during polarisation and migration should help to clarify this issue.

Outstanding questions

This chapter has defined separate, but closely linked roles for both actin and microtubule dynamics in regulating the stages of polarisation. Many areas remain unclear; however the successful completion of each of these stages requires tight regulation. Outstanding questions that are still to be answered include: the identity of the biochemical cue that signals spontaneous fibroblast polarisation, the exact molecular function of each of the actin rearrangements and how each is tightly spatially localised, the mechanism of tail formation and

the means by which it is stabilised by myosin-mediated cell body movement, and the mechanism whereby microtubules maintain a polarised protrusion. It remains for future studies to address these points and provide a more thorough picture of the molecular regulation of the cytoskeleton during polarity and polarisation.

Chapter VI: General Discussion

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To migrate productively, normally a cell needs to acquire a polarised morphology. Polarisation of a migrating cell can be split into two stages: acquisition of polarity, and subsequent maintenance of the polarised shape throughout migration. Central to both stages is the need for precise arrangement of the actin cytoskeleton: from the first assembly of actin into oriented arrays during polarisation, to the formation of the front-back oriented graded polarity bundles, and the maintenance of the lamellipodium of the migrating cell. This thesis has demonstrated two pivotal, and interlinked, roles for actin filament disassembly and AC family proteins in morphological cell polarity: First, in acquisition of a polarised morphology (formation of oriented actin bundles) and second, during cell migration (maintenance of a polarised lamellipodium and a continuous supply of actin monomer).

One issue is the question of whether AC actively affects cell polarity to set the direction of motility for a migration cell, or whether it just sustains the previously acquired polarised morphology by increasing actin turnover. Another is that the use of adenoviral-mediated over-expression of proteins throughout this work makes it difficult to state with absolute confidence which actin filaments AC activity affects under normal circumstances, and whether other cellular pathways are perturbed by the over-expression. Equally, the constructs used here rely on eliminating endogenous AC activity rather than attempting to swamp it out with exogenous protein, but nevertheless these experiments all affect a gross area of the cell. Ideally, further work would make use of such techniques as photoactivation of fluorescence or fluorescence photobleaching to perturb only a small cellular region such as the lamellipodium or a regional population of actin filaments.

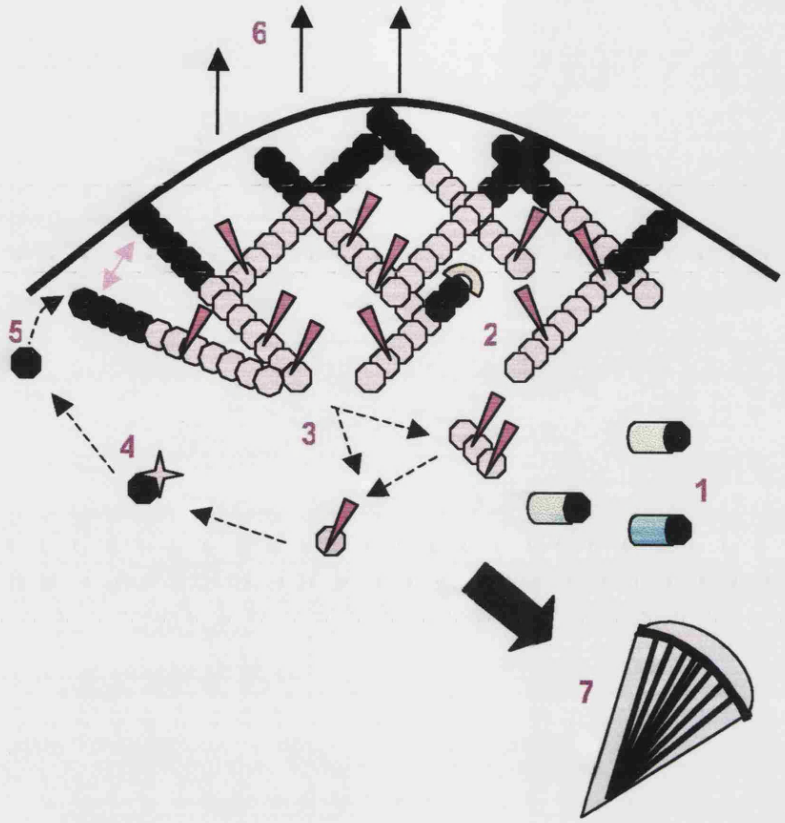
Is ADF, cofilin or both that act to regulate polarity in primary fibroblasts? To date, there is little evidence for separate roles for ADF and cofilin in cells. Differences exist, however these have not yet in the main been related to physiological relevance. ADF was named due to its actin depolymerising activity (Bamburg *et al* 1980) and subsequent formation of a 1:1 stoichiometric

complex with actin monomer while whereas cofilin was named for its ability to co-sediment with F-actin (Maekawa *et al* 1984). Despite this difference both proteins can disassemble/sever actin (Bamburg 1999), and cofilin null mutants in yeast can be rescued by adding back ADF (Moon *et al* 1993, Iida *et al* 1993). In addition to the regulatory mechanisms outlined in the introduction, cells can differentially regulate ADF and cofilin expression. ADF, but not cofilin, can be down regulated by a post-transcriptional, but pre-translational mechanism in response to increases in the level of actin monomer in cells (Minamide *et al* 1997). This provides a negative feedback mechanism whereby regulation of the amount of actin filament disassembly is linked to the concentration of the actin monomer pool. Thus, ADF may be a good candidate for providing on-going actin filament disassembly in the lamellipodium of migrating cells while ensuring that actin monomer remains limiting.

A limiting concentration of actin monomer in the lamellipodium appears to force the cell to use monomer derived from recently disassembled actin filaments in preference to the actin monomer pool and in so doing, maintains morphological cell polarity. However, why is the concentration of actin monomer so low in the lamellipodium? This remains an open question. One idea, posed in Chapter III, is that diffusion of actin monomer from the cell body into the lamellipodium is compromised. Why this should be the case remains unclear. It is possible that the density of the actin meshwork towards the extreme leading edge presents a spatial barrier to diffusion of actin monomer, especially if binding to other actin binding proteins holds it in an assembly-incompetent state and in so doing creates larger macromolecular complexes. Recent data on monomer diffusion in rat fibroblasts has shown that actin monomer is much more rapidly transported during cell protrusion events than can be accounted for by diffusion alone (Zicha *et al.* 2003). Thus, a form of active transport may well be required to deliver monomer to sites of actin filament assembly. A second, related, idea is that actin filament assembly in the lamellipodium is so rapid that it quickly "soaks up" available actin monomer (Bailly and Jones, 2002).

Since very different actin organisations exist in discrete regions of the cell and at different times, the process of their formation must be tightly spatially and temporally controlled. The mechanisms for formation and maintenance of most of these networks remain largely unknown and it is unlikely that the dendritic nucleation paradigm will hold in its entirety for other actin networks besides the meshwork of the leading edge. At the leading edge however, dendritic nucleation provides an appealing model that links both the theoretical and experimental data that have emerged over the last years. Central to this model is the part played by AC in regenerating the actin monomer pool, however the precise importance of AC activity has been largely unclear until recently. How does the evidence presented in this work for a role for AC in maintaining the persistent polarised protrusion of the leading edge fit into the dendritic nucleation model? Figure 6.1 shows a modified version of this model in which the distinction between polarised migrating cells and non-polarised, non-migrating cells is made. In the top panel, low levels of the actin monomer pool within the lamellipodium make the cell reliant on AC-mediated actin filament disassembly for a supply of monomer. All the AC is dephosphorylated, and hence potentially active. This high level of AC activity ensures the actin meshwork remains short and stiff, providing the force needed to push the membrane forward continuously. Restriction of the monomer supply to one spatial location curbs the ability to protrude multiple lamellipodia, and allows polarised migration to occur. In the bottom panel, much of the AC at the cell margin is phosphorylated. Less AC activity leads to less actin disassembly; the filaments therefore grow longer until they are unable to provide enough force to push the plasma membrane forward. Frequent retraction events ensue upon collapse of the actin filaments. These cause an increase in the amount of available actin monomer, which in turn is able to fuel production of a new lamellipodium. The protrusion-retraction cycle begins again with lamellipodia appearing wherever sufficient monomer is present and disappearing as the monomer is exhausted and the flexible filaments collapse. The result is a depolarised cell and no productive cell migration.

Many parts of this model remain unclear and several questions remain that require attention in the future. How are the actin filaments organised at the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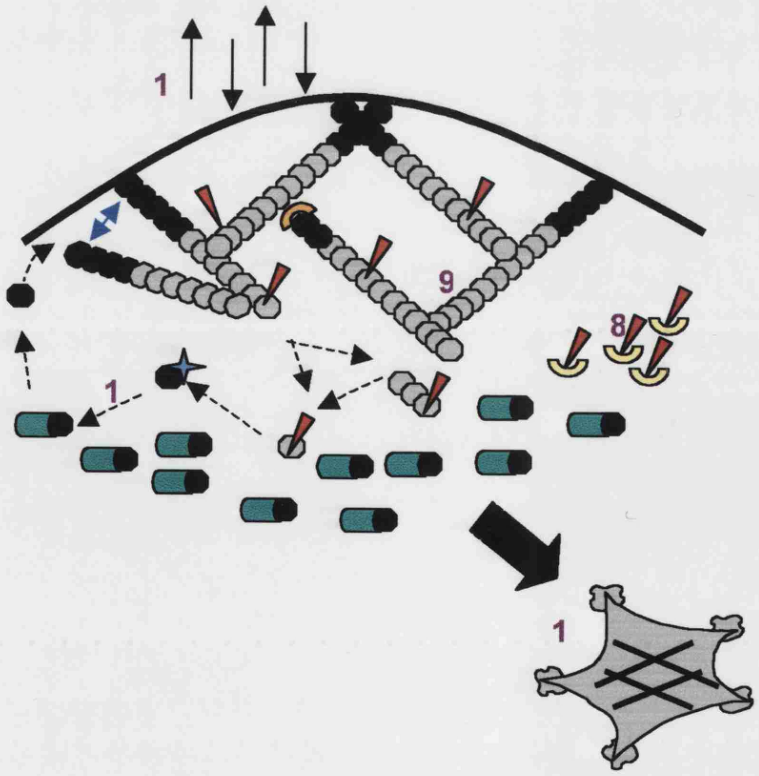
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Figure 6.1: AC activity and actin monomer supply at the leading edge. (top panel) (1) During cell migration, G-actin pool complexed to thymosin (black circles with green tubes) at the leading cell margin is highly limiting leading to increased importance of actin filament disassembly mediated by AC proteins. (2) Only the barbed ends at the plasma membrane are available for extension; barbed ends further back in the cell are capped (orange semi-circle). (3) AC (red triangles) disassembles and severs ADP actin (grey circles) but not ATP actin (black circles). The resulting actin monomer undergoes nucleotide exchange catalysed by profilin (blue star, 4) and is directly recycled to the cell margin. (5) Thermal fluctuations in the actin filaments (blue arrow) provide transient gaps between the filament and the plasma membrane that allow actin to polymerise at the barbed end and push the membrane forward. This continuous supply of actin monomer alongside high levels of AC activity ensure smooth protrusion of the cell margin (6) by maintaining a short, stiff and highly branched actin network. (7) Restriction of AC activity and actin disassembly to this one location provides a single region for protrusion of the leading edge and allows polarised cell migration. (bottom panel) (8) When a cell stops migrating, AC at the leading edge is inactivated by phosphorylation (yellow semi-circles on red triangles). (9) Less AC activity results in longer, less stiff actin filaments that are less able to withstand the force exerted by the cell membrane, leading to frequent retraction events (10), which result in a large actin monomer pool (11) that can fuel actin assembly. Uncoupling of assembly from disassembly means that assembly is no longer restricted to one spatial location, leading to multiple lamellipodia and no net cell migration (12).

leading edge under conditions of high and low AC activity? A role for AC in the transition from short stiff actin filaments to longer more flexible filaments is purely speculative, however given that cells in which AC is blocked behave in a similar manner to those in which Ena/VASP levels are increased, similarities may exist in the structure of the actin meshwork at the cell margin of both. As discussed previously, cells with high levels of Ena/VASP activity form longer actin filaments that are more prone to retraction and this correlates with both increased lamellipodium protrusion rate and reduced migration speed (Bear *et al.* 2002), reviewed by (Cramer 2002). It should, however, be noted that this scenario will only be effective if disassembly is no longer required to supply actin monomer.

How is actin monomer directly recycled to the leading edge for another round of polymerisation? Simple diffusion is unlikely to be fast enough and an active process requiring ATP has been shown to be required (Zicha *et al.* 2003). The nature of this active process requires further study and two possibilities have been put forward. One is the need for direct transport of actin monomer by a myosin; the second is that myosin-based contraction causes a "squeezing" of the cell that could generate a pressure gradient leading to hydrodynamic flow that carries actin monomer toward the leading edge (Zicha *et al.* 2003).

Once at the leading edge, how is the monomer recruited to a newly forming actin filament? The Arp2/3 complex appears to nucleate actin filaments and branches by mimicking an actin dimer (Volkmann *et al* 2001); actin monomer adds onto this pseudo-barbed end to create a filament. Arp2/3 can be activated in several different ways. The two protein families that have been best studied are the WASP/Scar proteins, and the cortactin family. Members from both families all have an acidic A domain, which binds Arp2/3 complex (reviewed in Weaver *et al* 2003), although cortactin also requires F-actin binding to activate Arp2/3 (Uruno *et al* 2001, Weaver *et al* 2001). The scaffolding protein CARMIL also has an A domain and can bind and weakly activate the Arp2/3 complex (Jung *et al* 2001) and in yeast an array of proteins, including the tail region of fission yeast myosin I have also been demonstrated to activate Arp2/3 complex (reviewed by Higgs and Pollard 2001, Weaver *et al* 2003). One major

difference between N-WASP and cortactin, that may explain why WASP/Scar is a more powerful activator of the Arp2/3 complex, is that N-WASP can bind actin monomer via the WH2 domain, which can then add to the Arp2/3 complex and promote daughter filament formation (Higgs and Pollard 2001). Finally, WASP/Scar proteins have a proline-rich region that binds profilin. This enhances nucleation (Yang *et al* 2000) and may provide a direct link between newly disassembled actin monomer and actin polymerisation by promoting a chain passing actin monomer from profilin to WASP and hence to Arp2/3. Many other proteins also bind profilin-actin and F-actin and hence have the capability to connect disassembly to assembly. These include Ena/VASP proteins, ERM (ezrin, radixin, moesin) proteins and the formins and the ways in which these can regulate actin polymerisation and recruit profilin-actin to the plasma membrane have been reviewed (Holt and Koffer 2001). A likely scheme for recruitment of actin monomer by profilin binding to proline-rich proteins is posited to consist of a series of protein-protein interactions (Holt and Koffer 2001). This hinges on the binding of profilin-actin to a proline-rich protein that contains additional binding sites for both F-actin and regulatory proteins. Unbranched actin filaments can be nucleated by formins. The formin homology (FH) domain 2 is both necessary and sufficient for actin nucleation (Pruyne *et al* 2002, Li and Higgs 2003) but the affinity of the FH2 domain for actin monomer is very low and co-factors that bind actin monomer and the formin, such as profilin (Severson *et al* 2002, Tolliday *et al* 2002) and VASP, needed for the increase in F-actin mediated by the formin mDia (Grosse *et al* 2003) are required. *In vitro*, the FH2 domain also binds to the barbed end of the actin filament and prevents capping by capping proteins and gelsolin (Zigmond *et al* 2003) and appears to "walk" along the actin filament with the addition of each actin monomer (Zigmond *et al* 2003). Thus, formin-mediated addition of actin monomer can occur not only during nucleation, as with Arp2/3 complex, but also during filament elongation.

What prevents AC from complete disassembly of the actin network at the leading edge? Migrating keratocytes have an AC-free region at the extreme leading cell margin (Svitkina and Borisy, 1999) that prevents total disassembly of the dentritic brush, however ADF and cofilin are found throughout the

lamellipodium of migrating fibroblasts. It remains probable that ATP hydrolysis provides an effective way of preventing disassembly towards the front of the lamellipodium as actin assembly is biased towards this region (Watanabe and Mitchison, 2002). The newer, ATP-containing parts of the filament will therefore be found at the extreme tip of the lamellipodium, and because AC binds ADP-actin with greater affinity than ATP-actin (Carlier *et al.* 1997), they will be resistant to disassembly. The mechanism that stops disassembly at the lamellipodium rear and maintains the integrity of the lamellipodium-lamella boundary remains an open question, as does the fate of any non-disassembled actin filaments toward the lamellipodium rear. Proteins such as tropomodulin, which contains two pointed end capping domains (Fowler *et al* 2003) may play a role in preventing end-wise depolymerisation while tropomyosin isoforms that are non-permissive for AC binding may stabilise the filament length. Perhaps stabilised filaments at the lamellipodium rear could act as "seeds" for the formation of graded polarity bundles in migrating fibroblasts. A related question is the identity of the kinase family that phosphorylates AC specifically outside the lamellipodium. Most attention has focussed on the LIM kinases (Arber *et al.* 1998; Yang *et al.* 1998), however it must not be forgotten that there are currently two more kinase families that can phosphorylate AC - TES (Toshima *et al.* 2001a; Toshima *et al.* 2001b) and NRK kinases (Nakano *et al* 2003) - and the discovery of these families shows that there may be further as yet unidentified kinases that can phosphorylate ser3.

What part, if any, does AC play in the turnover of actin filaments within filopodia? Filopodia consist of stable actin filaments that turn over only every 25 minutes (Mallavarapu and Mitchison, 1999), thus AC and actin disassembly would seem unlikely to play a major role in these structures. Recent evidence suggests that filopodia may originate from reorganisation of the dendritic network of the lamellipodium (Svitkina *et al* 2003, Vignjevic *et al* 2003). In this scenario, certain actin filaments within the lamellipodium are able to elongate beyond the normal length of filaments in the dendritic brush by acquiring a set of proteins including VASP that confer protection from barbed end capping. These elongated filaments are then bundled by proteins such as fascin to form the tightly packed actin bundle of a filopodium. There must be a way to protect

these privileged filaments not only from barbed end capping, but from being severed/disassembled by AC, inferring the existence of mechanisms that regulate AC activity not only in spatial regions of the cell, but also at the level of the single actin filament.

What is the nature of the switch that converts the cell from dependence on actin filament disassembly to allowing use of the actin monomer pool and how are other proteins beside AC regulated in order to effect this change? Whatever the mechanism, it must be both rapid and under certain circumstances reversible. While cells need to depolarise and stop migrating completely on reaching their destination, migrating CEF also transiently lose their polarised morphology during cell turning events. Under normal circumstances, CEF migrate outwards from the explant in a relatively straight line, however on entering the vicinity of a neighbouring cell they halt, presumably in response to secreted negative guidance cues from the neighbouring cell. The cell then transiently protrudes multiple lamellipodia from around the periphery, perhaps as a means of sensing the surrounding environment, and moves off in a new direction. This whole process can occur in a matter of a few minutes. Turning off AC activity is unlikely to be the only requirement for cessation of migration. Inhibiting myosin II and Arp proteins and increasing Ena/VASP activity are also very likely to play a part. Precisely how the activity of AC is so tightly spatially and temporally controlled, and the nature of the other proteins that undoubtedly act in concert with AC to define the location of, form and maintain each actin network remains for future work.

Elsewhere in the cell, AC activity is likely to be controlled differently. The level of AC activity in a particular cellular region must vary according to the behaviour of the cell. Evidence on the dynamics of actin filament networks has shown that each of these vary considerably in their stability (see Introduction, and reviewed by Cramer 1999a), however there is a clear requirement for a large degree of plasticity of actin filament networks if the cell is to be able to respond to environmental cues. For example, the graded polarity bundles found in the cell body of a migrating fibroblast are very stable, unlike the highly dynamic filaments of the lamellipodium. In the event of the cell needing to turn,

these stable filaments will be in the wrong orientation for the new direction, and hence must be disassembled. This requires a previously stable bundle network to become plastic, be disassembled, re-form, and become stable again. It is likely that AC activity is transiently increased in the cell body alongside a corresponding decrease in the activity of actin bundling and stabilising proteins for this to occur. Once the new direction is established, AC must once again be rendered inactive within the cell body, allowing the new actin network to be stabilised and migration to continue. Equally, when a migrating cell reaches its destination, there must be a way for it to stop. Turning off AC may provide a good mechanism for the cessation of migration: it would promote a switch in the behaviour of lamellipodium protrusion as the actin network changed from short, stiff filaments to longer filaments less able to withstand membrane tension. The subsequent increase in retraction events would make the actin monomer concentration non-limiting towards the cell edge, leading to the production of multiple lamellipodia and loss of polarity and migration capability in the cell. Loss of migration capability in CEF also correlates with the gradual formation of criss-crossed actin bundles in the cell. It is possible that each cell turning event puts a strain on the cell's ability to fully rearrange the graded polarity bundles, leading to residual bundles that are now facing in the wrong orientation for the direction of migration. The build up of these bundles may be one of the cues that eventually force the cell to stop moving. Over time, these bundles are likely to be oriented as stress fibres, the actin structures most commonly seen in non-migrating cells. A true stress fibre has alternating polarity within each actin fibre: i.e. the orientation of the barbed and pointed ends alternates from one filament to the next in the same organisation displayed by muscle sarcomeres (Byers *et al* 1984). Actin filaments arrayed in this fashion can therefore be expected to slide over each other in opposite directions to generate contractile force, as in muscle and this activity is thought to prevent cell locomotion (Byers *et al* 1984).

Variations in the patterning of actin filaments ultimately arise from differences in actin organisation and polarity, but variations in their function are often specified by the actin binding proteins to which they are bound. Many of these structures are associated with members of the myosin superfamily. Despite the

lack of any requirement for force provided by myosin in protrusion based motility as reconstituted by Loisel and colleagues (Loisel *et al.* 1999), at least 18 members of the myosin superfamily have been identified that are adapted for a wide variety of force-generating mechanisms in cells. All work using a conserved actomyosin ATPase cycle (reviewed in detail by Howard, 2001): myosin binds strongly to actin in the absence of ATP. ATP binding induces a conformational change in the myosin that weakens its affinity for actin and causes myosin to fall off the actin. A second conformational change allows ATP hydrolysis and re-binding of the myosin and the myosin power stroke occurs on release of the inorganic phosphate. Myosins are made up of three subdomains: the ATP-binding motor domain responsible for actin interaction, the neck domain that binds light chains and calmodulins, and the tail region, which anchors the motor domain and often contains protein-protein interaction motifs and coiled-coil regions that allow dimerisation of the myosin to form a two-headed molecule (Sellers, 2000). This tail region is likely to provide much of the functional diversity exhibited by different myosin isoforms, however it must also be noted that the kinetics of the ATPase cycle can also vary, with the consequence that some myosins spend more time bound to the actin filament than others. For example, myosin VI has a slow rate of Pi release and can therefore move long distances along an actin filament (De La Cruz *et al* 2001). In contrast, myosin I and some myosin II members spend only a small proportion of their time bound to actin (Ostap and Pollard 1996, Mezgueldi *et al* 2002, Kovacs *et al* 2003), and hence individual myosin I and myosin II molecules cannot provide much force to generate contraction or move cargo. On the other hand, these proteins may be ideally suited to mediating rapid contraction events such as fluctuations of the cell margin and teams of myosin II minifilaments have been proposed to mediate the dynamic network contraction model that is suggested to translocate the keratocyte cell body by remodelling the actin meshwork of the transition zone into contraction-generating boundary bundles and arcs (Svitkina *et al* 1997).

For much of the last 70 years, the cytoskeleton was perceived as a static structure that provided support to the cell and maintained cell shape. Improvements in microscopy techniques revealed it to be an incredibly dynamic

and tightly regulated network. It is now becoming apparent that both these ideas are oversimplifications; regions of the same cell vary in the plasticity of their actin networks and this variation not only occurs spatially but temporally as conditions change. A major challenge for the future is to understand how the cell can maintain such tight control over actin networks of vastly differing organisation and plasticity, and how these networks interact both with each other and with other components of the cytoskeleton to provided an integrated whole. What seems to be clear is that, despite the huge advances in our understanding of the cytoskeleton over the last 30 years, there is still much to learn concerning the spatial and temporal regulation of actin filament dynamics during cell migration.

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