A study examining the role of Rho family GTPases in the intracellular targeting of Src kinase during cell polarisation and migration.

By

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Abstract

The ability of a cell to polarise and move is governed by re-modelling of the cellular adhesion/cytoskeletal network that is in turn controlled by the Rho family of small GTPases. However, it is not known what signals lie downstream of Rac1 and Cdc42 during peripheral adhesion re-modelling that are required for directional migration.

We show here that individual members of the Rho family, RhoA, Rac1 and Cdc42, direct the specific intracellular targeting of c-Src tyrosine kinase to focal adhesions, lamellipodia or filopodia, respectively, and that the adaptor function of c-Src (the combined SH3/SH2 domains coupled to green fluorescent protein) is sufficient for targeting. Furthermore, Src's catalytic activity is absolutely required at these peripheral cell-matrix attachment sites for adhesion re-modelling that converts RhoA-dependent focal adhesions into smaller focal complexes along Rac1-induced lamellipodia or Cdc42-induced filopodia. Consequently, cells in which kinase-deficient c-Src occupies peripheral adhesion sites exhibit impaired polarisation towards migratory stimuli and reduced motility.

Our findings demonstrate that individual Rho GTPases specify Src's exact peripheral localisation, and that RhoA, Rac1 and Cdc42 cannot co-ordinate cell structural changes and directed cell migration when Src is present at adhesion sites in an inactive form. Src's role, which involves phosphorylation of adhesion substrates including FAK, is to induce the necessary adhesion re-modelling downstream of Rac1 and Cdc42 during migratory cell responses.

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Finally, I wish to thank the Beatson Institute, the CRC and Art Alberts for giving me the valuable opportunity to spend time learning about Diaphanous proteins at the Van Andel Institute in Michigan earlier last year.

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Declaration

I declare that all the work in this thesis was performed personally unless otherwise acknowledged. No part of this work has been submitted for consideration for any other degree or award.

Abbreviations

APS .....ammonium persulphate
ATP .....adenosine 5´-triphosphate
AFAP ..... actin filament associated protein
BSA .....bovine serum albumin
c- .....cellular
CSF-1R .....colony stimulating factor receptor
DH .....Dbl homology
DNA .....deoxyribonucleic acid
ECL .....enhanced chemiluminescence
ECM .....extracellular matrix
EDTA .....ethylenediamine tetraacetic acid, disodium salt
EGF(R) .....epidermal growth factor receptor
EGTA .....ethylene glycol-bis(β-aminoethyl ether)N,N,N´,N´-tetraacetic acid
ERK .....extracellular signal regulated kinase
ERM .....ezrin moesin radixin
F .....phenylalanine
FH .....forming homology
FAK .....focal adhesion kinase
FCS .....foetal calf serum
FITC .....fluorescein isothiocyanate
GAP .....GTPase activating protein
GDI ..... guanine dissociation inhibitor
GDF .....GDI displacement factor
GEF .....guanine nucleotide exchange factor
GFP .....green fluorescenct protein
GPCR .....g-protein coupled receptor
GTP .....guanosine 5′-triphosphate
HGF .....hepatocyte growth factor
Ig .....immunoglobulin
LPA .....lysophosphatidic acid
mAb .....monoclonal antibody
MBS .....myosin binding subunit
MLC(K).....myosin light chain kinase
MEM .....modified eagles medium
MRCK .....myotonic dystrophy kinase-related Cdc42 binding kinase
PAK .....p21-activated kinase
PAGE .....polyacrylamide gel electrophoresis
PBS .....phosphate buffered saline
PDGF(R) .....platelet derived growth factor receptor
PH .....Pleckstrin homology
PI3-kinase .....phophatidyl inositol 3-kinase
PIP .....phophatidyl inositol monophosphate
PIP2 ..... phophatidyl inositol- 4,5 bisphosphate
PIP3 ..... phophatidyl inositol- 3,4,5 bisphosphate
PIX .....PAK interacting exchange factor
PI5 kinase..... phophatidyl inositol 5-kinase
PKC .....protein kinase C
PLC .....Phospholipase C
POR .....Partner of rac
PTP .....protein tyrosine phophatase
RNA .....ribonucleic acid

ROCK .....rho kinase
SH2 .....src homology domain 2
SH3 ..... src homology domain 3
SDS .....sodium dodecyl sulphate
TEMED.....N,N,N´,N´-tetramethylethylenediamine
Tris .....tris-[hydroxymethyl]aminomethane
TRITC .....tetramethyl rhodamine isothicyanate
ts .....temperature sensitive
v- .....viral
WASP .....wiskott-Aldrich syndrome protein
w/v .....weight for volume
Y- .....tyrosine

Throughout this study, the names of genes are presented in italics (e.g. *fyn*) and the names of proteins are given in normal text beginning with a capital letter (e.g. Fyn).

Chapter 1

1 Introduction

1.1 Src

Tyrosine phosphorylation has been implicated in a variety of biological responses, including cell migration, proliferation, differentiation and cell survival (Bolen & Brugge, 1997). Many protein tyrosine kinases have been characterised that do not contain extracellular or transmembrane domains. Among these non-receptor tyrosine kinases, are the Src protein tyrosine kinase family which are capable of phosphorylating a diverse range of cellular targets and are the best characterised of all protein tyrosine kinases. c-Src was first isolated as the cellular homologue of v-Src, the transforming gene of Rous Sarcoma virus (Stehelin, 1976). As the first proto-oncogene described and through the finding that Src possesses tyrosine kinase activity (Collett & Erikson, 1978; Levinson et al., 1978), Src serves as a prototype in our understanding of molecular mechanisms behind cellular processes involving tyrosine phosphorylation and has led to the discovery of many other proto-oncogenes and kinase families.

The Src family kinases have been identified on the basis of their transforming phenotype (Yes, Frg and Lck), while others have been identified by regions of homology not found in the receptor family tyrosine kinases (Fyn, Yrk, Hck, Lyn and Blk). Most members of the Src family kinases demonstrate restricted distribution, primarily in hematopoetic cells, while Src, Yes and Fyn display almost ubiquitous expression (Bolen & Brugge, 1997).

1.1.1 Structure of Src

Topographically Src consist of a short membrane anchor domain of 15-17 residues, a poorly conserved unique domain of 40-70 residues which defines family members, a Src homology 3 (SH3) domain of 50 residues which can bind to specific proline rich sequences and a Src homology 2 (SH2) domain of 100 residues found carboxy terminal to the SH3 domain, which binds to tyrosine phosphorylated sequences. Following this the catalytic or kinase domain of Src encompasses 250 residues. Finally at the extreme termini of c-Src lies the carboxy-terminal regulatory region containing a conserved tyrosine residue (Figure 1).

Figure 1.

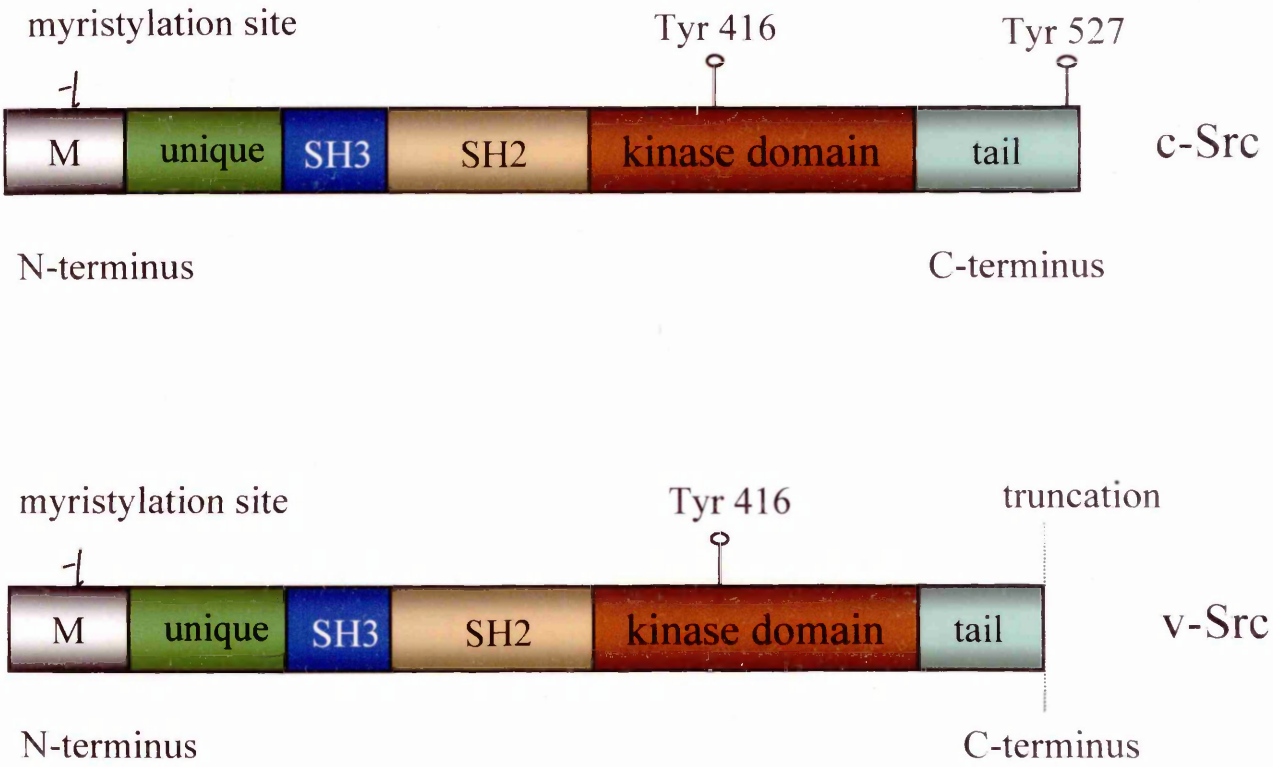


Figure 1. Schematic representation of cellular (c-) and viral (v-) Src. Amino acids numbers correspond to the chicken c-Src sequence The molecule is composed of an amino-terminal myristilation domain, a unique region, SH3 and SH2 protein interaction domains, a kinase domain that contains tyrosine-416 and a carboxy-terminal regulatory domain that contains tyrosine-527. In v-Src 19 C-terminal amino acids of c-Src are replaced by 12 amino acids in v-Src. The C-terminal amino acid deletions in v-Src includes tyrosine-527 which is critical for regulating the protein and as a result v-Src is rendered constitutively active.

1.1.2 Regulation of Src

Most family members of the Src family kinases are highly regulated, exhibiting limited kinase activity in the absence of an activating stimulus. This tight regulation and the methods by which this is achieved will be highlighted here with particular interest paid to the spatial regulation of Src as a method of localising Src's biological action and thus preventing aberrant effects of Src kinase activity within the cell. To begin addressing this, we describe the role of individual Src domains in regulating the enzymatic activity of Src and ultimately their collective role in Src's spatial regulation and biological action.

1.1.3 Allosteric regulation of Src

Initial insights into the important role phosphorylation of Src plays in regulating its kinase activity came from a study, showing that the removal of phosphotyrosine from normal c-Src enhanced the kinase activity of Src (Courtneidge, 1985). Later it was demonstrated that the reduced transforming ability of c-Src compared to v-Src, which is dependent on Src kinase activity (Kmiecik & Shalloway, 1987; Snyder et al., 1985), was due to the presence of tyrosine-527 in c-Src, whose phosphorylation was responsible for the negative regulation of c-Src kinase activity and thus transforming capacity (Cooper et al., 1986). v-Src lacks this residue and mutations in c-Src at tyrosine-527 to phenylalanine or serine augmented the protein tyrosine kinase and transforming activity of c-Src (Figure 1) (Kmiecik & Shalloway, 1987; MacAuley & Cooper, 1989). Moreover point mutations in the SH2 domain led to c-Src activation (Kato et al., 1986; Parsons & Weber, 1989), highlighting its importance in regulating c-Src activity, and led to the proposal of a model in which the SH2 domain participates in an intramolecular interaction with the phosphorylated tyrosine-527 carboxy domain, rendering the kinase domain inaccessible and therefore inactive in this 'closed' conformation (Figure 2). By conjecture, this model suggests that dephosphorylation of tyrosine-527 by cellular phosphatases for example, would abolish this interaction and allow c-Src to adopt an 'open' catalytically active conformation, as postulated for protein serine kinases (Soderling, 1990). When Src is in its 'open' conformation, where restraints from the intramolecular interactions are disrupted, the phosphorylation of the activation loop at tyrosine-416 in the kinase domain, which is an autophosphorylation site (see Section 1.1.8), is required to allow full activation of the molecule (Kmiecik & Shalloway, 1987; Snyder et al., 1983) (Figure 2). The transforming capacity of oncogenic c-Src mutants or v-Src are reduced in cells expressing point mutations in the activation loop (Kmiecik & Shalloway, 1987; Snyder et al., 1983).

Until the recent elucidation of the three-dimensional crystal structure of regulated Src, the role of the SH3 domain of Src was thought to be in co-operation with the binding of the SH2 domain to tyrosine-527 by binding proline rich sequences in the kinase domain (Courtneidge et al., 1993). However, it is now clear that the kinase domain is exposed during interactions between the SH2 domain and the carboxy-terminal region and that the SH3 domain participates in regulating the kinase activity of Src by interacting with the linker region between the SH2 and catalytic domain (Figure 2) (Sicheri & Kuriyan, 1997; Xu et al., 1997). Moreover, elimination of the SH3 domain-linker interaction has been shown to induce the activation of the Src family member, Hck, thereby providing a direct connection between the SH3 interaction and kinase activity (Moarefi et al., 1997). This, along with the SH2/tyrosine-527 interaction, results in constraints on the free movement within the kinase domain, rendering it inactive (Sicheri et al., 1997; Xu et al., 1997; Yamaguchi & Hendrickson, 1996). Interactions between the SH3 and upstream linker sequences between the SH2 and the kinase domain, therefore both contribute to the regulation of Src (Figure 2).

1.1.4 Factors influencing the enzymatic regulation of Src

Given the current model (Figure 2), there are several ways in which Src can be regulated and any one or more of these might be responsible for the enzymatic activity of Src. These include deletion of tyrosine-527 for example, which has previously been described for v-Src (Reynolds et al., 1987), resulting in its constitutive activation. Similar results were seen in the case of mutating tyrosine-527 to phenylalanine or serine causing the constitutive activation of c-Src (Kmiecik & Shalloway, 1987). More recently there has been reports of activating mutations in the carboxy terminal region in human c-Src on tyrosine-530 which have similar functional consequences as mutating or deleting tyrosine-527 in chicken c-Src, including links to enhanced metastasis and cell transformation (Irby et al., 1999). Other regulatory mechanisms include de-phosphorylation of tyrosine-527 by tyrosine phosphatases, negative regulation by the related regulatory kinase Csk, displacement of SH3/SH2- mediated intramolecular interactions by higher affinity ligands or by altering the binding affinity of the SH domains to these ligands and finally phosphorylation of tyrosine-416, all of which are described in more detail below.

Figure 2.

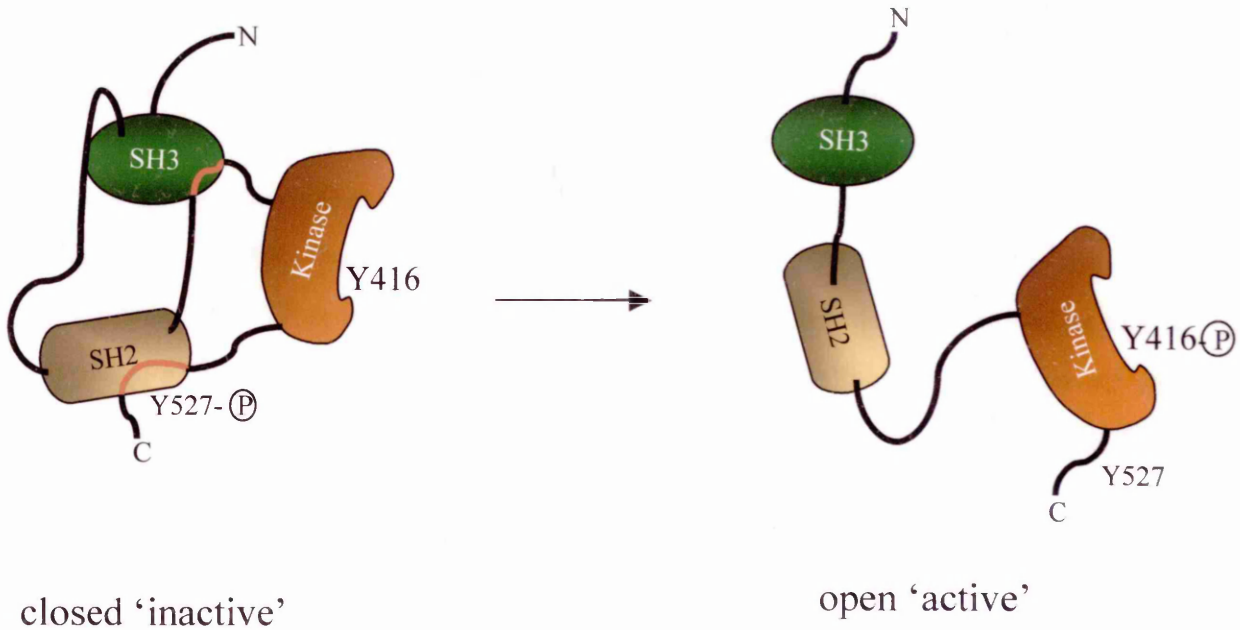


Figure 2. Schematic representation of c-Src activation by de-phosphorylation of tyrosine-527, which results in both the dissociation of intramolecular interactions with the SH2 and SH3 domains.

1.1.5 Regulation of Src by protein tyrosine phosphatases.

Several protein tyrosine phosphatases (PTPs) have been implicated in the regulation of Src family kinases serving to de-phosphorylate the C-terminal tyrosine-527 residue and thus displace the intramolecular interaction with the SH2 domain rendering Src 'open' and active. For example, Cd45 is able to dephosphorylate the C-terminal regulatory site of Lck and Fyn resulting in kinase activation (Mustelin & Altman, 1990) and Cd45-deficient mice have impaired T-cell signalling which correlates with decreased catalytic activity of Lck and Fyn and increased phosphorylation of the C-terminal tyrosine-527 (Mustelin et al., 1992; Ostergaard et al., 1989). However, it is not known whether Cd45-induced dephosphorylation of Lck or Fyn is primarily responsible for these effects, due to the fact that Lck and Fyn are already poorly phosphorylated in lymphocytes (Ostergaard et al., 1989). Perhaps a more likely phosphatase would be PTP-α, which dephosphorylates tyrosine–527 *in vitro* (Zheng et al., 1992) and in cells such as A431 carcinoma cells (Harder et al., 1998; Ponniah et al., 1999; Su et al., 1999). Consistent with this, overexpression of PTP-α in fibroblasts is oncogenic and causes dephosphorylation of Src on tyrosine-527 in these cells (Zheng et al., 2000). The likely-hood of PTP-α being a major physiological regulator of Src however is unlikely due to the lack of specificity of PTP-α for the carboxy-terminal tyrosine-527 over other residues, such as the autophosphorylation site at tyrosine-416 (Smart et al., 1981). Other phophatases that can dephosphorylate Src include PTP1B, SHP-1 and SHP-2. PTP1B was found to be up regulated in breast cancer cell and when purified, like PTP-α, can dephosphorylate and activate Src *in vitro* and in cells (Bjorge et al., 2000). The regulation of Src may be achieved by the activity of a variety of protein tyrosine phosphatases and the level of PTP expression in different cell types may serve to regulate Src kinase family activity, on a cell-type specific basis (O'Connor et al., 1995; Zhao et al., 1992).

1.1.6 Regulation of Src by C-terminal Src kinase Csk.

In addition to the actions of phosphatases, the tyrosine kinase Csk and its homologue Chk are thought to be involved in controlling the activation status of Src family kinases by phosphorylating the C-terminal tyrosine-527 (Nada et al., 1993). Csk is structurally similar to Src comprising an SH2 and SH3 domain, but it lacks the regulatory tyrosine residue that critically controls Src activity (Nada et al., 1991). It also does not have a membrane anchorage domain and thus Csk is not membrane bound but is found predominantly in cytoplasmic pools (Bergman et al., 1995; Okada & Nakagawa, 1988;

Okada & Nakagawa, 1989). One mechanism proposed for Src regulation by Csk is that access to Src by alterations in Csk localisation may influence its effect on Src regulation (Chow et al., 1994; Chow et al., 1993; Ford et al., 1994). Csk has a high affinity for sequences around tyrosine-416 of activated Src which may target Csk to areas of Src activation and thereby act in a negative feedback loop to regulate Src activity (Sabe et al., 1995; Songyang et al., 1994b). Interestingly, Csk phosphorylates native Src more effectively than C-terminally mutated or denatured Src, suggesting that it recognises the tertiary structure of Src and that it is in close proximity to the SH2/SH3 and C-terminal domains before phosphorylation occurs (Okada & Nakagawa, 1988; Okada & Nakagawa, 1989). It can therefore be postulated that protein interactions with the SH2/SH3 domain of Src could influence the likely-hood of Csk-induced Src phosphorylation and repression of its kinase activity (see Section 1.1.10). Finally as with PTPs, the level of Csk expression between different cell types may serve to regulate the relative activity of Src. As demonstrated in hepatocellular carcinomas, where Csk levels have been reported to be reduced compared to normal liver tissue, this altered level of expression correlates with enhanced Src activity (Masaki et al., 1999). Loss of Csk activity or reduced expression leads to activation of Src family kinases and results in the deregulation of many cellular and developmental processes (Masaki et al., 1999; Nada et al., 1993). Consistent with this, Csk null mice cause constitutive activation of Src family kinases (Nada et al., 1991; Nada et al., 1993). Moreover, overexpression of Csk has been reported to suppress metastasis of colon cancer in an animal model correlating to repression of Src kinase activity (Nakagawa et al., 2000). Another related mechanism of negatively regulating Src is the autophosphorylation of carboxy-terminal tyrosine-527, which has been demonstrated *in vitro* (Cooper et al., 1986; Osusky et al., 1995) and in fibroblasts (Bagrodia et al., 1991; Bagrodia et al., 1993) however the kinetics of this suggest that other kinases along with Csk are more physiologically relevant regulators of Src tyrosine-527 phosphorylation.

1.1.7 Regulation of Src by altered protein stability or expression levels.

As discussed above, control of the steady state levels of PTPs or Csk serve to regulate their activity and in turn regulate Src activity (Nada et al., 1991; Nada et al., 1993). Recent evidence suggests that Src is subject to ubiquitin-dependent degradation. Specifically, activated forms of Src, including that activated as a consequence of Csk loss, are turned over more rapidly than wild type or kinase-inactive Src proteins (Harris et al., 1999; Yokouchi et al., 2001). Consistent with this, previous work has demonstrated an increased susceptibility of dephosphorylated, 'open' Src, compared to repressed or 'closed' Src, to a spectrum of protease-induced degradation conditions (MacAuley & Cooper, 1989).

Therefore, if the ubiquitin-proteosome degradation system in general is altered this could potentially effect the level of Src activity in the cell (Kamei et al., 2000). The ubiquitously expressed protease calpain (Fox et al., 1985) may also co-operate with proteosomes to regulate Src by calpain-induced cleavage of Src (Jang & Choi, 1999). Calpain is proposed to impair Src dependent activity by decreasing its kinase activity due to a reduction in its association with the cell membrane. Association with the cell membrane, as described later in Section 1.1.9, is essential for many of Src's activities and therefore impairing this association may constitute a further mechanism of regulating Src within the cell (Oda et al., 1993). Irrespective of the mechanism involved, down regulation of Src activity by proteolytic pathways represents an additional mechanism by which Src family kinases can be regulated.

1.1.8 Regulation of Src by the Src kinase domain.

The kinase domain of Src is the most conserved region between family member and plays a role in substrate selection along with the SH3 and SH2 domains. Moreover, the preferred substrate sequence of the kinase domain shows similarities to the preferred binding sequence of the SH2 domain, suggesting cross-talk between these domains during substrate binding (Zhou et al., 1995). As discussed earlier (see Section 1.1.3) when Src is in its 'open' conformation, where restraints from the SH2/SH3 intramolecular interactions are disrupted, the phosphorylation of the activation loop at tyrosine-416 is required to allow full activation of the molecule (Kmiecik & Shalloway, 1987; Snyder et al., 1983). Auto-phosphorylation appears to occur mainly by intramolecular interactions and it is thought that phosphorylation of tyrosine-416 serves to stabilise the catalytically active conformation of Src (Feder & Bishop, 1990; MacAuley & Cooper, 1988; Osusky et al., 1995; Sugimoto et al., 1985). Moreover, recent studies have also proposed a bi-directional regulatory mechanism involving the phosphorylation of tyrosine-416 in the kinase domain, where phosphorylation or mutations in the activation loop result in the increased availability of the SH3 domain for binding. By this mechanism the Src residues within the kinase domain control the accessibility of the regulatory domains (SH3/SH2), while as described previously (see Section 1.1.3), the regulatory domains control the accessibility of the kinase domain. The lysine residue at position 295 in the kinase domain is strictly conserved between family members and plays a role in the phosphotransfer reaction during ATP binding (Hunter & Cooper, 1985). Mutation of this residue results in a kinase-defective form of Src (Kamps & Sefton, 1986).

1.1.9 Regulation of Src by the unique domain.

Carboxy-terminal to the myristylation domain lies the unique domain (Figure 1). This domain is highly divergent and the least conserved between family members. There are protein kinase (PKC) dependent phosphorylation sites at residue 12 and 48 in this domain, and furthermore, serine-17 in the unique domain, is a major phosphorylation site for cyclic AMP-dependent protein kinase (Brown & Cooper, 1996; Courtneidge et al., 1993). Moreover, there have also been reports of cdc2/cyclin-dependent phosphorylation of Src's unique domain (Kaech et al., 1993; Shenoy et al., 1992). The overall function and significance of the phosphorylation events in the unique domain however have still to be fully understood. Some studies have revealed potential functions of this domain in terms of protein interactions and in regulating Src family kinase activity itself. Cdc2/cyclin-dependent phosphorylation of Src's unique domain for example, seems to disengage the C-terminal regulatory domain from the SH2 domain, rendering tyrosine-527 more accessible to phosphatases and therefore activation (Chackalaparampil & Shalloway, 1988). Interesting in the case of Lck, there are indications that serine-49 may regulate the specificity of the SH2 domain for ligands (Park et al., 1995; Shenoy et al., 1992), which, as will be discussed in more detail later, may serve to regulate Src kinase activity by interfering with the association of phosphorylated tyrosine-527 and the SH2 domain, rendering it 'open' and in the active conformation. Recently, auto-phosphorylation of tyrosine-29 in the Src family member, Hck's, unique region has been shown to activiate Hck (Johnson et al., 2000). Therefore the unique domain may play a key role in regulating Src kinase activity during specific processes.

1.1.10 Regulation of Src by molecular displacement.

Carboxy-terminal to the unique domain is the SH3 domain followed by the SH2 domain (Figure 1). The SH3 domain binds proline-rich ligands with a left handed helical conformation and the SH2 domain preferentially binds to phosphotyrosine containing sequences with the consensus sequence YEEI (Songyang et al., 1994a; Yu et al., 1994). In both cases these domains are highly conserved amongst Src family members and other non-related proteins, however variations in the binding affinity of the SH2 and SH3 domains between Src family members has been demonstrated. For example the SH3 domain of Src, Fyn and Yes have been shown to recognise a common motif, while Lyn demonstrates a distinct binding specificity (Rickles et al., 1995). Moreover, like the SH3 domain, the binding affinity of the SH2 domain for its ligand has been demonstrated to be regulated by post-translational modification. For example, tyrosine phosphorylation at

tyrosine-192 increases the binding affinity of the SH2 domain for phospholipase C (PLC) (Couture et al., 1994).

Displacement of intramolecular SH3/SH2 interactions constitutes another mechanism for activating and thereby regulating Src kinase activity within the cell. A diverse class of Src interacting partners has been identified that could compete effectively with the intramolecular interactions and thereby disturb Src's inactive state. The first indication that Src kinase as well as other family member such as Fyn (Ralston & Bishop, 1985) could be regulated by this mechanism, demonstrated that binding of the SH2 domain of Src or Fyn to the platelet-derived growth factor receptor (PDGFR) or to the colony stimulating factor receptor (CSF-1R) activates Src or Fyn's kinase activity (Mayer et al., 1999; Ralston & Bishop, 1985; Songyang et al., 1994a). As stated above the SH2 domain preferentially binds to phosphotyrosine containing sequences and activation of Src has previously been observed following binding to a tyrosine phosphorylated peptide derived from the PDGFR (Mayer et al., 1999; Ralston & Bishop, 1985; Songyang et al., 1994a). In addition to actively competing for the Src SH2 domain, the PDGFR may activate Src by phosphorylation of the SH3 domain. Tyrosine-136 phosphorylation in the SH3 domain by the PDGFR has been shown to reduce the affinity of the SH3 domain for peptide ligands (Broome & Hunter, 1996), analogous to the effect of the unique domain of Lck on SH2 binding (see Section 1.1.9). As the SH3 domain is partially involved in maintaining the 'closed' conformation of Src this could lead to destabilisation and therefore activation of Src kinase activity. Interestingly, another non-receptor tyrosine kinase family member, focal adhesion kinase (FAK), that was first identified as a tyrosine phosphorylated protein in Src transformed cells (Kanner et al., 1990), is an example of a Src effector that can bind to the SH2 domains of Src and lead to Src kinase activation (Schaller et al., 1994; Thomas et al., 1998). Recently it was demonstrated that the FAK binding partner, p130 Cas (Crk associated substrate), could activate Src via binding to its SH domains and displace the intramolecular interactions that regulate Src activity (Burnham et al., 2000). As described previously, (see Section 1.1.5), PTPα can activate Src by dephosphorylation of the C-terminal tyrosine-527 of Src. Recently PTPα has also been shown to regulate Src by the phosphotyrosine displacement mechanism described above (Zheng et al., 2000).

1.2 Spatial regulation of Src.

Conserved domains of Src are not only involved in regulating Src kinase activity by regulating the 'open,' or 'closed' conformation of Src but also serve a purpose in the

correct intracellular localisation of Src during various cellular processes. For example early observations demonstrated that Src-family kinases are commonly found associated with the plasma membrane, as well as intracellular membranes including endosomes and the endoplasmic reticulum (Courtneidge et al., 1980; Garber et al., 1983). The amino-terminal myristylation of Src is required for the association with cellular membranes and is essential for the transforming function of oncogenic Src mutants (Resh, 1994). Consistent with this, myristylation defective alleles of v-Src, which are no longer associated with the membrane, are no longer transforming (Hamaguchi & Hanafusa, 1987). Similarly the perinuclear distribution of Src has been shown to be dependent on the myristylation domain and is thought to be retained there by association with the microtubule network, as demonstrated, by co-localisation studies in which both Src and tubulin co-localised in the perinuclear region of the cell (Fincham et al., 1996). Moreover the switch from a perinuclear to a cytoplasmic distribution of Src upon treatment with the microtubule disrupting drug nocodazole also demonstrated the microtubule association of Src in this region of the cell (Fincham et al., 2000; Fincham et al., 1996). Myristylation is an irreversible process mediated within the first seven residues of Src where the myristylation group is added to glycine at position two of the protein (Figure 1). In general myristylation is necessary for membrane localisation (Bagrodia et al., 1993) however some myristylated Src molecules are found to be cytoplasmic (Buss et al., 1984) suggesting that myristylation is not the only domain involved in membrane localisation of Src. Besides the myristylation domain providing a signal for membrane association, all Src family members except Src itself and Blk (Resh, 1994), undergo palmitylation of amino-terminal basic cysteine residues, which serves to stabilise membrane association by compensating for the absence of a positive charge on the N-terminal region of the protein (Lock et al., 1991; Robbins et al., 1995). Palmitylation is a reversible process and control of palmitylation has been proposed as a potential mechanism for the regulation of Src family kinase localisation within different membrane compartments such as caveolae in response to the certain stimuli (Kaplan & Simon, 1988; Robbins et al., 1995). Recently alterations in the palmitylation status of Lck, has been shown to block its normal function in T-cell signalling (Kabouridis et al., 1997) analogous to alterations in the myristylation of Src blocking oncogenic transformation (Buss et al., 1984; Resh, 1994). Therefore the myristylation domain plays an important role in c-Src distribution, by anchoring c-Src to various membrane locations. Furthermore, in association with other domains and modifications, myristylation regulates the trafficking of Src to specific membrane micro-environments (Buss et al., 1984; Kaplan et al., 1994).

figure3legend

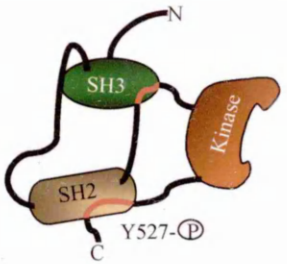
Figure 3

A schematic representation of Src's spatial localisation during various cellular processes. Following translocation to the cell periphery Src can be found in focal adhesions where it may be involved in a number of signal transduction pathways or in focal adhesion turnover during cell migration. Src may also be localised to cell-cell junctions inducing cell-cell disruption.

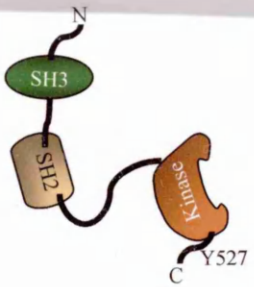
Figure 3.

Spatial Regulation

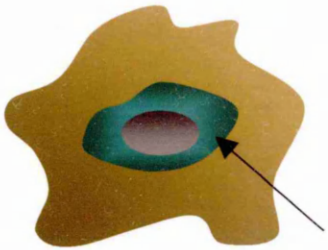
Src closed
'inactive'



Src open
'active'

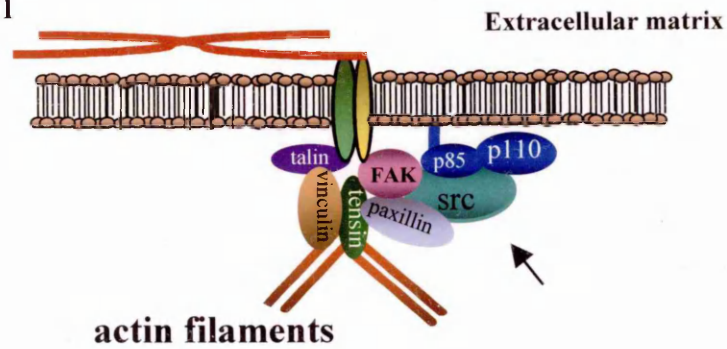


Perinuclear

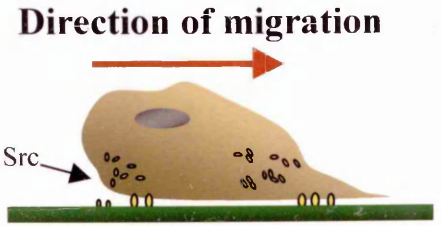


Translocates

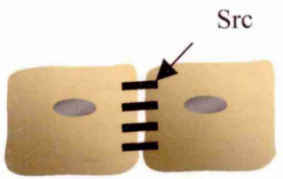
Focal adhesion
signalling



Focal adhesion
turnover



Cell-cell
junction disruption



1.2.1 Src domains involved in spatially regulating Src.

The finding that the transforming activity of Src involves interactions between multiple domains and that myristylation-induced membrane association is necessary for this process, emphasises the importance of spatial regulation of Src in the biological action of Src. It is also clear however, that domains other than the myristylation domain of Src, besides contributing to Src's allosteric regulation, also provide a role in Src localisation.

As stated previously, early observations demonstrate that Src is readily found in the perinuclear region of the cell where co-localisation and biochemical fractionation studies demonstrated that Src localises with endosomal markers and associates with endosomal membrane structures (David-Pfeuty & Singer, 1980; Kaplan et al., 1992; Nigg et al., 1982). v-Src however, is abundant in both the perinuclear region of the cell and in focal adhesions, which are areas of adhesion, where the extracellular matrix can interact with transmembrane receptors and the actin cytoskeleton (see Section 1.3.2) (Burr et al., 1980; Kaplan et al., 1992; Nigg et al., 1982). The apparent difference in sub-cellular localisation of c-Src and v-Src led to the proposal that deletion of the C-terminal tyrosine-527 in v-Src may be responsible for this altered distribution of v-Src (Figure 1). In v-Src, 19 C-terminal amino acids of c-Src are replaced by 12 unique amino acids in v-Src (Takeya & Hanafusa, 1983). This includes tyrosine-527, which, as mentioned above, is critical for the regulation of the protein. This altered sub-cellular distribution of v-Src along with the fact that myristylation can confer membrane association led to investigations examining the contribution of each domain in controlling the intracellular targeting of Src. The role of each domain in this process was investigated by examining a series of deletions and point mutations of these sites in c-Src.

Mutation of tyrosine-527 to phenylalainine in c-Src, which results in catalytic activation, as well as release of constraints on the SH3 and SH2 domains (see Section 1.1.4), led to c-Src being constitutively associated with focal adhesions (Kaplan et al., 1994). Similarly, temperature sensitive *ts* variants of v-Src that are temperature sensitive for kinase activity, located to focal adhesions when activated by switch to permissive temperature (Fincham & Frame, 1998; Fincham et al., 1996; Welham & Wyke, 1988). Although the mechanism for temperature-induced activation of ts v-Src is not established, it is believed to mimic the conformational changes in c-Src upon release of the regulatory intramolecular interactions between the SH3/SH2 domains and the catalytic domain (Abram & Courtneidge, 2000; Thomas & Brugge, 1997).

Subsequently, the role of the kinase domain in c-Src targeting to focal adhesions was examined in this context. Using the SrcY527F mutant with a point mutation in the ATP binding domain (K295M) (see Section 1.1.8) the translocation of c-Src to focal adhesions was shown to occur independently of kinase activity (Kaplan et al., 1994), consistent with the kinase-independent targeting of (ts) v-Src to focal adhesions (Fincham & Frame, 1998). Moreover, in addition to activating the kinase activity, mutation of tyrosine-527 to phenylalanine also alters the tertiary structure of c-Src to expose the N-terminal regulatory domains of Src (Figure 2) (Gonfloni et al., 2000).

The ability of focal adhesion targeting by the N-terminal domains of Src comprising the first 251 residues was therefore examined. The first 251 residues of c-Src readily associate with focal adhesions (Kaplan 1994), indicating that the N-terminal region of Src contains sufficient information to allow targeting of Src to focal adhesions. Deletion of the SH3 domain residues 92-144 abolished Src targeting to focal adhesions along with a reduction in the detergent-insoluble (or membrane) fraction of c-Src (Kaplan et al., 1994). This, along with further mutational analysis of both c- and v-Src, including a single amino acid change in the SH3 domain which ablates SH3 binding (Erpel et al., 1995), revealed a critical role for the SH3 domain in the translocation of Src to focal adhesions (Fincham et al., 2000; Fincham & Frame, 1998; Kaplan et al., 1994). Mutations of the SH2 domain to block phosphotyrosine binding (Bibbins et al., 1993), or deletion of the C-terminal residues in the SH2 domain (residue 169-204, Figure 1), revealed that impairment of the SH2 domain function has no significant effect on Src localisation to focal adhesions, resulting in only a slight increase in perinuclear staining. In contrast, previous reports by Fukui, demonstrated that deletions of the N-terminal residues (residues 149-169, Figure 1) of the SH2 domain reduced the association of v-Src with the membrane fraction of the cell (Fukui et al., 1991). This apparently paradoxical result can be explained however by the likely-hood that such large deletions in the N-terminal region of the SH2 domain (as opposed to the C-terminal region) may effect the conformational stability of the adjacent SH3 domain (Figure 1), which has been demonstrated to be necessary for Src translocation (Fincham et al., 2000; Kaplan et al., 1994). Furthermore, mutations in the SH2 domain have also been reported to effect the overall stability of the molecule making data interpretation difficult (Kaplan et al., 1994). It is likely that the SH2 domain plays a role in peripheral targeting by modulating the association of the SH3 domain with candidate mediators of Src's peripheral targeting, such as the p85 regulatory domain of phophatidyl inositol 3-kinase (PI3-kinase). v-Src binds to PI3-kinase in normal growing cells via its SH3 domain, however upon activation of v-Src there is a rapid induction of binding to PI3-kinase via the SH2 domain of Src which is thought to serve in regulating the recruitment of

PI3-kinase to Src which is required during Src translocation to the cell periphery (Fincham et al., 2000; Haefner et al., 1995). Furthermore the N-terminal region of the SH2 domain has been implicated as having a role in Src's association with the actin cytoskelton, as N-terminal but not C-terminal deletions in this domain reduce cytoskeletal association (Fukui et al., 1991; Okamura & Resh, 1994). Deletions in the N-terminal region of the unique domain residues (residue 17-30, Figure 1) had no effect on focal adhesions targeting, while deletion in the C-terminal end of the unique domain (residues 30-92, Figure 1), disrupted the localisation of Src to focal adhesions, which, as above, was suspected to be due to interference in the SH3 related targeting processes. Clearly multiple domains in Src are involved in the targeting of Src to the cell periphery, whether it be via membrane association or by allowing recruitment of high affinity binding partners to specific sites. By this means, activation of Src induces the formation of SH2- and SH3- dependent complexes with proteins or lipids (Kaplan et al., 1990; Rameh et al., 1995) that can specify the targeting of Src during suitable cellular responses. As discussed previously for the activation loop (see Section 1.1.8), a bi-directional regulatory mechanism has been proposed, in which the intramolecular interactions involving the SH2/SH3 domains and the C-terminal region of Src, regulate kinase activity, as well as serving to regulate the availability of these N-terminal protein-protein interaction domains. By this mechanism the localisation of Src and the kinase activity can be simultaneously regulated (Gonfloni et al., 2000).

As discussed earlier in Section 1.1.6, one mechanism proposed for Src regulation by Csk is that access to Src by alterations in Csk localisation may influence its effect on Src regulation (Guan & Shalloway, 1992). In particular, Csk has been shown to translocate to focal adhesions and co-localise with Src during cell spreading and adhesion formation (Kaplan et al., 1995). It has been proposed that Csk may bind to various tyrosine phosphorylated chaperon proteins such as FAK or paxillin and therefore be brought to the cytoskeletal or focal adhesions fraction of the cell and locally inhibit Src, indicating that the spatial regulation of proteins such as Csk may serve a role in their biological function or efficacy within the cell.

1.2.2 Potential binding partners involved in spatial regulation of Src

The finding that the Src SH3 domain along with potential contributions from the SH2 domain directs peripheral targeting of Src (Fincham et al., 2000; Kaplan et al., 1994), led to investigations to examine the nature of the SH3 binding partners involved in the peripheral targeting of Src. The binding of potential proteins involved in Src targeting to

an SH3 binding mutant of (ts) v-Src, which inhibits SH3 binding (W118A) (Erpel et al., 1995) and the targeting of Src to focal adhesions, was compared to the binding of normally targeting (ts) v-Src, to examine a variety of potential regulators of this process. The actin filament associated protein (AFAP) which is a Src substrate and binding partner (Flynn et al., 1993), the cytosolic chaperon HSP90 that binds to both c-Src and v-Src and is reported to be essential for membrane association of Src (Bijlmakers & Marsh, 2000; Brugge, 1986; Xu et al., 1999) and FAK which contains a C-terminal focal adhesion targeting sequence and binds to Src via the Src SH3 and SH2 domains (Hildebrand et al., 1993; Thomas et al., 1998) were all examined. Both AFAP and HSP90 were ruled out as contributing to this process due to their ability to associate with v-Src independently of SH3 binding, as demonstrated by their binding to (ts) v-Src containing the SH3 domain binding mutation (W118A) (Erpel et al., 1995; Fincham & Frame, 1998). Similarly FAK was ruled out as a chaperon in the translocation of Src to focal adhesions as v-Src could translocate to focal adhesions in FAK -/- cells and over expression of FAK mutants that impair Src binding to FAK did not inhibit v-Src targeting (Fincham & Frame, 1998). PI3-kinase however, which is involved in regulation of vesicular transport (Clark & Brugge, 1993; Schuh & Brugge, 1988) and has been recently detected in focal adhesions with similar dynamics as the activation of Src (Gillham et al., 1999; Guinebault et al., 1995), was shown to only associate with the targeting version of v-Src and not the targeting defective mutant (Fincham et al., 2000). This is consistent with previous work demonstrating the binding of PI3-kinase with the SH3 domain of activated Src *in vitro* (Haefner et al., 1995; Liu et al., 1993). Subsequently, PI3-kinase dependent targeting of Src was demonstrated using the PI3-kinase inhibitor LY294002 which prevented the translocation of v-Src to focal adhesions (Fincham et al., 2000). This however does not rule out other possible binding partners involved in this process.

1.2.3 Spatial location of Src during various cellular processes.

Regardless of how Src kinase activity is regulated, Src's contribution to cellular processes is almost certainly not confined to whether it is active or not. For example, upon activation, Src has been shown to translocate from a perinuclear localisation to the cell periphery within the cell. Src has been found in focal adhesion structures at the cell periphery where it is associated with various proteins resulting in the activation of a number of signal transduction pathways (Figure 3). Moreover Src's localisation in focal adhesions is thought to lead to focal adhesion turnover and remodelling, a prerequisite for cell migration (see Figure 3 and Section 1.6.1). Src has also been shown to localise to areas of cell-cell contact in epithelial cells and its kinase activity at these sites is thought to

function in the disruption of cadherin mediated cell-cell junctions during their normal dynamic regulation and upon cell scattering (Behrens et al., 1993; Matsuyoshi et al., 1992; Owens et al., 2000). Other roles for Src kinase activity at cell-cell junctions include the breakdown of gap junctions as demonstrated by the phosphorylation of connexin 43 leading to gap junction dissociation (see Figure 3) (Crow et al., 1990; Filson et al., 1990). Src has also been shown to be re-located from cell-cell junctions to integrin adhesions during epidermal growth factor (EGF)-induced invasion (Brunton et al., 1997). Src induced disruption of cell-cell contacts has implications for cell migration and metastasis allowing cells to disperse from an epithelial sheet (Owens et al., 2000). Src can also be found associated with secretary vesicles indicative of a role in directed secretion (Grandori & Hanafusa, 1988; Linstedt et al., 1992). Moreover, Src is found in the nuclear compartment along with other Src family kinases such as Fgr and the related kinase Frk where it is thought to be involved in mRNA processing in association with Sam68 during mitosis (Taylor & Shalloway, 1994). Src as well as other family member such as Fyn and Lck can also be found associated with microtubules or pericentriolar vesicles respectively (Ley et al., 1994). The distinctive localisations of Src and Src family members, suggest that localisation is partially regulated by individual domains of Src and also highlights that intracellular localisation plays a major role in the biological action of Src family kinases.

1.2.4 Src's intracellular targeting within the cell.

Despite our increasing understanding of the regulation of Src, and the importance of the SH3 and SH2 domains in binding to potential chaperon molecules, there is still little information on the mode of intracellular targeting of Src to the cell periphery and how this targeting is controlled. Earlier observation however, demonstrated that Swiss 3T3 cells expressing high levels of v-Src were surprisingly unable to induce morphological transformation or induce mitogenic signalling when deprived of serum (Fincham et al., 1996). Resistance to the normal transforming effects of v-Src was found to be due to impaired peripheral targeting of Src, consistent with myristylation defective mutants of Src which fail to transform (see Section 1.2). The reason for the impaired peripheral targeting however, originates from previous observations by Ridley and Hall, that Swiss 3T3 cells lose their actin stress fibres upon serum starvation (Ridley & Hall, 1992). This observation in conjunction with demonstrations that activated Src in response to PDGF, EGF or thrombin translocates to the actin cytoskeleton (Burr et al., 1980; Clark & Brugge, 1993; Weernink & Rijksen, 1995), led to investigations into the actin dependence of Src targeting to focal adhesions. Addition of serum or microinjection of a constitutively active form of RhoA (see Section 1.3.2), both of which induce actin stress fibre formation, into serum-

deprived Swiss 3T3 cells, restored actin stress fibres and the translocation of v-Src to focal adhesions thereby restoring the oncogenic potential of v-Src in these cells (Fincham et al., 1996). v-Src initially localised along re-formed stress fibres and there was a time dependent re-distribution of Src to the stress fibre termini at the cell periphery (Fincham et al., 1996). Furthermore, treatment with the actin disrupting drug cytochalasin D prevented Src translocation in Swiss 3T3 cells (Fincham et al., 1996). Src has previously been shown to bind to polymerised actin (Lakkakorpi et al., 1997) and interestingly the v-Src SH3 mutant that was unable to target to focal adhesions was unable to bind to polymerised actin, implying that a potential role of the SH3 domain is to link Src to cellular actin filaments allowing translocation to the cell periphery (Fincham et al., 2000). Similar effects have been seen for the intracellular targeting of Src and other ubiquitously expressed family members such as Fyn and Yes in epithelial cells, in which treatment of cells with cytochalasin D prevented translocation to cadherin mediated cell-cell junctions (see Figure 3) (Owens et al., 2000). From this it has been concluded that regulation of Src's intracellular targeting within the cell requires an intact actin cytoskeleton. Interestingly the PI3-kinase inhibitor LY294002 used to assess the role of PI3-kinase in Src's peripheral targeting also reduced the number of actin stress fibres in cells, although peripheral actin was still observed, implying that one potential role of PI3-kinase as a chaperon is to maintain actin stress fibres in a state permissive for Src translocation (Fincham et al., 2000). In this regard, the report of PI3-kinase, Src and RhoA translocation to the actin cytoskeletal fraction upon osteoclast attachment helps implicate this action of PI3-kinase in Src's targeting to the cell periphery (Lakkakorpi et al., 1997).

Based on the actin dependence of Src's peripheral targeting we set out to examine whether intracellular regulators of the actin cytoskeleton (RhoGTPases, see below) could influence the intracellular location of Src and thereby serve to spatially regulate its biological action within the cell.

1.3 RhoGTPases

Over the past decade there has been a remarkable increase in our knowledge of the role RhoGTPases play in a diverse spectrum of cellular processes including regulation of the actin cytoskeleton, cell migration, membrane trafficking, cell growth and transformation (Ridley, 1996; Van Aelst & D'Souza-Schorey, 1997). Here we focus on actin cytoskeletal organisation and remodelling induced by RhoGTPases and the role they play in processes such as cell motility, cell-substratum adhesion and disruption, and highlight their potential role in facilitating the local effects of Src during cell migration.

RhoGTPases are a subfamily of proteins belonging to the Ras superfamily of small GTPases (Hall, 1998; Van Aelst & D'Souza-Schorey, 1997). A great deal of our knowledge regarding RhoGTPases, stem from work on the prototype Rho family members RhoA, Cdc42 and Rac1, each demonstrating distinct functions within the cell. As a result RhoGTPases can be subdivided into the following groups based on cellular function and sequence homology. The first of these include RhoA, RhoB and RhoC, which share similar cellular functions including the formation of stress fibres (see Section 1.3.2). The Rac family subgroup includes Rac1, Rac2, Rac3 and RhoG which are thought to be related in function and have been shown to induce lamellipodia (see Section 1.3.2). A third class of RhoGTPases includes Cdc42 and TC10 which induce filopodia (see Section 1.3.2). A fourth class of RhoGTPases include the RhoE related GTPases RhoE, Rho6/Rnd1 and Rho7/Rnd2 which have been shown to reduce actin stress fibres (Guasch et al., 1998; Nobes et al., 1998). A final subclass of RhoGTPases include RhoD and TTF which are thought to be distinct from the other proteins and have been implicated in vesicular transport (Murphy et al., 1996). There are many more family members rapidly being identified (Ellis & Mellor, 2000), their function and role within the cell however remain poorly understood. Although members within these subgroups share similar cellular function they can also differ as a result of post-translational modification, such as alterations in lipid association (Adamson et al., 1992), which may regulate their subcellular location within the cell thereby serving to facilitate unique roles between members. Similarly, expression levels can also vary between cell types and family members, again adding to the diversity between members (Fritz et al., 1995; Hunter & Cooper, 1985; Jahner & Hunter, 1991).

1.3.1 Regulation of RhoGTPases

The Rho family GTPases are regulated in a similar manner as Ras family GTPases, serving as molecular switches by cycling between an inactive (GDP)-bound form and an active (GTP)-bound form, which in turn controls a number of cellular processes described above (Figure 4). The ratio of GTP bound (active) or GDP bound (inactive) RhoGTPases, is determined by three distinct regulatory proteins which include guanine nucleotide exchange factors (GEFs), which enhance the exchange of bound GDP for GTP (Ridley, 1996; Whitehead et al., 1997); the GTPase activating proteins (GAPs), which serve as negative regulators of Rho family GTPases by increasing the rate of intrinsic hydrolysis of bound GTP (Cerione & Zheng, 1996); and guanine nucleotide dissociation inhibitors (GDIs), which inhibit both GTP exchange and the hydrolysis of GTP (Ueda et al., 1990a).

There has been a great increase in the number of GEFs discovered recently, the individual role of many in RhoGTPase signalling however, has yet to be fully established due to their cell type-specific expression and altered specificity for different RhoGTPases (Adra et al., 1997; Zheng et al., 1996). GEFs share a tandem motif of Dbl homology (DH) domains (Cerione & Zheng, 1996), which is essential and sufficient for guanine exchange activity (Hart et al., 1992) and a pleckstrin homology (PH) domain, which is involved in intracellular localisation of activated GEFs via specific lipid or protein-protein interactions (Zheng et al., 1996). There are a number of Rho family specific GEFs such as Lsc for RhoA, Tiam for Rac1 and Fgd1 for Cdc42 (Glaven et al., 1996; Habets et al., 1994; Olson, 1996), while others display activity towards many Rho family proteins, such as Vav which activates both RhoA and Rac1 (Crespo et al., 1997). Many GEFs have domains other than the DH and PH domains including SH3 domains, which suggests functions other than GEF activity (Cerione & Zheng, 1996). In this regard several GEFs have been reported to have effector functions such as PI3-kinase which via Vav may also act as an effector for Cdc42 during the hierarchical cascade between Rho family members (Zheng et al., 1994a) (see Section 1.4.3).

In a similar manner many GAPs have been discovered with differences in their Rho family protein specificity (Ellis et al., 1990; Ridley et al., 1993). For example, p122RhoGAP has specific activity towards RhoA (Homma & Emori, 1995), while α-chimerin (Leung et al., 1993) and Ralbp1 (Cantor et al., 1995) demonstrate specific activity towards Rac1 and Cdc42 respectively. Others however have a more promiscuous nature and effect a number of Rho family proteins, such as Abr, which has activity towards both Rac and Cdc42 (Tan et al., 1993). Moreover GAPs have been documented as having downstream effector functions as well as increasing the hydrolysis of GTP. For example expression of N-and β-chimerin have been documented as inducing lamellipodia and filopodia (see Section 1.3.2) (Kozma et al., 1996).

Interestingly as well as maintaining the GDP-bound state of RhoGTPases by inhibiting both GTP exchange (Ueda et al., 1990b), RhoGDIs have a role in the spatial regulation of RhoGTPases. Active RhoGTPases are found associated with the cell membrane however RhoGDIs partially control their effects within the cell by maintaining them in the cytosol in the inactive state as opposed to the membrane (Isomura et al., 1991). RhoGDIs associate with GDP-bound Rho in the cytosol and upon activation it has been reported that Rho is released from RhoGDIs with the subsequent translocation of Rho to the membrane (Takahashi et al., 1997). RhoGDIs can also bind to GTP-bound Rho family proteins and serve to reduce the intrinsic or GAP-induced GTPase activity and hence protect the

activated state of RhoGTPases (Hart et al., 1992). Until more recently the molecular mechanism underlying the dissociation of GDIs was unclear. However, RhoGDI dissociating factors (GDFs) such as the ERM (ezrin, radixin and moesin) family protein family have been implicated in this process (Hart et al., 1992; Tsukita et al., 1994). Radixin for example has been shown to bind to the RhoGDI/RhoGDP complex and induce dissociation thereby enhancing the ability of GEFs to induce GDP/GTP exchange (Takahashi et al., 1997).

It is clear that the role of individual GEFs, GAPs or GDIs can greatly influence many cellular processes via their control of RhoGTPases, although our understanding of their role in individual processes remains to be fully established. More is known however about the role of individual RhoGTPases in regulating the actin cytoskeleton and structures during various processes (Hall, 1998).

Figure 4.

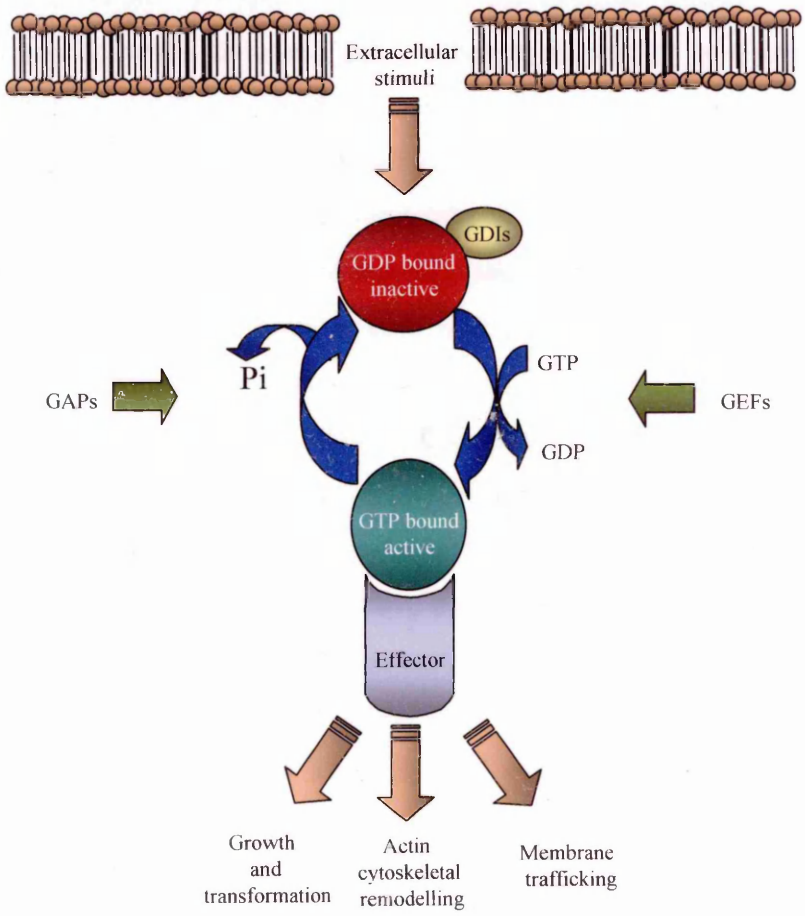


Figure 4. Regulation of RhoGTPases. RhoGTPases act as molecular switches by cycling between an inactive GDP-bound form and an active GTP-bound form. This is regulated by a number of different proteins including GEFs, GAPs and GDIs. In the GTP bound state RhoGTPases can interact with their effector or target proteins and play a role in a diverse spectrum of cellular processes including actin remodelling.

1.3.2 RhoGTPases act as a link between extracellular stimuli and the remodelling of the actin cytoskeleton

Several investigations have implicated the Rho family of small GTPases in linking external stimuli to the assembly and remodelling of the actin cytoskeleton. This was initially explored in Swiss 3T3 fibroblasts where the external stimulus, lysophosphatidic acid (LPA) induced stress fibre and focal adhesion formation in quiescent Swiss 3T3 cells (Ridley & Hall, 1992). Stress fibres consist of bundled contractile actin-myosin filaments that traverse the cell and provide cell attachment via focal adhesions to the extracellular matrix (Figure 5) (Chrzanowska-Wodnicka & Burridge, 1996). Focal adhesions can be defined as points of contact between cells and their extracellular environment, consisting of clustered integrins and proteins such as α-actinin, vinculin, paxillin cortactin and FAK that link the extracellular matrix to the actin cytoskeleton (Nobes & Hall, 1995; Schoenwaelder & Burridge, 1999). This stress fibre formation induced by LPA was ablated by the addition of C3-transferase which ADP-rybosylates and inactivates RhoA, B and C (Aktories & Hall, 1989; Narumiya et al., 1988; Ridley & Hall, 1992). Furthermore experiments using constitutively active and dominant negative RhoA proteins implicated RhoA in this process (Figure 5) (see section 3.2.4) (Paterson et al., 1990; Ridley & Hall, 1992; Ridley et al., 1992). Subsequent investigations demonstrated that RhoA links other extracellular stimuli to actin assembly and organisation to form stress fibres, such as sphingosine-1-phosphate and thrombin (Jalink et al., 1994; Postma et al., 1996; Seufferlein & Rozengurt, 1995).

In similar experiments, PDGF was reported to induce actin polymerisation at the cell periphery resulting in the formation of lamellipodia (Nobes & Hall, 1995; Ridley et al., 1992). Lamellipodia consist of rapidly turning over protrusive sheets of cross-linked actin filaments at the cell periphery, which develop between finger like protrusion known as filopodia (Figure 5). The formation of lamellipodia was blocked in cells expressing dominant negative Rac1 (Nobes & Hall, 1995; Ridley et al., 1992). Furthermore, microinjection of constitutively active Rac1, induced lamellipodia formation (Ridley et al., 1992), therefore establishing that Rac1 was responsible for relaying extracellular stimuli from the cell membrane to cause localised and co-ordinated actin rearrangements to form lamellipodia (Figure 5) (Ridley et al., 1992). Other growth factors such as insulin, EGF and bombesin have been reported to induce lamellipodia by a Rac1-dependent mechanism in Swiss 3T3 and other cell types (Rodriguez-Fernandez & Rozengurt, 1996).

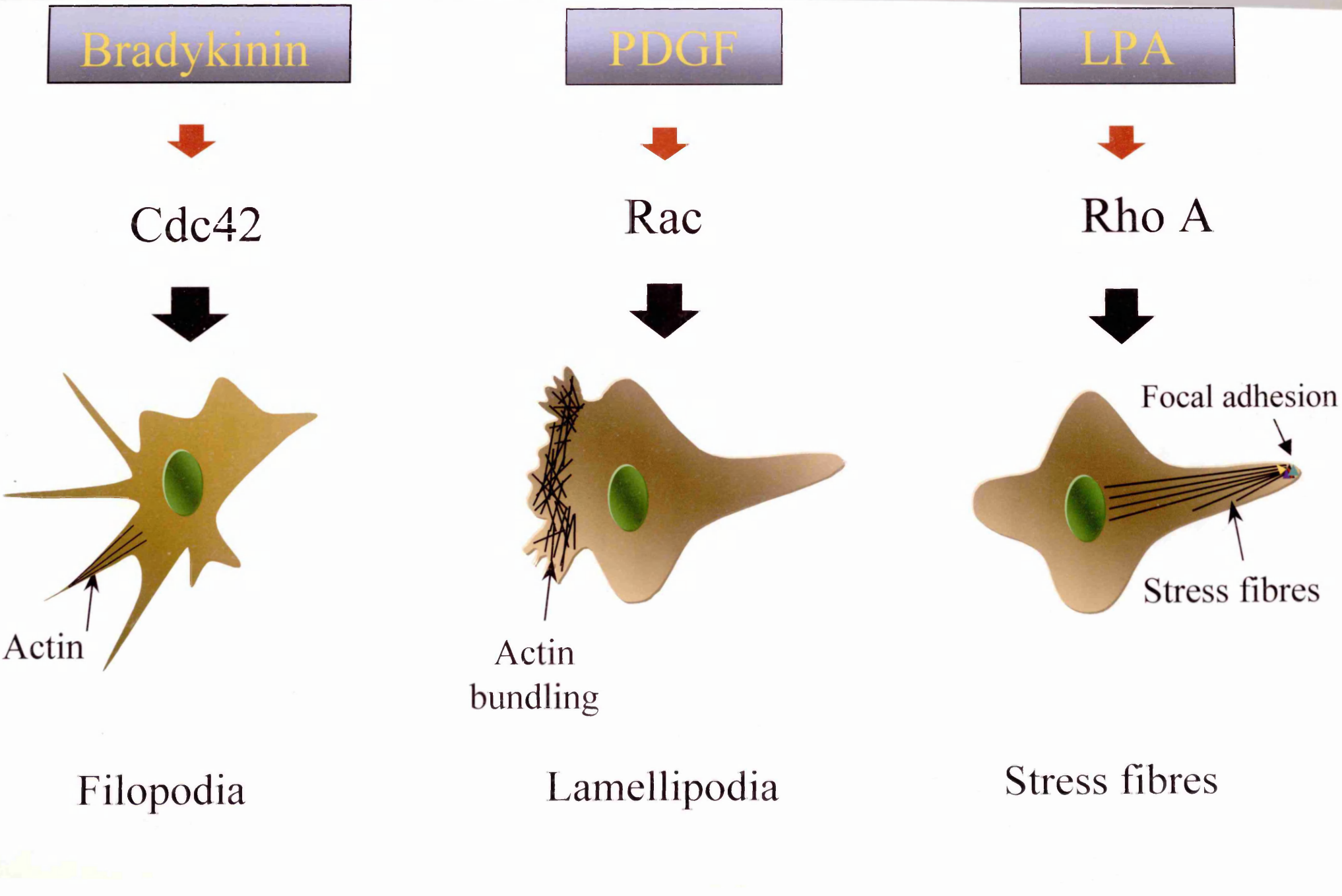
Similarly, Bradykinin has been reported to induce actin polymerisation at the cell periphery resulting in the formation of filopodia (Kozma et al., 1995; Nobes & Hall, 1995). Filopodia, are finger like, transient structures protruding from the cell periphery, consisting of thin bundled actin filaments (Figure 5). Filopodia production was demonstrated to be a Cdc42 dependent mechanism as dominant negative Cdc42 ablated filopodia production while constitutively active Cdc42 caused induction of filopodia (Figure 5) (Kozma et al., 1995; Nobes & Hall, 1995). Subsequent studies led to the discovery that bombesin stimulation is also linked to the rearrangement of the actin cytoskeleton in a Cdc42-dependent manner to induce filopodia formation (Rodriguez-Fernandez & Rozengurt, 1996).

figure5ledend

Figure 5

Schematic representation of the specific morphological changes induced by activation of the RhoGTPases in Swiss 3T3 cells. Bradykinin activates Cdc42 to induce filopodia formation, while PDGF and LPA activate Rac1 or RhoA to form lamellipodia or stress fibres respectively.

Figure 5.


Bradykinin
PDGF
LPA
Cdc42
Rac
Rho A
Actin
Actin bundling
Focal adhesion
Stress fibres
Filopodia
Lamellipodia
Stress fibres

1.3.3 Link between extracellular signalling and RhoGTPases

Investigations were carried out to determine how LPA, PDGF and bradykinin transduce signals to Rho family proteins resulting in organisation of the actin cytoskeleton. The manner in which LPA activates RhoA to create stress fibres, appears to involve a tyrosine kinase, as LPA, but not microinjection of constitutively active RhoA-induced stress fibres can be blocked by the tyrosine kinase inhibitor, tyrophostin (Nobes et al., 1995). Thus a tyrosine kinase may be acting upstream of RhoA during LPA-induced stress fibre formation. It has been proposed that the tyrosine upstream of RhoA maybe involved in RhoA activation by altering the activity of RhoGEFs although not know yet. This type of regulation however has been documented for the Rac1 GEF, Vav, which has been reported to be regulated by tyrosine phosphorylation via Lck and Sck (Gulbins et al., 1993; Han et al., 1997; Miranti et al., 1998). Furthermore, as the LPA receptor is a member of the hetrotrimeric G-protein coupled receptor family (GPCRs), GPCRs were potential mediators in relaying extracellular signals to the respective RhoGTPase. Consistent with this idea, microinjection of activated G protein sub-units of the LPA receptor, Gα13 and Gα12, induced stress fibres formation in Swiss 3T3 cell (Buhl et al., 1995; Hooley et al., 1996). Subsequently a direct link between Gα13 and RhoA has been established in which the α sub-unit, Gα13, interacts with and activates the RhoGEF, Lsc/p115 RhoGEF (Figure 6) leading to RhoA activation and stress fibres formation (Gohla et al., 1998; Hall, 1998; Hart et al., 1998; Kozasa et al., 1998). PDZ RhoGEF also act as a direct target of Gα 12/13 to induced RhoA activation (Figure 6) (Fukuhara et al., 1999). Finally it has also been shown that the Gα13 subunit associates with the tyrosine kinase PYK2 to induce RhoA activation, while the Gβγ subunit which normally induces stress fibre disruption has been reported to play a role in signalling to RhoA to induce stress fibre formation in epithelial-like HeLa cells (Figure 6)(Shi et al., 2000; Ueda et al., 2000).

The bradykinin receptor is also a member of the GPCR family and a similar mechanism has been proposed as a potential link between the bradykinin receptor and Cdc42, however this has yet to be established. It has also previously been reported that during the pheromone signalling pathway in yeast, the Dbl related family protein Cdc24 acts upstream of Cdc42 activation (Zhao et al., 1995; Zheng et al., 1994b). Similarly, microinjection experiments have demonstrated that the GEF, Fgd1, specifically induces Cdc42 activation and filopodial extensions (Figure 6) (Olson et al., 1996).

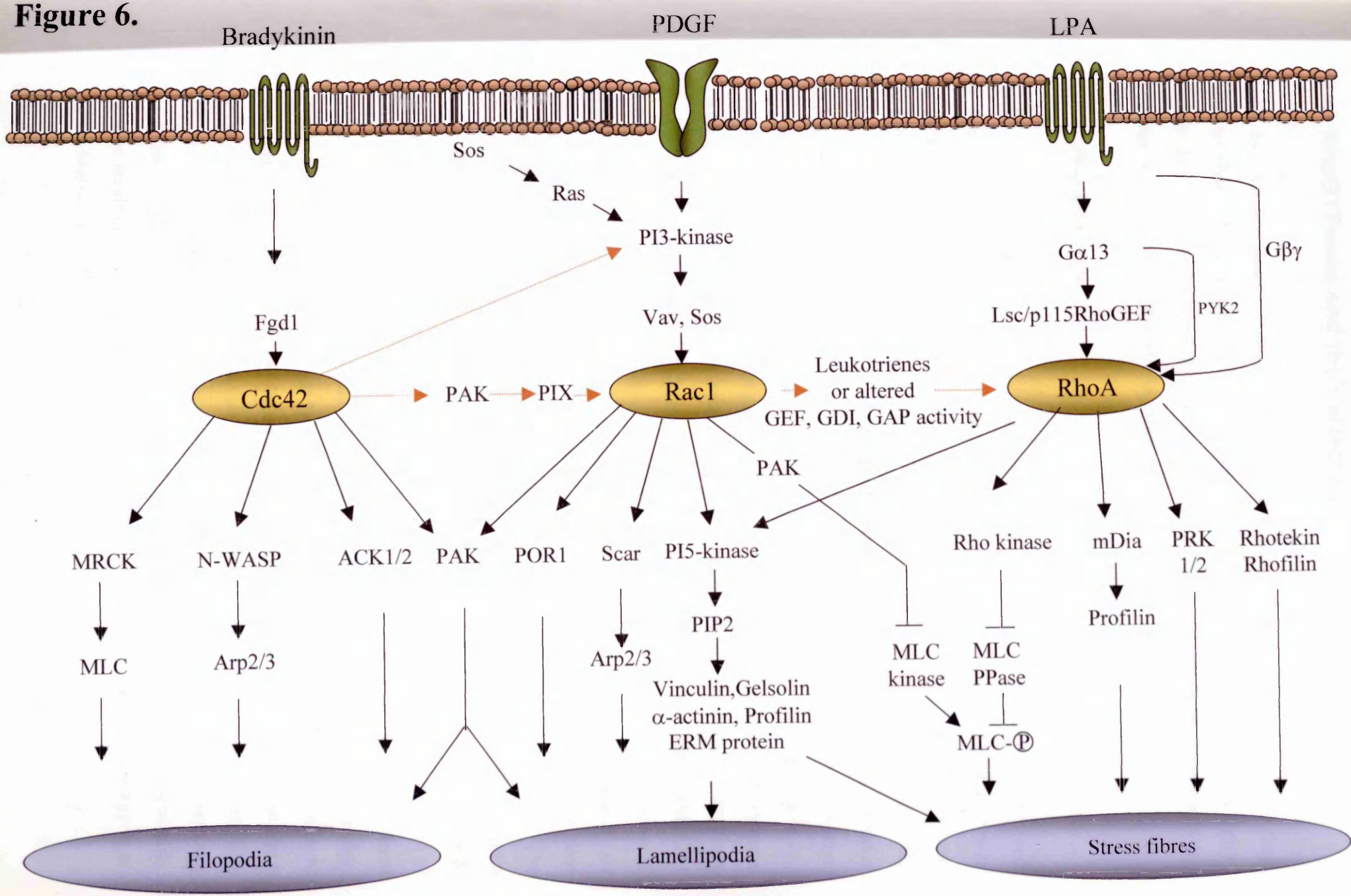
A number of studies have proposed that PI3-kinase is involved in PDGF cytoskeletal remodelling during lamellipodia formation (Nobes et al., 1995; Wennstrom et al., 1994). This originates from studies using the PI3-kinase inhibitor wortmannin (Nobes et al., 1995), which inhibits PDGF induced lamellipodia but not lamellipodia induced by microinjection of constitutively active Rac1, indicating that PI3-kinase is acting upstream of Rac1 in this process (Figure 6) (Nobes et al., 1995; Wennstrom et al., 1994). Following this, it was demonstrated that constitutively active PI3-kinase could cause lamellipodia formation in a Rac1 dependent manner (Reif et al., 1996). The mechanism of PI3-kinase induced Rac1 activation is thought to be mediated by the Rac1 GEF, Vav (Figure 6) (Nobes et al., 1995).

figure6ledgend

Figure 6

Signalling pathways involved in actin cytoskeletal remodelling induced by RhoGTPases. Red dotted lines represent potential cross-talk between RhoGTPases.

Figure 6.


Bradykinin
PDGF
LPA
Sos
Ras
PI3-kinase
Gα13
Gβγ
Fgd1
Vav, Sos
Lsc/p115RhoGEF
PYK2
Cdc42
PAK
PIX
Rac1
Leukotrienes
or altered
GEF, GDI, GAP activity
RhoA
PAK
MRCK
N-WASP
ACK1/2
PAK
POR1
Scar
PI5-kinase
Rho kinase
mDia
PRK
1/2
Rhotekin
Rhofilin
MLC
Arp2/3
Arp2/3
PIP2
Profilin
MLC
kinase
MLC
PPase
Vinculin,Gelsolin
α-actinin, Profilin
ERM protein
MLC-Ⓟ
Filopodia
Lamellipodia
Stress fibres

1.4 RhoGTPases and their effectors.

Over the past few years the characterisation and discovery of a number of proteins that bind to RhoGTPases in the GTP-bound state and act as effectors has provided a major insight into the molecular mechanisms by which RhoGTPases affect the actin cytoskeleton and regulate a variety of cellular processes.

1.4.1 Rho effectors

There are a large number of RhoA-binding proteins identified that are potentially involved in actin stress fibres formation or maintenance. The homologous serine/threonine protein kinases p160 ROCK (also called ROCK-1 or ROKβ) and ROKα (also called Rho-kinase or ROCK Ð) are major downstream effectors of RhoA (Ishizaki et al., 1996; Leung et al., 1995; Matsui et al., 1996). Initial studies demonstrated that expression of ROKα induced stress fibre-like clusters of actin, emanating from the middle of the cell and that expression of a kinase-deficient mutant could disrupt these stress fibre-like structures (Leung et al., 1996). Similar results were subsequently observed for Rho-kinase in other cell systems (Amano et al., 1997). A further understanding of how Rho-kinase functions, stems from the finding that the myosin binding subunit (MBS) of myosin light chain kinase (MLCK) was a Rho-kinase substrate (Kimura et al., 1996; Matsui et al., 1996). Phosphorylation of MBS by Rho-kinase ablates myosin light chain phosphatase activity, resulting in an increase of phosphorylated MLC (Kimura et al., 1996). Moreover, Rho-kinase also directly phosphorylates MLC at the same site as MLCK (Amano et al., 1997). The conformational change induced by phosphorylation increases the binding of MLC to actin filaments and subsequently triggers induction of stress fibres (Chrzanowska-Wodnicka & Burridge, 1996; Fernandez et al., 1990; Lamb et al., 1988). In agreement with a role of MLC phosphorylation in stress fibres formation RhoA-induced stress fibres and focal adhesions could be blocked by MLCK inhibition (Chrzanowska-Wodnicka & Burridge, 1996; Fincham et al., 2000). It has been proposed that a balance between MLCK induced phosphorylation and MLC phosphatase activity partially determines whether stress fibres form (Amano et al., 1996). Furthermore ERM proteins have been implicated in Rho-kinase induced stress fibres formation as Rho-kinase is thought to promote the interaction of ERM proteins with actin and transmembrane receptors by inducing a conformational change resulting in the 'unfolding' of ERM proteins via phosphorylation (Mackay et al., 1997; Matsui et al., 1998).

Other RhoA effector are the diaphanous related formin proteins mDia1/2 proteins which are the mammalian homologue of Bnip in yeast (Kohno et al., 1996; Watanabe et al., 1997). mDia1/2 belong to a family of formin-related proteins, including the *drosophila* diaphanous proteins and share two proline-rich, formin homology domains FH1 and FH2. These proteins have been shown to be involved in many actin based processes including cell migration, cytokinesis and cell morphology. These proteins can interact with the actin monomer binding protein, profilin and selectively bind to activated RhoA (Watanabe et al., 1997). The co-localisation of mDia and profilin at the plasma membrane and at the tips of actin stress fibres has lead to the proposal that mDia1/2 may be involved in the formation and maintenance of stress fibres. It has been suggested that profilin and mDia are involved in this process by localising ATP-bound actin monomers to areas of actin assembly and by interacting with other actin binding proteins such as VASP (Wasserman, 1998; Zigmond, 1996). Moreover, phosphatidylinositol 4,5-bis phosphate (PIP2) production via PI5-kinase can result in the removal of actin capping proteins such as gelsolin (Toker, 1998) and this together with the recruitment of mDia1/2 to these sites has been suggested to be involved in stress fibre formation. This may be necessary for the linkage of actin filaments during stress fibre formation or the long-term maintenance of stress fibres due to the intrinsic turnover of actin filament within the cell (Kirschner, 1980; Wegner, 1976). The role of mDia in RhoA-induced stress fibres has been demonstrated by work in which expression of activated mDia1 causes thin stress fibre-like actin structures, which are disorganised in the absence of Rho-kinase activity (Watanabe et al., 1999). Furthermore the Rho-kinase induced stress fibre structures described above, can be altered by the co-expression of activated mDia1 to form stress fibres that are more reminiscent of RhoA-induced stress fibres (Watanabe et al., 1999). It has therefore been proposed that mDia and Rho-kinase work together to co-ordinate actin stress fibre assembly. Interestingly, mutations in the human homologue hDia encoded by DFNA1 results in deafness, which is thought to be caused be a defect in the actin organisation in hair cells of the inner ear (Lynch et al., 1997).

While Rho-kinase, as described earlier, serves in the maintenance of MLC contractility which is required for RhoA-induced stress fibres, expression of Rho-kinase induces stress fibre-like structures are disorganised and form in clusters from the centre of the cell (Leung et al., 1996) (Amano et al., 1997; Watanabe et al., 1999). Recently, this was shown to be due to the spatial regulation of MLC phosphorylation by Rho-kinase (Totsukawa et al., 2000). It was shown that in the centre of the cell, Rho-kinase acts to phosphorylate MLC and inhibit MLC phosphatase activity, while at the cell periphery it acts only to inhibit MLC phosphatase activity, where MLCK phosphorylation of MLC can induce stress fibre

formation. Thus the spatial regulation of RhoA effectors or actin binding proteins may serve to regulate the actin cytoskeleton during RhoA-induced stress fibre formation. Interestingly, mDia binds to Src's SH3 domain via the FH1 domain of mDia and inhibition of Src/mDia binding blocks the co-operative effects of mDia and Rho-kinase, suggesting a role for Src and mDia in RhoA-induced processes (see Section 1.6.2) (Tominaga et al., 2000).

As the Rho-kinase and mDia induced actin fibres were not identical to RhoA-induced stress fibres, it was proposed that other effectors may be involved in stress fibre formation (Hall, 1998; Sahai et al., 1998; Watanabe et al., 1999). PIP2 production by RhoA-induced PI5-kinase activity has profound effects on the actin cytoskeleton by regulating various actin binding proteins and controlling actin polymerisation (Figure 6) (Toker, 1998). In this regard, it is interesting that microinjection of an anti-PIP2 antibody ablates LPA or RhoA-induced stress fibre and focal adhesion formation suggesting that PI5-kinase has a role in the assembly of stress fibres (Gilmore & Burridge, 1996). This suggests that PIP2 induced control of actin polymerisation may play a role in actin stress fibre formation. Interestingly, work by Machesky and Hall indicated that actin polymerisation although not ultimately responsible for stress fibres formation, is required for the long-term maintenance of RhoA-induced stress fibres (Machesky & Hall, 1997).

In addition to Rho-kinase, mDia1/2 and PI5-kinase many other RhoA targets have been identified as potential effectors involved in RhoA-induced stress fibre formation. These include PRK1 (also known as PKN) and PRK2, which are both serine/threonine kinases, related to PKC (Amano et al., 1996; Palmer et al., 1995; Watanabe et al., 1996) having a broad spectrum of expression. PRK1 was thought to have a role in stress fibre formation as staurosporine, a potent inhibitor of PKC and possibly PRK1, blocks focal adhesion formation (Mosch et al., 1996). Moreover LPA stimulation results in the RhoA-dependent activation of PRK1 (Watanabe et al., 1996). Further experiments illustrate that PRK1 can bind to α-actinin, which provides a link between PRK1 and the actin cytoskeleton (Mukai et al., 1997). Similarly PRK2 was initially reported to bind to activated RhoA and Rac1 (Quilliam et al., 1996; Vincent & Settleman, 1997). The expression of a kinase-defective PRK2 caused disruption of actin stress fibres in NIH 3T3 cells thereby suggesting that PRK2 may serve as a Rac1/RhoA effector, inducing the re-modelling of the actin cytoskeleton (Vincent & Settleman, 1997). Rhotekin and Rhophilin are two additional Rho effectors that lack a kinase domain but share homology with PRKs in the Rho-binding domain (Reid et al., 1996; Watanabe et al., 1996). Both interact with GTP-bound RhoA but their cellular function has yet to be fully established. One possibility is that they

contain other putative binding domains such as PH and protein-protein interacting domains which may serve as docking regions or serve a purpose in the localisation of other effectors during RhoA induced actin re-modelling. No one effector, to date, seems capable of inducing stress fibres reminiscent of those caused by RhoA and therefore it is more likely that a combination of effectors work concurrently to form stress fibres.

1.4.2 Cdc42 and Rac effectors.

Rho family protein effectors have a number of consensus binding motifs including a minimal region of approximately 16-18 residues which has been identified as being required for the binding of Cdc42 or Rac1, called the CRIB motif (Cdc42 /Rac1 interactive binding motif) (Burbelo et al., 1995). Both Cdc42 and Rac1 bind to some common effector which do not bind to RhoA as a result of the sequence homology between Cdc42 and Rac1. Using a computer based search a number of Cdc42/Rac1-binding proteins were identified that contain the CRIB domain including the Wiskott-Aldrich syndrome protein (WASP) (Figure 6). WASP binds to GTP-bound Cdc42 and is preferentially expressed in hematopoietic cells. It is thought to be a major component in Cdc42-induced actin rearrangements, as WASP induced actin polymerisation is inhibited by expression of dominant negative Cdc42 (Ramesh et al., 1999; Symons et al., 1996). WAS patients have thrombocytopenia, which is thought to be due to abnormalities in T and B cell function as a result of impaired Cdc42 function (Kolluri et al., 1996; Snapper et al., 1998; Zicha et al., 1998). Cdc42 is thought to be essential for T-cell polarity towards antigen presenting cells and WASP-deficient cells display both impaired cell migration and polarity in this process (Snapper et al., 1998; Stowers et al., 1995; Zicha et al., 1998). N-WASP, the ubiquitously expressed form of WASP also serves a similar function in other non-hematopoietic cell types (Miki et al., 1996). Over-expression of N-WASP induces filopodia and has been proposed to be involved in bradykinin-induced filopodia formation as an effector of Cdc42 (Miki et al., 1996; Miki et al., 1998a). Both WASP and N-WASP bind to F-actin and profilin as well as the Arp2/3 complex. The Arp2/3 complex consists of seven components including Arp2, Arp3, p41-Arc, p34-Arc, p21-Arc, p20-Arc and p16-Arc resulting in the stimulation of actin nucleation and polymerisation to allow actin remodelling to occur (Figure 6) (Machesky et al., 1997; Miki et al., 1998a; Rohatgi et al., 1999). Furthermore, N-WASP and WASP have a PH domain which is thought to serve in their localisation to the cell membrane and thereby function at this region of the cell (Rohatgi et al., 1999). Recently further members of the WASP family proteins such as Scar/WAVE, have been shown to be involved in Rac1-dependent cytoskeleton remodelling events, serving to couple Rac1 activity to the Arp2/3 complex and thereby actin nucleation and lamellipodia

formation (Machesky et al., 1997; Miki et al., 1998a). Various other proteins have been implicated in Cdc42 cytoskeleton re-organisation, including the non receptor tyrosine kinase, ACK and myotonic dystrophy kinase-related Cdc42 binding kinase (MRCK). Both ACK-1 and ACK-2 have been reported to bind to activated Cdc42 and can be phosphorylated as a results of bradykinin and EGF stimulation, implying that ACK may mediate the actions of Cdc42 (Kato-Stankiewicz et al., 2001; Yang & Cerione, 1997). MRCK has both a PH and a CRIB domain and over-expression of MRCK reflects Cdc42 induced filopodia, while expression of kinase-dead MRCK blocks filopodia formation (Leung et al., 1998). Moreover, MRCK, like Rho-kinase (see Section 1.4.1) preferentially phosphorylates MLC at serine-19 in vitro (Leung et al., 1998) and it has been suggested that this regulates, in part, an acto-myosin contractile force at the site of filopodia formation.

Amongst the Cdc42 and Rac1-binding protein that have been identified, p21 activated kinases (PAK1-5) have attracted a great deal of attention (Figure 6) (Manser et al., 1994; Martin et al., 1995a; Sells et al., 1997). PAK possesses a CRIB motif and an SH3 domain, and in general bind to Rac or Cdc42 in a GTP-dependent manner inducing PAK re-localisation and PAK kinase activity. Expression of activated PAK induces remodelling of the actin cytoskeleton in a similar manner as activated Cdc42 or Rac1 (Manser et al., 1997; Sells et al., 1997). Moreover, expression of dominant negative PAK blocks Cdc42 or Rac1 induced actin rearrangements implicating PAK as a downstream effector of Cdc42 and Rac1 (Obermeier et al., 1998). More recent studies indicate that there may be isoform and cell type specific differences in PAK signalling with regards to Cdc42 and Rac1-induced actin rearrangements. For example, PAK3 induced effects on the actin cytoskeleton can be inhibited by dominant negative Rac1 in PC12 cells, while PAK1 seems to induce its effect on the actin cytoskeleton in a Rac1 and kinase-independent manner in fibroblasts (Obermeier et al., 1998; Zhao et al., 1998). Moreover, PAK4 a novel effector of Cdc42, only binds to Cdc42 and has been implicated in Cdc42-induced actin remodelling to form filopodia (Abo et al., 1998), while the brain-specific PAK isoform PAK5, like Cdc42, promotes neurite outgrowth in N1E-115 cells (Dan et al., 2002). Recently, cortactin, which has actin cross-linking activity, is recruited to sites of cortical actin polymerisation in a PAK-dependent manner (Weed et al., 2000). Furthermore, PAK phosphorylates LIM-kinase, which phosphorylates and inactivates the actin depolymerising protein cofilin thereby promoting the stabilisation of actin filaments and providing a mechanism by which PAK can indirectly influence the formation of cytoskeletal strucutures such as filopodia or lamellipodia (Daniels & Bokoch, 1999). Interestingly expression of mutant forms of Cdc42 or Rac which cannot bind to PAK can still induce filopodia or lamellipodia

respectively indicating that PAK may not be involved in Cdc42 or Rac1-induced actin rearrangements (Lamarche et al., 1996; Westwick et al., 1997). Further studies demonstrate that GTPase binding-deficient mutants of PAK can still induce actin remodelling, thereby arguing against the requirement of PAK kinase activity and GTPase binding of PAK during PAK induced actin remodelling (Obermeier et al., 1998). These paradoxical results regarding PAK's involvement in Rac1 or Cdc42-induced actin rearrangements may be partially explained by the involvement of an SH3 domain binding protein. As stated above, PAKs also contain a SH3 domain and it has been postulated that the actin remodelling induced by PAK may in some cases be mediated by the SH3 domain at the N-terminal of PAK, rather than via the CRIB motif (Manser et al., 1997; Sells et al., 1997). PIX (PAK interacting exchange factor) was able to cause lamellipodia formation by interacting with the SH3 domain of PAK and acts as a Rac1-specific GEF thereby providing a GTPase binding and kinase independent pathway for PAK induced actin remodelling (Obermeier et al., 1998; Sells et al., 1999), which may help explain the contradictory results described above regarding PAK's role in Rac1 or Cdc42 induced actin remodelling.

Rac1 and Cdc42 -induced actin remodelling not only involves the formation of lamellipodia or filopodia respectively but is also associated with the disassembly of RhoA-induced stress fibres and focal adhesions. Cross-talk between RhoGTPases is thought to be involved in this process and is described in more detail later (see Section1.4.3) . In this regard, PAK is thought to not only be involved in the formation of actin structures induced by Cdc42 or Rac1 but may also play a key role in the disassembly of stress fibres and focal adhesions associated with Rac1 and Cdc42 (see section 1.4.3). Rac1 and Cdc42 -induced stress fibre and focal adhesion disruption is thought to be achieved via PAK induced phosphorylation and inhibition of MLCK, which results in the inhibition of MLC phosphorylation and the subsequent loss of actin stress fibres contraction (Figure 6) (Frost et al., 1998; Sanders et al., 1999). PAK is therefore thought to have an effector and antagonistic role in Rac1 and Cdc42 induced actin remodelling.

With respect to the induction of lamellipodia, several other effectors have been implicated including POR (partner of Rac), which was identified in a yeast-two hybrid screen (Van Aelst et al., 1996). POR interacts with activated Rac1 but not Cdc42 or RhoA. Consistent with a role for POR in lamellipodia production, mutants of POR1 blocked Rac1-induced lamellipodia. Moreover Ras induced lamellipodia formation was synergistically enhanced by co-expression of POR. Finally mutants of Rac1 that do not bind to POR block lamellipodia formation (Joneson et al., 1996).

Another effector likely to play a key role in actin re-modelling and polymerisation is PI5-kinase, which binds to both Rac1 and RhoA and has been reported to be regulated by these Rho family proteins (Figure 6) (Hartwig et al., 1995; Ren et al., 1996). It has been demonstrated that Rac1 induces the uncapping of barbed ends of actin filaments, a prerequisite for actin re-modelling and polymerisation associated with lamellipodia (Hartwig et al., 1995). Furthermore this was shown to be enhanced by PI5-kinase induced PIP2 production (Hartwig et al., 1995). PIP2 has been shown to alter the folding and activity of many scaffolding and actin binding proteins such as α-actinin, ERM proteins, profilin, vinculin and WASP (Gilmore & Burridge, 1996; Rohatgi et al., 1999; Toker, 1998). For example, PIP2 binds and results in the unfolding of vinculin allowing it to associate with talin, which in turn can bind to the actin cytoskeleton (Gilmore & Burridge, 1996). PIP2 also increases α-actinin cross-linking activity and enhances uncapping of actin filaments from gelsolin (Toker, 1998), while profilin induces dissociation of actin monomers. Moreover activation of ERM proteins which serve as key regulators of both Rac1 and Rho-induced actin re-modelling, suggests that PI5-kinase-induced regulation of ERM proteins provides some role in lamellipodia and for that matter stress fibre formation (Gilmore & Romer, 1996; Mackay et al., 1997; Tsukita et al., 1994; Tsukita & Yonemura, 1997).

1.4.3 Cross-talk between RhoGTPases

As well as having individual roles in actin organisation there has been a great deal of evidence demonstrating cross talk between Rho family GTPases. Work in Swiss 3T3 cells in particular has suggested that they can act in concert forming a hierarchical cascade that links their activities, such that Cdc42 can activate Rac1 which in turn can activate RhoA (Figure 6) (Nobes & Hall, 1995). Expression of constitutively active Cdc42 results in the formation of filopodia as well as Rac1 induced lamellipodia (Kozma et al., 1995; Nobes & Hall, 1995). Furthermore dominant negative Cdc42 blocks bradykinin induced filopodia and lamellipodia, while dominant negative Rac1 only blocks lamellipodia (Nobes & Hall, 1995). On a similar note activated Rac1 causes lamellipodia and RhoA-induced stress fibres, while dominant negative Rac1 blocks PDGF-induced lamellipodia and stress fibres, but dominant negative RhoA only blocks stress fibre formation (Nobes et al., 1995). Furthermore microinjection of constitutively active Cdc42, while blocking Rac1 by co-expressing dominant negative Rac1, or blocking RhoA, using C3-transferase (Narumiya et al., 1988), enhanced the rate of filopodia production in both cases, demonstrating a hierarchical cascade from Cdc42>Rac1>RhoA (Nobes & Hall, 1995). This has also been demonstrated in neuroblastoma cells, where inactivation of RhoA, leads to increased

activation of Cdc42 and Rac1 (Kozma et al., 1997). Similar hierarchical cascades have been observed in other cell types such as macrophages in relation to cell migration (Allen et al., 1997) as well as between other Rho family members. For example, RhoG-induced lamellipodia have been reported to form via a Rac1-dependent mechanism (Roux et al., 1997). Cross-talk has also been reported to occur between small G protein family subgroups such that Ras can activate Rac1 to induce lamellipodia and subsequently cause RhoA-induced stress fibres (Figure 6) (Fan et al., 1998; Hall, 1998; Nimnual et al., 1998).

The precise nature of how these GTPases relay signals to each other is poorly understood. The versatility of GEFs, GDIs and GAPs in controlling RhoGTPases and their ability to act as effectors along with their promiscuous nature, implicates their involvement in RhoGTPase cross-talk. Evidence for this potential link comes from a number of different sources including, the regulation and link between Cdc42 and Rac1. In particular a mechanism for Cdc42-induced activation of Rac1 has been proposed in which the Cdc42 effector PAK (see Section 1.4.2) regulates Rac1 by increasing the GEF activity of PIX for Rac1 (Figure 6) (Manser et al., 1998; Obermeier et al., 1998). Similarly, PI3-kinase could serve as an effector of Cdc42 and activate Rac1 via phophatidyl inositol- 4,5 bisphosphate PIP3-induced GEF activity towards Rac1 (Figure 6) (Zheng et al., 1994a). Leukotrienes have also been proposed to be involved in the cross-talk between Rho family proteins, where EGF or Rac-induced arachidonic acid production subsequently leads to the generation of leukotrienes which are sufficient for RhoA activity and stress fibre formation (Figure 6) (Peppelenbosch et al., 1995).

Even though a hierarchical cascade exists in Swiss 3T3 and other cell types, variations of this theme have been documented involving separate pathways or antagonistic behaviours between RhoGTPases. Cdc42 and Rac1 have been noted to disrupt both stress fibres and focal adhesions. The antagonism between Rac1 and RhoA has been demonstrated in both NIH3T3 and neuronal cells where Rac1 activity is able to downregulate RhoA activity (Leeuwen et al., 1997; Sander et al., 1999). In particular over-expression of Tiam1, a Rac1 GEF, or PDGF-induced Rac1 activation, impaired RhoA-induced actin rearrangements in NIH3T3 cells (Sander et al., 1999). Moreover, downregulation of RhoA via Rac1 activation impairs their migratory phenotype and behaviour (Sander et al., 1999). In the same study, over-expression of constitutively active RhoA restored RhoA activity and resulted in the reversion of the epithelial-like morphology and behaviour induced by Rac1 activation (Sander et al., 1999). Similarly, in further studies down regulation of Rac1 activity has been reported to cause an increase in RhoA activity which was accompanied by an epithelial to mesenchymal transition in cell morphology (Zondag et al., 2000).

Interestingly, expression of V14RhoA or N19RhoA had no effect on Rac1 activity indicating the hierarchical cascade from Rac1 towards RhoA (Sander et al., 1999). In neuronal cells, neurite retraction and cell rounding, both of which are known RhoA-induced effects, were blocked by Rac1 activation and conversely, Rac1 induced neurite formation has been shown to be enhanced by RhoA inactivation (Leeuwen et al., 1997). Moreover, Cdc42-induced filopodia have also been observed to form at the expense of RhoA-induced stress fibres (Dan et al., 2002; Kozma et al., 1995; Sander et al., 1999) (Manser et al., 1997; Qiu et al., 1997).

It has been suggested that a reduction in GEF activity towards RhoA induced by Cdc42 or Rac1 could influence RhoA activity (Zohn et al., 1998). Similarly, alterations in both Rho GAP or GDI activity could be involved in the down regulation of RhoA. Interestingly CIP4, which was discovered as a Cdc42-binding protein has sequence homology with a number of RhoGAPs and causes a breakdown of stress fibres (Aspenstrom, 1997). CIP4 does not induce filopodia and it has therefore been proposed that some effectors may serve in the formation of morphological actin structures while other act as antagonists towards other RhoGTPases during this process (Aspenstrom, 1997). Others propose that the antagonism is a product of competition between family members for a common pool of actin, actin binding partners or effectors. In this regard it has been suggested that the Cdc42 and Rac1 effector PAK may play a role in this antagonistic process. Expression of constitutively active PAK induces stress fibre and focal adhesion breakdown (Frost et al., 1998) (Sanders et al., 1999; Zhao et al., 1998) which is thought to occur due to PAK induced MLCK phosphorylation leading to inhibition of MLC phosphorylation, contractility of actin-myosin and therefore inhibition of stress fibre formation (Figure 6). Others have shown the antagonism between RhoGTPases to occur further upstream, as down regulation of RhoA in NIH3T3 cells by Rac1 was shown to occur independently of Rac1 induced cytoskeletal rearrangements. Rac1 mutants that were defective in mediating cytoskeletal changes could still down regulate RhoA, suggesting that the antagonism observed here between Rac1 and RhoA occurs at the Rho family GTPase level (Sander et al., 1999; Zondag et al., 2000). Furthermore, evidence of antagonism between other Rho family members and RhoA has been documented to occur, for example, activated Rad1 and RhoE/Rad3 have been shown to cause stress fibre disruption (Guasch et al., 1998; Nobes et al., 1998). It has also been proposed that effectors can act as negative regulators of RhoGTPases in a negative feedback loop. One such effector, p116(Rip) identified as a RhoA-binding protein has similar effects as dominant negative RhoA or C3-transferase, indicating not only that RhoGTPase-induced effectors can influence the activity of other family members but that they can act in a negative feedback loop to reduce the effect of

their respective RhoGTPases (Gebbink et al., 1997). Co-ordination between family members has also been reported to occur in controlling other cytoskeletal based processes such as the co-operation between Rac1 and RhoA in the formation of cadherin mediated cell-cell junctions (Braga et al., 1997; Hordijk et al., 1997; Kuroda et al., 1998; Takaishi et al., 1997). The cross-talk and co-operation between family members during processes such as cell migration will be discussed in more detail below.

1.4.4 Cross-talk between small family subgroups

A similar scenario of cross-talk has been proposed to be involved in cross talk between small G protein family subgroups. As stated earlier many GEF, GAPs and GDIs have a variety of functions that could potentially lead to cross talk between GTPases. It has been shown for example that Ras can activate Rac1 via the Ras-induced effector PI3-kinase, which enhances the activity of the Rac1 GEF Vav, by p56 Lck-induced tyrosine phosphorylation. Which increases GDP/GTP exchange activity of Vav and thereby enhances Vav induced Rac1 activity (Crespo et al., 1997; Han et al., 1997; Han et al., 1998). Furthermore Ras is thought to be able to activate Rac1 via the Rac1 exchange factor activity of Sos (Nimnual et al., 1998). The PH domain of Sos normally inhibits its Rac1 GEF activity, however binding of the PH domain to PIP3 blocks this inhibitory effect, allowing Sos to have GEF activity for Rac1 (Nimnual et al., 1998). A further indication that this is a likely mechanism for cross-talk is that Sos and other Ras GEFs such as, Ras-GRF and Ras-GRF2 have been reported to have either Cdc42, Rac1 or RhoA GEF DH and PH domains (Fan et al., 1998; Nimnual et al., 1998).

1.5 Cell motility and migration

Cell migration is a key aspect of many cellular functions, including leukocyte migration in response to inflammation, cell migration during development or wound healing, and tumour cell metastasis and invasion (Lauffenburger & Horwitz, 1996). Cell movement has been described as a crawling motion, mediated by the dynamic remodelling of both cell-substratum sites and the actin cytoskeletal network. Here, we focus on how the co-ordinated regulation of the actin cytoskeleton by RhoGTPases contributes to cell migration, acknowledging that other cytoskeletal networks including the microtubule network and intermediate filaments interact and play a role in this process.

1.5.1 A role for Rac1 in cell migration.

Rac is thought to serve a role in cell migration by stimulating the formation of lamellipodia, which initially form weak attachment sites to the substratum referred to as focal complexes. Focal complexes are thought to be rapidly turning over focal adhesions (Schoenwaelder & Burridge, 1999; Small et al., 1995) composed of various actin binding, structural and signalling proteins such as α-actinin, vinculin, paxillin cortactin and FAK which to date have been reported to have the same protein composition as focal adhesions (Nobes & Hall, 1995). The rapid turnover and supply of free actin monomers and scaffolding proteins provides the protrusive force for lamellipodia formation and cell migration (Borisy & Svitkina, 2000). The supply of actin to new adhesion sites at the barbed end of actin filaments pointing towards the leading lamellae is thought to provide a protrusive force and cause extension of the membrane resulting in lamellipodia (Borisy & Svitkina, 2000). At the same time, real time microscopy using GFP-tagged vinculin demonstrated that as cells move small complexes that form at the base of lamellipodia remain static in relation to the cell substratum as the cell migrates forward over them (Figure 7) (Small et al., 1995; Small et al., 1999). It has been postulated that Rac1 mediated focal complex formation at the leading edge provides new immature adhesion sites in the direction of cell locomotion and as the cell moves these focal complexes gradually develop into focal adhesions as a result of their localisation behind the base of lamellipodia. This focal complex to focal adhesion transition is thought to provide an adhesive force required for traction during cell migration at the leading edge, while MLC contraction at the rear induces tail-retraction and cell translocation (Figure 7) (Chrzanowska-Wodnicka & Burridge, 1996; Small et al., 1999; Stossel, 1993). Rac1 induced actin remodelling is thought to be essential for cell migration as cells expressing dominant negative Rac1 fail to form a leading edge and migrate into the wound (Nobes & Hall, 1999).

Similarly the Rac GEF, Tiam1 induces invasion of T-lymphoma cells and produces cell metastasis in nude mice (Habets et al., 1994; Michiels et al., 1995). Others however have shown Tiam1 to block migration of NIH3T3 cells on fibronectin and laminin (Sander et al., 1998). This can be explained by work demonstrating that the ability of Tiam1 to induce or inhibit migration is dependent on the extra cellular matrix (ECM) components present (Mitchison & Cramer, 1996; Sander et al., 1998). For example, on collagen Tiam1 increases migration, while on laminin or fibronectin Tiam1 inhibits cell migration (Sander et al., 1998). Further evidence for Rac1 in migration can be seen as alterations in Rac1 activity increase human breast cell motility and invasion (Keely et al., 1997; Zohn et al.,

1998). Moreover macrophage and fibroblast chemotaxis towards CSF-1 and PDGF has been documented to require Rac1 activity and lamellipodia formation (Allen et al., 1997; Anand-Apte & Zetter, 1997). It should be noted that in cells with focal adhesions and no lamellipodia, subsequent stimulation to form these structures induces the dynamic remodelling of focal adhesions to focal complexes as visualised using GFP tagged paxillin (Laukaitis et al., 2001), which provides the initial lamellipodia and leading edge required for directional migration. It is thought that Rac1 antagonises RhoA activity in cells via PAK (see Section 1.4.3) allowing these membrane extensions to form. It is therefore clear that both focal adhesions and focal complexes are transient structures whose function within the cell is critically governed by RhoGTPases during cell migration.

1.5.2 A role for Cdc42 in cell migration.

Cdc42 serves a role in cell migration by stimulating the formation of protrusive structures known as filopodia (Figure 7) (Hall, 1998; Kozma et al., 1995), which also contain focal complexes at the base and along their length. Filopodia are transient structures and like lamellipodia they are rapidly turning over (Borisy & Svitkina, 2000; Stossel, 1993). The supply of new actin and structural proteins provide the key components to extend the cell membrane and form these vital structures during directed cell migration (Allen et al., 1997; Borisy & Svitkina, 2000). Cdc42-induced filopodia are not essential for cell migration but are required for maintaining cell polarity during fibroblast movement and macrophage chemotaxis (Allen et al., 1997; Allen et al., 1998; Carney & Couve, 1989; Nobes & Hall, 1999). Using a Dunn chamber chemotaxis assay to examine directionality and persistence of cell movement, cells expressing dominant negative Cdc42 fail to maintain directed cell migration towards a chemotactic gradient (Allen et al., 1998). Interestingly, cells expressing dominant negative Cdc42 demonstrated an increase in migration velocity which has been proposed to be as a result of alleviated directionality normally induced by Cdc42 (Allen et al., 1997; Allen et al., 1998). In this regard aberrant activity of Cdc42 has been linked to the increased motility of human breast epithelial cells (Keely et al., 1997). Thus factors other than cell contraction and cell adhesion are involved in controlling cell migration rates. Cdc42 activity has been proposed to direct lamellipodia protrusions induced by Rac1 to the leading edge during wound healing (Nobes & Hall, 1999). This orientation of a leading edge can also be seen in individually migrating fibroblasts where a number of filopodia eventually coalesce to form a leading edge and extend lamellipodia in the direction of cell locomotion (Figure 7). Similarly, in yeast, Cdc42 co-ordinates actin cytoskeleton remodelling and polarisation of budding yeast (Stowers et al., 1995). Interestingly the Cdc42 and Rac1 effector PAK has been demonstrated to be required for

sustained polarisation of cells, as kinase-defective PAK expressing cells form lamellipodia but fail to sustain polarised cell migration (Sells et al., 1999). Finally as discussed in Section 1.4.1, Cdc42 activity is essential for T-cell polarity towards antigen presenting cells and cells deficient in the Cdc42 effector WASP display both impaired migration and polarity during this process (Snapper et al., 1998; Stowers et al., 1995; Zicha et al., 1998).

1.5.3 A role for RhoA in cell migration.

It has been suggested that RhoA may play a role in cell migration via myosin-based bundling of actin filaments into stress fibres and the maintenance of focal adhesions during cell movement. The contractile forces exerted by stress fibres results in the aggregation of integrins and their associated proteins to form focal adhesions which serve to stabilise adhesions and allow cell traction to occur from behind the leading edge (Chrzanowska-Wodnicka & Burridge, 1996). The contractile force also causes retraction of the cell rear and allows the cell to detach and translocate the cell body forward (Figure 7) (Chen, 1981). The extent of RhoA induced retraction has been clearly seen in neuronal cell lines, where RhoA-induced contraction leads to cell rounding in NE115 neuroblastoma cells and neurite collapse (Kozma et al., 1997; Leeuwen et al., 1997). This contractility generally antagonises cell extension, as demonstrated by the introduction of C3-transferase into cells, which blocks RhoA-induced contraction and has been reported to stimulate lamellipodia formation (Rottner et al., 1999). Similarly, expression of constitutively active RhoA or wild type RhoA blocks membrane protrusion and extension (Cox et al., 2001). Both temporal and spatial regulation of this antagonism is proposed to be co-ordinated during cell migration. Lamellipodia formation is a prerequisite for a cell to produce a leading edge however RhoA has been suggested to have a role in the selective orientation of the leading edge and plays a role in the contraction and collapse of lateral lamellipodia during directed cell motility (Stossel, 1993). Moreover, contractility can itself contribute to cell extension when sufficient adhesions are present and this has been proposed to partially be involved in the formation of both filopodia and lamellipodia (see Section 1.4.1) (Schoenwaelder & Burridge, 1999). Interestingly, as described earlier (see Section 1.4.3), Rac1 activity has been shown to lead to an increase in RhoA activity and in some cell types this increase in RhoA activity has been linked to the epithelial-mesenchymal transition which is thought to be one of the initial steps that occur during epithelial cell migration (Zondag et al., 2000).

Various experiments have resulted in an apparently paradoxical view on whether RhoA activity is important for cell motility. For example it has been reported that over-

expression of constitutively active RhoA has inhibitory effects on migration, possibly due to the inhibition of the dynamic remodelling and turnover of focal adhesions and stress fibres during this process (Nobes & Hall, 1999; Ridley et al., 1995; Takaishi et al., 1994). Similarly inactivation of RhoA activity can enhance cell migration rates, as demonstrated using the Rho-kinase inhibitor Y-27632, which augments the migration rate of rat embryo fibroblasts during a wound healing assay (Nobes & Hall, 1999). In keeping with this, Rnd3/RhoE promotes cell migration in response to hepatocyte growth factor (HGF) (Guasch et al., 1998), consistent with the antagonism of RhoA by Rnd3/RhoE (Guasch et al., 1998; Nobes et al., 1998).

On the other hand, others have reported that inactivation of RhoA hinders cell migration, as treatment of cells with C3-transferase blocks T-cell lymphoma invasion as well as LPA-induced migration and invasion of hepatoma cells (Verschueren et al., 1997; Yoshioka et al., 1995), while expression of constitutively active RhoA in NIH3T3 cells confers the potential of cells to metastasise to the lung in a tail vein metastatic assay (del Peso et al., 1997). Recently, RhoA has been implicated in colon carcinoma cell migration via the formation of lamellipodia as opposed to stress fibres and cell contraction, as demonstrated by the suppression of $\alpha 6\beta 4$-dependent lamellipodia formation and cell migration by over expression of N19RhoA and not N17Rac1 (O'Connor et al., 2000). Therefore, the role of RhoA in cell migration is not as clear as was first thought and seems to be cell type or model specific. Nevertheless, as both activation and inhibition of RhoA activity can hinder migration, it has been proposed that the precise regulation of RhoA activity is required for efficient cell migration. In this regard, the temporal regulation of RhoA activity within the cell has recently been suggested to allow cell migration to occur. For example RhoA has been documented to be transiently inhibited during integrin engagement and it is thought that this inhibition may allow initial protrusion at the leading edge of the cell which would otherwise be antagonised by the contractile forces induced by sustained RhoA activity (Arthur & Burridge, 2001; Arthur et al., 2000; Ren et al., 1999). Co-ordination of this RhoA-induced contractility along with co-ordinated activity of other RhoGTPases (as previously discussed) has been postulated to allow cells to extend in the direction of cell migration while maintaining the ability to contract and lift the rear end of the cell body during cell locomotion. The spatial regulation of RhoGTPase activity is also evident, such that cell extension occurs at the leading edge while traction forces induced by RhoA occur directly behind the leading edge and along the bottom of the cell in contact with the substratum. RhoA-induced MLC contraction also provides the driving force behind tail-retraction at the rear of the cell to allow full translocation of the cell body. Recently, a

related member of the RhoA family proteins, RhoC (see Section 1.3), was identified using microarray analysis, as a gene upregulated in metastatic melanoma cells in contrast to their poorly metastatic counterparts (Clark et al., 2000). Moreover, inhibition of RhoC blocked metastasis, while over-expression of RhoC in non-metastatic cells conferred an increased migratory and invasive behaviour in this cell line.

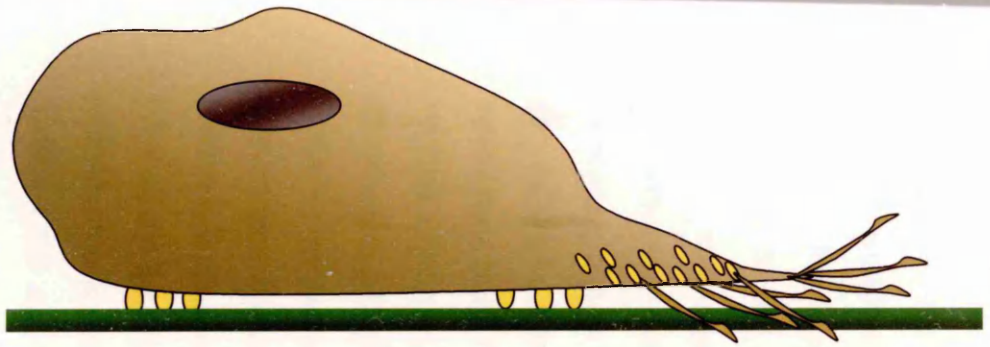
The precise temporal and spatial regulation of morphologically distinct structures induced by RhoGTPases has yet to be fully established. It does however provide an explanation as to why complete inactivation or sustained activation in various cell systems can lead to a misinterpretation regarding the importance of individual RhoGTPases in cell migration. A more likely explanation is that they all play a role in this process through their co-ordinated effect on contraction and extension at different times and places during cell migration.

figure7ledgend

Figure 7

Schematic representation of adhesion dynamics during migration induced by Cdc42, Rac1 and RhoA.

Figure 7.

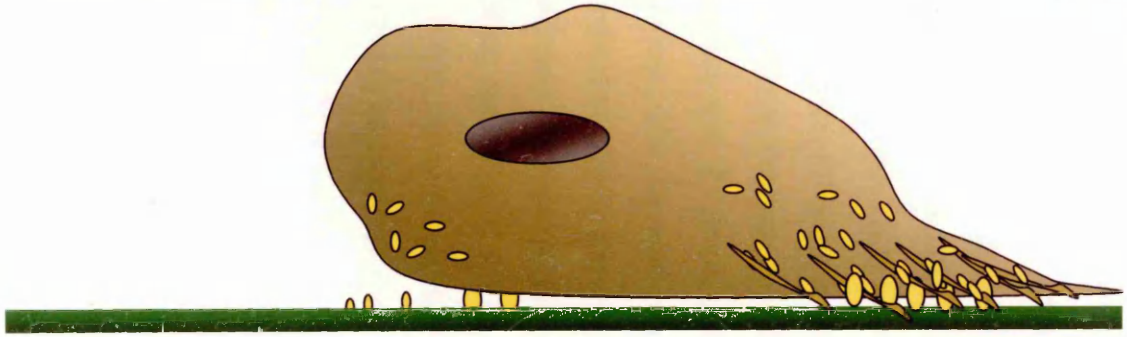


Cdc42

Chemoattractant

Substrate

Focal adhesion remodelling – Filopodia formation
(initiating directional cell migration).



Rac1

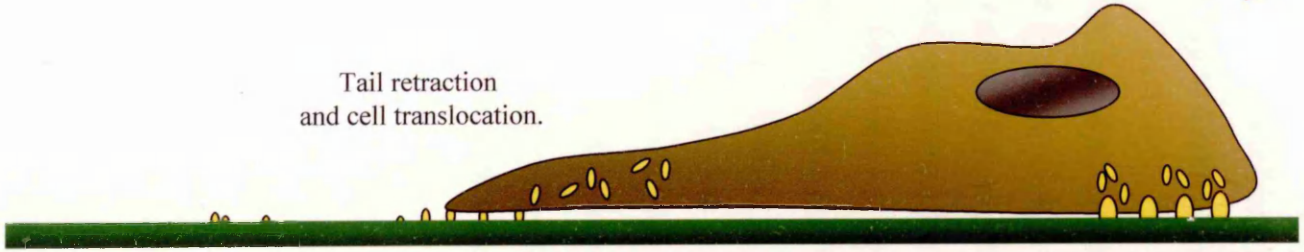
Chemoattractant

Substrate

Simultaneous, focal adhesion remodelling
and disruption at the rear of the cell begins to occur.

Focal adhesion remodelling – Lamellipodia formation between filopodia
(providing a leading edge for directional cell migration).

Direction of migration



RhoA

Tail retraction
and cell translocation.

Chemoattractant

Substrate

Acto-myosin based cell contraction

1.6 Regulation of adhesion dynamics during cell migration.

Cell locomotion ultimately relies on the spatial and temporal regulation of adhesion strength as well as the contractile or expansion forces induced on the cell body by RhoGTPases. The resistance that contractile forces have to overcome in order to accomplish translocation of the cell body is predominantly due to adhesive interactions between the cell and the substratum at focal adhesions or focal complexes. Alterations therefore by protein interactions in focal adhesions and focal complexes during this process will have profound effects on the overall adhesion of the cell to the substratum. Importantly the spatial and temporal localisation of proteins involved in focal adhesion dynamics, such as Src, will therefore serve a fundamental role in controlling cell migration.

Disruption and disassembly of focal adhesions occur at the rear of the cell during cell retraction, while the initiation of new attachment sites or the rapid turnover of focal complexes has to occur at the leading edge of the cell. Moreover, in order for efficient cell migration to occur there has to be an equilibrium between adhesion strength during cell traction and adhesion disruption during cell detachment. Finally, there has to be a spatial imbalance between these adhesion forces such that adhesions at the leading edge of the cell remain while cell attachments at the rear are released (Sheetz, 1994). Interestingly, the front of migrating neuronal and fibroblast cells have a significantly greater link to cytoskeleton based adhesions than the rear of the cell (Schmidt et al., 1993). A clear illustration of how cell adhesion effects migration can be seen in fish keratinocytes which generally have a low adhesion threshold to the substratum and as a result migrate much faster than their fibroblast counterparts which demonstrate a much greater adhesion strength (Harris et al., 1980; Oliver et al., 1994). In this regard, neutrophils have an intermediate adhesive strength and consequently exhibit a migration rate between that of fish keratinocytes and fibroblasts (Evans et al., 1993). It is clear then that an optimal cell-substratum adhesion strength exists for different cell types and that simple alterations in relative adhesion to the substratum at different times during cell migration can govern whether and how fast a cell migrates. As discussed earlier contractile forces by RhoA and directional influences via Cdc42 are also critical determinants of cell migration speed (see Section 1.5.2 and 1.5.3). We will focus here however, on the role and link between proteins present at adhesion sites, such as Src, and discuss the implication of molecular interactions at these sites with regards to cell motility.

1.6.1 A role for Src in cell migration.

Several studies have suggested that Src family kinases play a role in cell motility. For example, c-Src deficient fibroblasts have a decreased random migration (Hall et al., 1996; Kaplan et al., 1995), and cells deficient in all three ubiquitously expressed family members, Src, Fyn and Yes are unable to migrate into a wound (Klinghoffer et al., 1999). Furthermore, Src -/- fibroblasts have a defect in cell spreading (Kaplan et al., 1995), while Src -/- osteoclasts have a defect in cytoskeletal organisation (Schwartzberg et al., 1997). Others have shown c-Src kinase activity to be required for growth factor induced migration and scattering in a number of cell types, providing definitive evidence that Src contributes to the control of cell migration (Liu et al., 1999; Rahimi et al., 1998; Rodier et al., 1995). The ability of Src to influence cell motility in fibroblasts has been primarily linked to the regulation of focal adhesion turnover. Cells lacking Src family members are able to assemble focal adhesions (Klinghoffer et al., 1999), indicating that Src is not required for their formation. However, cells in which kinase defective mutants of Src are expressed have enlarged focal adhesions (Fincham & Frame, 1998; Kaplan et al., 1994), suggestive of a role for Src kinase activity in the turnover of these structures that is needed for cells to move (Fincham & Frame, 1998). Moreover, linkages between integrin vitronectin receptors and the actin cytoskeleton are strengthened in Src-deficient fibroblasts (Felsenfeld et al., 1999), indicating that Src activity is required to weaken, or disrupt, links at the integrin-cytoskeleton interface thus modulating cell motility. The presence of functional SH2 and SH3 domains in these Src proteins suggests that, in addition to phosphorylating key substrates, Src may be acting as an adaptor protein allowing its recruitment into focal adhesion structures and/or the recruitment of cellular partners to these sites. In line with this, kinase-deficient Src mutants can rescue both defects in cell spreading and cytoskeletal organisation in fibroblasts and osteoclasts, as mentioned above (Kaplan et al., 1995; Schwartzberg et al., 1997), implying that at least some effects of Src on cell adhesions and the cytoskeleton are kinase-independent and may rely on the adaptor function of Src.

Src's activity at adhesions however, ultimately results in their turnover, an effect that has been attributed to Src's ability to influence cell migration. Much of the mechanistic information on how Src affects adhesions and regulators of the actin cytoskeleton has been generated from studies with v-Src. v-Src for example, leads to the disruption of cellular adhesions and loss of actin organisation, resulting in cell rounding and detachment (Fincham et al., 1999; Fincham et al., 1995). It is possible that the v-Src mediated cell detachment and rounding is an extreme version of a tightly controlled process that triggers

adhesion turnover when cell/matrix adhesions are being dynamically regulated during Src dependent cell migration. Src has been reported to localise with a variety of adhesion components, including structural regulatory and signalling proteins such as, FAK, cortactin, paxillin and ERM proteins. The exact function of each individual protein during cell migration is not fully understood however the phosphorylation of many of these adhesion components may also be linked to cytoskeletal remodelling and adhesion turnover during cell motility.

There is convincing evidence for example that the linked activity of Src and FAK, control adhesion turnover and cell migration. In support of a role for FAK in cell migration, inhibition of FAK signalling by expression of dominant negative FAK constructs has been shown to decrease migration rates (Gilmore & Romer, 1996). Furthermore, cells derived from FAK -/- mouse embryos have enlarged focal adhesions and are relatively non-motile due to impaired focal adhesions turnover (Ilic et al., 1995; Ilic et al., 1996). Direct evidence of a link between FAK and Src comes from studies where over-expression of exogenous FAK in CHO cells stimulates cell migration, which is dependent on its ability to associate with Src family members (Cary et al., 1996). Furthermore, expression of FAK mutants unable to bind Src, hinder cell migration (Cary et al., 1996). Evidence that dominant-inhibitory Src proteins and the absence of FAK both give rise to enlarged cellular adhesions and impaired migration, strongly suggests that the critical role of their activity is to induce adhesion turnover. Recently, regulatory events that lead to Src-mediated adhesion disassembly via FAK have come from studies on v-Src-induced focal adhesion disruption in which v-Src induced tyrosine phosphorylation of FAK precedes calpain-mediated proteolytic cleavage of FAK, leading to the cleavage and loss of FAK protein, adhesion disassembly and cell detachment (Carragher et al., 2001; Carragher et al., 1999; Fincham et al., 1995). In this regard mutants of Src that suppress cell motility, including kinase and myristylation defective Src, do not induce FAK proteolysis and adhesion turnover (Fincham & Frame, 1998; Fincham et al., 1995).

In addition, since FAK is unlikely to be the sole protein involved in cell migration, other potential Src substrates such as cortactin could be involved in Src induced focal adhesion turnover and remodelling during cell migration. Cortactin has been reported to localise to residual focal adhesions (Wu et al., 1991) and Rac1 activity has been documented to cause cortactin to localise to lamellipodia in Swiss 3T3 cells (Weed et al., 1998). Cortactin also binds to actin filaments (Wu et al., 1991) and has actin cross-linking activity that is regulated by Src (Huang et al., 1997). Furthermore, cortactin has been shown to co-localise and interact with Arp2/3 at sites of actin assembly (Weed et al., 2000).

Interestingly, tyrosine phosphorylation of cortactin is linked to Src and cell motility (Huang et al., 1998; Kanner et al., 1990) and recently tyrosine phosphorylation of cortactin has been reported to promote its susceptibility to calpain cleavage (Huang et al., 1997). These properties suggest that cortactin, like FAK, is a potential substrate involved in Src-induced focal adhesions turnover and remodelling during cell motility.

Several studies have also suggested that paxillin may also be an important substrate in Src induced focal adhesion turnover during cell migration. For example, paxillin -/- fibroblasts have impaired cell migration and display defects in cortical actin and focal adhesions (Hagel et al., 2002). Moreover, tyrosine phosphorylation of paxillin has been demonstrated in a number of studies to be associated with cell migration, while VEGF induced cell migration was shown to enhance the binding of Src and paxillin (Burridge et al., 1992; Kintscher et al., 2001; Munshi et al., 2000; Petit et al., 2000). Finally, Src induced phosphorylation of ezrin may expose the actin binding site on ezrin and thereby effect membrane/cytoskeletal interactions leading to lamellipodia formation, as previously demonstrated by over-expression of ezrin (Krieg & Hunter, 1992; Martin et al., 1995b). There may be many other Src substrates potentially involved in cell migration which will be discussed in more detail later.

1.6.2 Src induced modulation of RhoGTPases during cell migration.

As well as mediating the adhesion and actin cytoskeletal dynamics during cell migration by interacting with focal adhesion proteins, Src has also been proposed to modulate the actin cytoskeleton dynamics during cell motility by directly regulating RhoGTPases. This can occur at several different levels including regulation of GEFs, GAPs and RhoGTPase effectors. Src family kinases such as Lck have been shown to influence the activity of the Rac1, by tyrosine phosphorylation of Vav (Gulbins et al., 1993; Han et al., 1997). On the other hand, Src may also lead to the antagonism of RhoA via phosphorylation of p190RhoGAP (Ellis et al., 1990), leading to its association with p120RasGAP (Chang et al., 1995; McGlade et al., 1993). This association, in turn, results in an increase in RhoA GTPase activity, inhibition of RhoA activity and the disruption and disassembly of actin stress fibres (Chang et al., 1995; Fincham et al., 1999). The antagonism of RhoA as a key event in Src's de-regulation of the actin cytoskeleton was highlighted by the RhoA-induced reversion of the Src-mediated actin changes in rat fibroblasts (Mayer et al., 1999). In keeping with this, the complex between p190RhoGAP and p120RasGAP is required for stress fibre turnover and efficient cell migration (Kulkarni et al., 2000). Interestingly, integrin signalling induces early Src-dependent tyrosine phosphorylation and activation of

p190RhoGAP that is thought to relieve RhoA-mediated contractile forces thus allowing protrusions to form at the leading edge of motile cells (Arthur et al., 2000). In keeping with this, further studies have demonstrated that inhibition of RhoA by p190RhoGAP enhances cell spreading and migration by promoting cell protrusion and polarity (Arthur & Burridge, 2001). Src has also been implicated in modulating the activity of mDia1/2 with regards to stress fibre formation. As discussed previously in Section 1.4.1 (Figure 6), mDia1/2 co-operate with Rho-kinase to mediate actin remodelling downstream of RhoA (Watanabe et al., 1999). Recently a direct association between c-Src and mDia, mediated by the SH3 domain of Src and the proline rich FH1 domains of Dia proteins, has been reported to be necessary for the co-operative effects of Dia and Rho-kinase in mediating stress fibre formation (Tominaga et al., 2000). Inhibition of Src and mDia binding by expression of a dominant inhibitory Src protein, or deletion of the FH1 domain of Dia proteins, was shown to block the co-operation between Dia and Rho-kinase and thereby block stress fibre formation (Tominaga et al., 2000). It is therefore possible that Src may influence actin/adhesion dynamics during cell migration by functionally interacting with and regulating RhoGTPases at a specific time and place during cell migration.

1.7 Aim.

Src can have both positive and negative effects on adhesion formation and may act as a switch between their assembly and disassembly placing it in a central position from which to co-ordinate the multi-faceted process of directed cell motility. The functional interplay between Src family members and the actin cytoskeleton with regards to RhoGTPases lead us to probe this relationship further and examine the link between these proteins and their function. Here we addressed how RhoA-, Rac1- or Cdc42-induced changes in the actin cytoskeleton influence the intracellular localisation of c-Src, and Src's biological effects within the cell. Furthermore, we address whether cross-talk between RhoGTPases is involved in Src's intracellular targeting. Finally, as Src is known to be required for cell motility, we aim to establish whether Src's catalytic activity functions in this targeting process and whether it plays a role in cell polarisation and migration.

Chapter 2

2 Materials and methods

2.1 Cell culture reagents

Supplier: Beatson Institute Central Services

Sterile dH_20
Sterile PBS (Dulbecco 'A')
Sterile PBS/1mM EDTA
Penicillin solution (10,000 units/ml)
Streptomycin solution (10mg/ml)

Supplier: Gibco Europe Life Technologies Ltd., Paisley, UK.

10× Dulbeccos MEM concentrate
1x sodium pyruvate
200mM L-glutamine
2.5% trypsin solution

Supplier: Qiagen, Crawley, UK

Polyfect transfection reagent

Supplier: Sigma Chemical Co., Poole, UK..

Foetal calf serum (FCS)

2.2 Cell culture plasticware

Supplier: BD Biosciences, Oxford, UK

Falcon tissue culture dishes

Supplier: ERIE scientific, BDH Merke eurolab, Poole Dorset

Supercell culture slides

Supplier: TCS biologicals, Botolph Claydon, UK.

Nunc Delta tissue culture flasks

Nunc cryotubes

2.3 Inhibitors

Supplier: CN biosciences, Nottingham, UK

PP2 Src-family kinase inhibitor

Supplier: Cytoskeleton, Inc, Northampton UK

C3 -transferase

Supplier: Sigma Chemical Co., Poole, UK.

Cytochalasin D

Supplier: Sigma pool,UK

Emetine

Supplier: Welfide Corporation, Japan

Y-27632 Rho-kinase inhibitor

2.4 Growth factors

Supplier: Sigma Chemical Co, Poole, UK

Bradykinin, LPA

Supplier: TCS Biologicals, Botolph Claydon, UK

PDGF

2.5 Antisera and associated materials

Supplier: Amersham International, Little Chalfont, UK.

Anti-mouse/horseradish peroxidase conjugate
Anti-rabbit/horseradish peroxidase conjugate

Supplier: BioRad Labs, Hercules, CA

Confocal microscope MRC 600

Supplier: Biosource International, CA

Anti-FAK tyrosine-925 mAb
Anti-phospho-Src-Y416 polyclonal

Supplier: Eppendorf Hamburg, Germany

Microinjector eppendorf 5171

Microinjector Femtotips

Supplier: Jackson ImmunoResearch, Luton, UK.

FITC labelled sheep anti-mouse IgG
TRITC labelled goat anti-rabbit
TRITC labelled goat anti-mouse
FITC labelled goat anti-rabbit
FITC labelled goat anti-rat IgG

L machesky, Birmingham University, UK

Anti-Arp3 mAb

Supplier: PIERCE

DMP

Supplier: Research Diagnostics, Inc, Flanders, NJ

Rhodamine conjugated anti-paxillin antibody

Supplier: Sigma chemical Co., Poole, UK.

2mM phenylmethylsulphonyl fluoride
0.1% (v/v) aprotinin
Protein A-sepharose beads
Rhodamine-conjugated phalloidin
Anti-vinculin mAb
saponin

T Hunter, Salk Institute, CA

Anti-Src mAb N2-17

Supplier: Transduction Laboratories, BD Biosciences, Oxford, UK

Phosphotyrosine antiserum, mouse monoclonal (PY20)
Anti-FAK mAb

Supplier: Upstate Biotechnology, Lake Placid, NY

Anti-Grb2 mAb
Anti-Scar polyclonal
Anti-Src mAb EC10
Anti-myc tag polyclonal Ab 9E10
Anti-myc tag mAb 9E10

Supplier: Vector Laboratories Ltd., Peterborough, UK.

Vectashield mounting medium for immunofluorescence.

2.6 Protein reagents and materials

Supplier: Amersham, Little Chalfont, UK.

High molecular weight rainbow markers (14.4kDa to 220kDa)
ECL reagent

Supplier: Beatson Institute Workshop.

Semi-dry blotting apparatus

Supplier: Genetic Research Instrumentation, Dunmow, UK.

Atto protein electrophoresis apparatus

Supplier: PIERCE, CLB perbio Ltd.

Micro BCA protein assay kit

Supplier: Schleicher and Schuell, London, UK.

Nitrocellulose membrane

Supplier: Severn Biotech Ltd., Kidderminster, UK.

Design-a-Gel 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide solution

Supplier: Sigma Chemical Co., Poole, UK.

TEMED (N, N, N′, N′-Tetramethylethylenediamine)
0.1% (w/v) Ponceau S solution in 5% acetic acid
Bovine serum albumin (BSA), fraction V
Ammonium persulphate (APS)

Supplier: Unipath, Basingstoke, UK.

'Oxoid' phosphate buffered saline tablets (Dulbecco 'A')

Supplier: Whatman, Maidstone, UK

3MM filter paper

2.7 Stock solutions and buffers

Cell culture solutions

Cell culture medium

1× DMEM supplemented as follows:
10% (v/v) foetal calf serum
1mM sodium pyruvate
2mM L-glutamine
50 units/ml penicillin
10μg/ml streptomycin

Trypsin solution for routine passaging

0.25% v/v Gibco trypsin in sterile PBS/1mM EDTA

2.8 Protein extraction and processing

RIPA buffer

50mM Tris/HCl, pH 7.6
150mM NaCl
1% Triton X-100
0.5% deoxycholate
0.1% sodium dodecyl sulphate (SDS)
2mM EGTA
10mM sodium pyrophosphate

100μm sodium orthvanidate

2mm phenylmethlsolphonylfluoride

10μg/ml aprotinin

3× sample buffer100μM sodium orthovanadate Glycine

150mM Tris/HCl, pH 6.7

6% (w/v) SDS

30% (v/v) glycerol

15% (v/v) 2-mercaptoethanol

bromophenol blue to colour

SDS-PAGE stacking gel buffer

0.5M Tris/HCl, pH 6.7

0.4% (w/v) SDS

Stacking gel

4.0ml 30% acrylamide/0.8% bisacrylamide solution

6.0ml stacking gel buffer

14.0ml dH_2O

250μl 10% APS solution

20μl TEMED

SDS-PAGE resolving gel buffer

1.5M Tris/HCl, pH 8.9

0.4% (w/v) SDS

Resolving gel (for a 7.5% gel):

9.0ml 30% acrylamide/0.8% bisacrylamide solution

9.0ml resolving gel buffer

18.0ml dH_2O

250μl 10% APS solution

20μl TEMED

Protein electrophoresis tank buffer (10× concentrate)

0.05M Tris
0.05M glycine
0.1% (w/v) SDS

Semi-dry transfer buffer for immunoblotting

50mM Tris
40mM glycine
0.037 % (w/v) SDS
20% methanol

Immunoblot wash buffer

0.2% Tween 20 in PBS

Immunoblot stripping buffer

0.2M glycine
1% (w/v) SDS
pH adjusted to 2.5 with hydrochloric acid

2.9 Cells and plasmids

CA10-Src251-GFP and CA10-wtSrc were kind gifts from P Schwartzberg (NIH, Bethesda, MD) and CA10–SrcY527F and CA10-SrcMF (K295M,Y527F) were a kind gift from K Kaplan (MIT, Cambridge, MA). All Src constructs were chicken c-Src. pEGFP (Clontech, Basingstoke, UK) was used for expression of green fluorescent protein. N19- and V14RhoA, V12- and N17Cdc42, and L61- and N17Rac, all in the pRK5myc plasmid were kindly provided by Alan Hall (UCL, London, UK).

2.10 Methods

2.10.1 Experimental protocol for serum starvation

Swiss 3T3 cells were routinely grown in DMEM supplemented with 10 % foetal calf serum. Cells were grown for one day after plating and subsequently serum starved for 48 h in serum free medium to induce stress fibre disassembly. To reduce the heterogeneity in the population after growth factor stimulation, cells were placed in serum-free conditions overnight (16 h), which did not abolish stress fibres but acted to reduce the heterogeneity amongst the cell population. The transfection efficiency of cells was typically between 20-30 % and in each figure the cells shown are representative of the cell morphology observed in over 80 % of cells examined. In each case experiments were repeated at least 3 times.

2.10.2 Microinjection and transfection of plasmids

Plasmids were introduced into the cells by nuclear microinjection or transient transfection as indicated in the text. Following microinjection of the plasmids at 100 ng μl^{-1}, the proteins were expressed within 3 to 5 h after which the analysis was carried out. Transfection of the cells was carried out using Polyfect according to the manufacturer's protocol with 0.5 μg DNA per chamber of an 8 well chamber slide. Typical transfection efficiencies were around 30 %.

2.10.3 Immunofluorescence

Cells were plated onto glass chamber slides at a cell density of $1x10^4$ 24 h prior to transfection or onto glass coverslips 24 h prior to microinjection. For experiments in which cells were serum starved to remove stress fibres prior to expression of the proteins, the cells were grown for 48 h in serum-free conditions prior to microinjection of the appropriate plasmids and analysis carried out 3-5 h later after expression of the proteins. For all other experiments the proteins were expressed by transient transfection. Growth factor treatment of the cells were as follows, PDGF, 25 ng ml^{-1} for 30 min, bradykinin, 100 nM for 5 min or LPA 200 ng ml^{-1} for 30 min. The Rho-kinase inhibitor Y-27632 (10 μM), PP2 (5 μM), cytochalasin D (0.1 μg ml^{-1}) or emetine (55 μg ml^{-1}) were added as indicated.

Cells were fixed and stained for confocal microscopy as follows:

1. Cells were washed twice with PBS and fixed in 3 % paraformaldehyde (20 min).

3. Cell were then permeabilised with PBS containing 0.1% saponin and 20 mM glycine (20 min).

4. Cells were then blocked with PBS containing 0.1 % saponin and 10 % foetal calf serum for 1 h.

5. Subsequently, cells were incubated with the appropriate primary antibodies, rhodamine-conjugated phalloidin or rhodamine conjugated paxillin antibody for 60 min.

6. Non-conjugated antibody detection was by reaction with a species specific FITC- or TRITC-conjugated antibodies for 45 min.

7. Cells were visualised using a confocal microscope.

2.10.4 Preparation of protein extracts from adherent fibroblasts.

Swiss 3T3 fibroblasts were lysed in RIPA buffer to extract cellular proteins as follows:

The flasks or dishes were transferred directly from the 37°C incubator onto ice and were rinsed twice with ice-cold PBS. The culture vessels were then inclined and allowed to drain for 10 min to minimise the amount of residual PBS on the cells. Ice-cold lysis buffer was then pipetted onto the cells (0.5ml per 9cm plate) which were allowed to lyse for 10 minutes on ice. At the end of this time, the cells were scraped off the tissue culture plastic using a disposable cell scraper and the resulting suspension was transferred into microcentrifuge tubes. The lysates were then cleared of insoluble cell debris by centrifugation at 14,000g at 4°C for 15 min. The total protein concentration of the lysates was estimated using the BCA protein assay kit according to the manufacturers directions. The aborbance of the samples was then measured at 562nm using a Beckman DU 650 spectrophotometer. Experimental values were compared to a standard curve (obtained using BSA) and the total protein concentration in each sample calculated.

2.10.5 Protein electrophoresis.

Lysates prepared in RIPA buffer were standardised for total protein content before adding 3× sample buffer. The lysates were then boiled for 5 minutes and allowed to cool before loading onto SDS-polyacrylamide gels for electrophoresis. Protein samples were separated

on discontinuous 2mm SDS-polyacrylamide gels comprising a short stacking gel for the initial stage of the electrophoresis and a longer resolving gel to separate the proteins. The resolving gels contained 10% or 7.5 % acrylamide. Gels were typically run at either 180V for 3-4 hours or 30V overnight under constant-voltage conditions until the dye-front had reached the bottom of the gel. All gels were run with molecular weight markers.

2.10.6 Immunoblotting

After electophoresis, proteins were transferred onto nitrocellulose membrane by electrophoretic semi-dry blotting. Briefly, the SDS-PAGE gel, nitrocellulose membrane and 12 pieces of 3MM paper were soaked in transfer buffer and arranged between the blotter electrodes with 6 sheets of 3MM paper on either side of the gel/membrane sandwich oriented with the membrane on the cathode side of the gel. After gently excluding air bubbles from the paper/gel/membrane stack, the proteins were transferred for 1 h at a maximum current of 200mA and a maximum voltage of 20V for a gel of 140cm^2.

2.10.7 Antibody incubation and detection by ECL

Following the transfer, the nitrocellulose membrane was blocked in the appropriate blocking solution (either 5% (w/v) non-fat milk or 3% (w/v) BSA in wash buffer) for 1 hr at room temperature on a shaker. After blocking, the blots were probed with primary antibody diluted in blocking solution for either 1 h at room temperature or overnight at 4°C. After incubation in primary antibody, the blots where washed 3 times in wash buffer (15 min per wash) and were then incubated in horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. After washing as described above, the blots were drained and incubated in fresh ECL reagent for 1 min with gentle agitation and were then wrapped in Saran-Wrap. The blots were placed in contact with X-ray film for a period of time sufficient to detect the light produced by the ECL reaction and the film was then processed in a Kodak automated processor.

2.10.8 Removing bound antisera from immunoblots.

Nitrocellulose blots which had been previously incubated in antisera were treated as follows to remove these bound antibodies prior to incubation with a different antiserum. The blot was firstly washed with immunoblot wash buffer to remove spent ECL reagent and was then incubated in immunoblot stripping buffer for 30 minutes at room temperature

on a gyratory shaker. At the end of this incubation, the blot was washed again and then blocked and incubated with the desired antibodies.

2.10.9 Immunoprecipitation of extracted proteins

Lysates prepared in RIPA where standardised for total protein content (0.25-1.0 mg). The IgG complexes were the collected by incubating for 1 hr at 4°C with 50µl of a 50% slurry of protein-A sepharose beads (coated with rabbit anti-mouse IgG for normal mouse IgG or left uncoated for rabbit serum). The immunoprecipitating IgG was incubated overnight at 4°C before collecting the IgG complexes with coated or uncoated protein-A sepharose beads as described above. The collected immune complexes were washed 5 times in RIPA lysis buffer before resuspending in 20µl of 3× sample buffer prior to electrophoresis and blotting.

2.10.10 Random cell migration

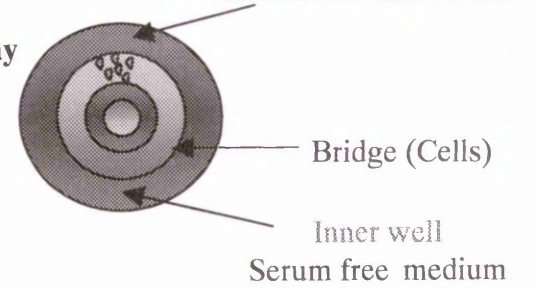
Cells grown on glass cover slips were transfected with Src251-GFP or GFP or co-transfected with SrcY527F and GFP or SrcMF and GFP to allow detection of the transfected cells. Following expression of the proteins microscopic images of the cells were acquired from a charged couple device camera and captured by Open Lab (improvision Software, UK) every 15 min over a 5 h period. The processed images were displayed rapidly as a movie and interactive tracking of cells with a mouse pointer resulted in the generation of cell trajectory. Calculations for the rate of cell migration were derived from the data set, with individual cell speed being calculated for each of the 20 consecutive images. The mean cell speed was then calculated for each lapse interval, and an overall figure derived for the 5 h period. Values are mean $\pm$ S.E.M. of between 30 and 60 cells taken from at least 5 experiments.

2.10.11 Dunn chamber polarisation assay

Cells were co-transfected with SrcY527F and GFP or SrcMF and GFP for detection, or transfected with Src251-GFP or GFP alone. Cells were trypsinised 24 h post-transfection and plated onto coverslips at 1 x 10^5 in 35 mm plates for 1 h. Cells were then serum starved for 1 h and mounted on Dunn chambers with PDGF 10ng ml^{-1} as chemoattractant (Allen et al., 1997; Allen et al., 1998). Microscopic images of the cells were acquired from

a charged couple device camera and captured by Open Lab every 15 min over a 5 h period. Criteria for calculating cells that remodelled their actin cytoskeleton were as follows. Cells that undergo a morphological shape change to form a leading lamellipodia and elongated tail directed towards the chemical gradient were included in the data set as positive polarising cells. Cells already demonstrating a cell polarity towards the chemical gradient were not included in the data set to reduce any false positives. Only cells that had random polarisation i.e, any cell out with the above criteria, or those with no obvious polarity at the beginning of the experiment were assessed over the 5 h period. The mean percentage of cells polarising along the chemical gradient for each transfected cell type was used to construct an overall figure for the 5 h period. The percentage of cells able to polarise was calculated from at least 5 independent experiments

Dunn chamber chemotaxis assay



2.10.12 Primary antisera dilutions used for immunofluorescence

Vinculin Clone VIN-11-5……..1:100

N2-17 (endogenous Src)……1:1000

Grb2 clone 81 ……1:400

Scar ……...1:400

EC10 (Chicken specific Src)…..1:500

Myc 9E10……...1:500

Anti-phospho FAK-Y925……1:500

FAK clone 77……..1:500

Conjugated Paxillin……1:400

2.10.13 Secondary antisera dilutions used for immunofluorescence

Anti-mouse/FITC conjugated.....1:100

Anti-mouse/Texas red conjugated.....1:100

Anti-Rabbit/FITCconjugated.....1:100

Anti-Rabbit/Texas red conjugated.....1:100

2.10.14 Primary antisera dilutions used for immunoblotting and immuonprecipitation

Phosphotyrosine (PY20).....1:1000 (w)

Scar ....1:1000 (w), 4ug (IP)

Anti-phospho-Src-Y416 .....1:1000 (w)

EC10.....1:1000 (w) 2ug (IP)

9E10 Conjugated mAb, was cross linked using DMP as described by manufacturer (PIERCE) (V Brunton) (200ug/ml).....10ug (IP)

FAK.....1:1000 (w) 5ul (IP)

Grb2.....1:1000 (w)

2.10.15 Secondary antisera dilutions used for Immunoblotting

Horseradish peroxidase (HRP) conjugated anti - mouse or rabbit.....1:5000

2.10.16 Bacterial transformation and DNA extraction

20µl of competent DH5α bacterial cells were aliquoted into chilled microfuge tubes. 1ul of diluted DNA was added to the cells and shaken to mix. The tubes were then paced on ice for 30 min, heat shocked for 40 seconds in a 42°C water bath, then placed back on ice. 80µl of L Broth was added to the cells and the microfuge tubes were left to shake at

225rpm for 1 Hour at 37°C. Afterwards, the cells were plated onto a L Broth plate containing the antibiotic for which the resistence gene is carried by the the transfected DNA.

DNA extraction was carried out using Qiagen plasmid prep kits. The concentration and purity of solutions of isolated DNA fragments or plasmids was determined spectrophotometrically using a Beckman DU650 spectrophotometer.

2.10.17 Routine Swiss 3T3 propagation

Swiss 3T3 fibroblasts were routinely grown on tissue culture treated dishes or flasks using DMEM supplemented with 10% foetal calf serum with tice-weekly medium changes and were never grown to confluence. These cells were maintained in a humid 37°C/5% CO_2 incubator. Adherent Swiss3T3 cells were subcultured as follows:

Fristly, the medium was aspirated off the cells and the monolayer was rinsed with PBS. The cells were thenincubated in 10 % trypsin solution andwith occasional gentle agitation until the cells had detached. The cells were then resuspended in DMEM, counted and plated into cell culture flasks.

2.10.18 Swiss 3T3 fibroblast cyropreservation and recovery

Cyropreserved Swiss 3T3 fibroblasts were prepared as follows:

Swiss 3T3 were allowed to proliferate until they reached 70-80 % confluence. They were then trypsinised to remove then from culture plates. The cells were then rised with PBS and couted before resuspending in culture medium plus 7% DMSO at $1x10^6$ cells/ml. This suspension was trasferred into cryotubes (1ml per vial) which were then wrapped in cotton wool and placed in a -70°C freezer overnight. The following day the cryotubes were immersed in liquid nitrogen for long term storage.

For recovery from storage in liquid nitrogen, cryotubes were rapidly immersed in water at approximately 37°C to thaw cells. The cell suspension was then immediately made up to a volume of 10 ml with culture medium and plated into a culture flask.

Chapter 3

3 Characterisation of Swiss 3T3 cells and Src251-GFP as tools to monitor c-Src targeting within the cell.

3.1 Purpose

The temporal and spatial localisation of Src has implications for the biological actions of Src kinase within the cell. As described previously (Kaplan et al., 1992) c-Src normally associates with endosomal membranes in the perinuclear region of the cell and upon activation translocates from there to its site of action in cellular adhesions by an actin-dependent mechanism. The aim of this study was to examine whether the distinct rearrangements of the actin cytoskeleton mediated by Rho-GTPases could influence the intracellular localisation of c-Src within the cell. The regulation of the actin cytoskeleton by the Rho family of GTPases RhoA, Rac1 and Cdc42 is well documented and readily manipulated in Swiss 3T3 cells (Hall, 1998; Nobes & Hall, 1995), making them an ideal model system in which to study their role in the intracellular targeting of c-Src. In this chapter we set out to determine whether the Swiss 3T3 fibroblast model was appropriate for studying the actin dependent targeting of c-Src.

3.2 Results

3.2.1 Manipulation of the actin cytoskeleton in Swiss 3T3 cells

Activation of RhoA stimulates actomyosin-based contractility, which leads to the assembly of actin stress fibres and focal adhesions found at the end of stress fibres (Ridley & Hall, 1992) (Chrzanowska-Wodnicka & Burridge, 1996; Nobes & Hall, 1995). As documented previously (Ridley & Hall, 1992), we found that Swiss 3T3 fibroblasts grown in the presence of serum contain actin stress fibres that traverse the cell, as visualised following incubation with conjugated phalloidin (Figure 8a). This was also associated with the presence of prominent focal adhesions which are tethered at the ends of stress fibres and contain a number of proteins including, vinculin, paxillin and FAK, as visualised here using an anti-vinculin antibody (Figure 8b, solid arrows). Previous reports have demonstrated that deprivation of serum in Swiss 3T3 cells results in the loss of actin stress fibres (Ridley & Hall, 1992). In our system, removal of serum form Swiss 3T3 cells for 48 h induced the loss of actin stress fibres within the cells (compare Figure 8 c and d) and loss

of focal adhesions (compare Figure 8b and 8e). We then used a molecular probe in cells serum starved for 48 h to confirm that stress fibre formation in Swiss 3T3 cells was a RhoA dependent event. Microinjection of a constitutively active Myc-tagged RhoA protein (V14RhoA), which is insensitive to RhoGAPs resulted in the formation of stress fibres in the absence of serum (Figure 9a,d compare broken arrows indicating cells expressing V14RhoA to solid arrows indicating non-injected cells). Cells expressing Myc-tagged V14RhoA were identified using an anti-Myc antibody (Figure 9a). It should be noted that manipulation of the actin cytoskeleton by RhoA had to be carried out in serum-free conditions as serum results in stress fibre assembly. We introduced RhoA, Rac1 and Cdc42 expression plasmids and Src251-GFP by transient transfection or microinjection and found there was no difference in the outcome between experiments using either method. Therefore, for convenience, transient transfection was used throughout, except in the experiments where we expressed the V14RhoA and N19RhoA proteins. Deprivation of serum in Swiss 3T3 cells results in the loss of actin stress fibres whose assembly is regulated by RhoA and manipulation of the actin cytoskeleton by Rho proteins was therefore carried out in serum-free conditions in which the transfection efficiency was very low. Expression of RhoGTPases was typically observed within 3-5 h post-microinjection.

Rac1 activation has been reported to lead to a distinct rearrangement of the actin cytoskeleton resulting in localised actin polymerisation at the cell periphery, and the formation of lamellipodia (Nobes & Hall, 1995; Ridley et al., 1992). Transfection of constitutively active Myc-tagged Rac1 (L61Rac1) protein in growing cells (Figure 9b,e broken arrows) resulted in the rearrangement of the actin cytoskeleton to produce lamellipodia while non-transfected cells (Figure 9e, solid arrow) had no lamellipodia. Similarly, Cdc42 activation is known to stimulate the formation small actin rich protrusions known as filopodia which emanate from the cell body (Kozma et al., 1995; Nobes & Hall, 1995) and transfection of a constitutively active Myc-tagged Cdc42 (V12Cdc42) protein in growing cells (Figure 9c,f broken arrows) resulted in the rearrangement of the actin cytoskeleton to produce filopodia (Figure 9f, broken arrow) while non-transfected cells (Figure 9f, solid arrow) had no filopodia.

Figure 8

Disruption and breakdown of the actin cytoskeleton by serum starvation. Cells grown in the presence of 10 % serum (a-c) or were serum starved for 48 h to remove stress fibres (d,e). Solid arrows indicate vinculin staining. Actin was visualised with rhodamine-phalloidin (a,c,d) and vinculin using an anti-viculin antibody (b,e). Scale bars 25 μm.

Figure 8

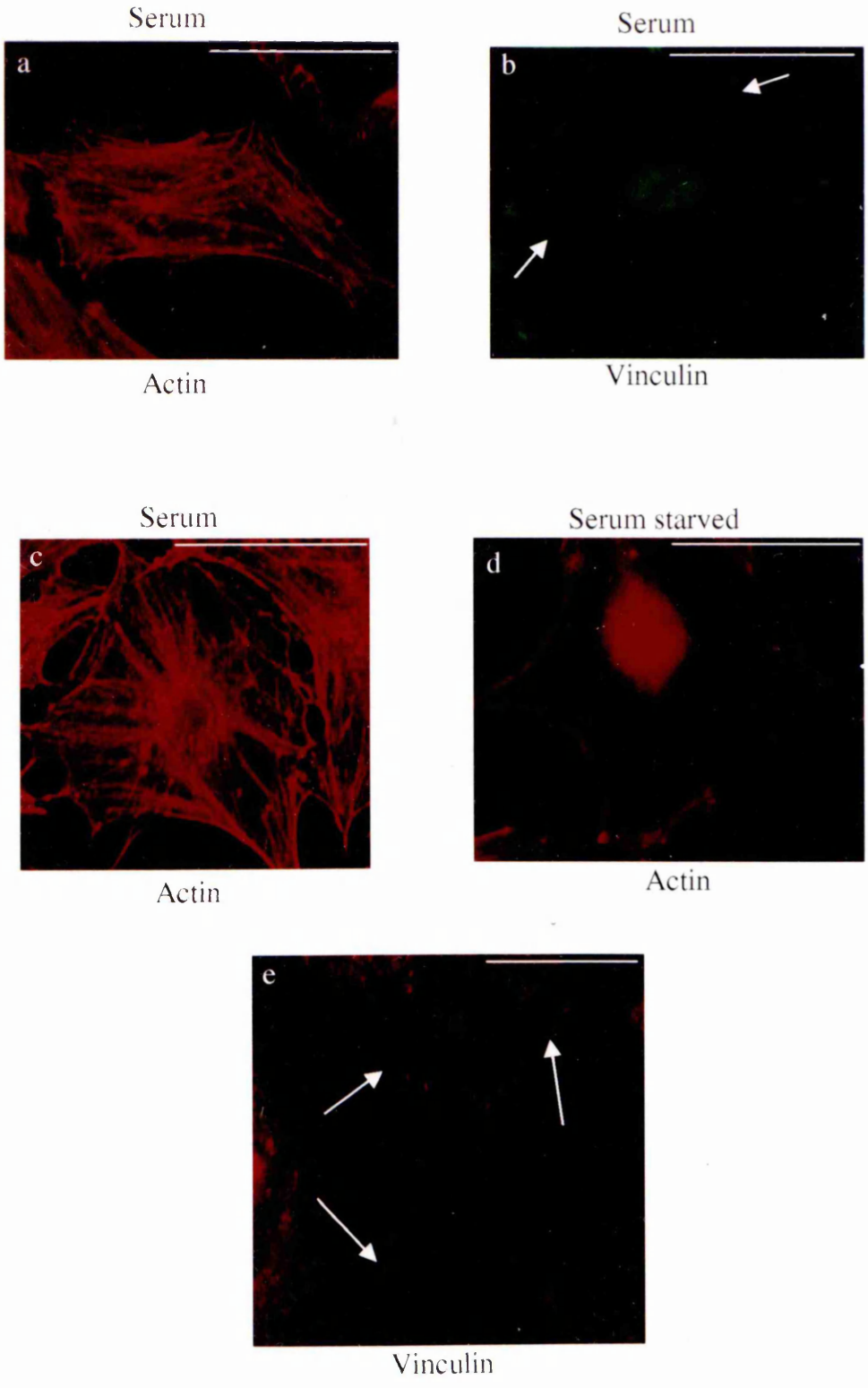
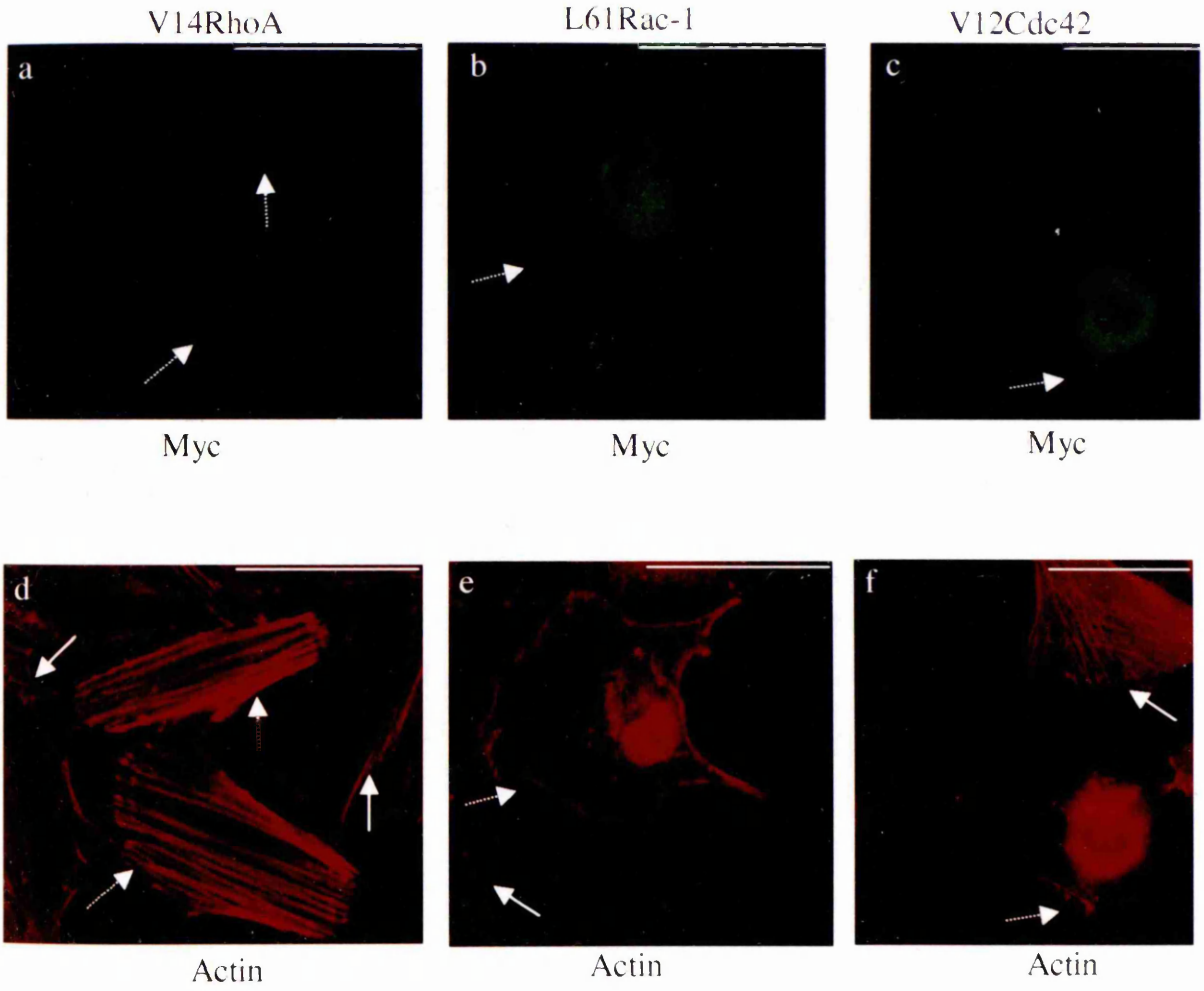

Serum
a
Actin
Serum
b
Vinculin
Serum
c
Actin
Serum starved
d
Actin
e
Vinculin

Figure 9

Specific activation of RhoGTPases using constitutively active RhoGTPases leads to distinct actin rearrangements. Cells were serum starved for 48h to remove stress fibres prior to microinjection of V14RhoA (a,d). Solid arrows indicate non-injected cells, while broken arrows indicate injected cell. Cells in the presence of serum were transfected with L61Rac (b,e), or transfected with V12Cdc42 (c,f). Broken arrows indicate expressing cells, while solid arrows indicate non-expressing cells. Cells expressing Rho-constructs were detected with a Myc-tag antibody (a-c), while actin was visualised with rhodamine-phalloidin (d-f). Scale bars 25 μm.

Figure 9


V14RhoA
L61Rac-1
V12Cdc42
a
b
c
Myc
Myc
Myc
d
e
f
Actin
Actin
Actin

In fibroblasts activation of RhoGTPases by different transmembrane receptors has been reported to lead to distinct morphological rearrangements of the actin cytoskeleton (Hall, 1998). Lysophosphatidic acid (LPA) for example, which is reported to be the active constituent in serum, causes stress fibre formation in a RhoA dependent manner (see section 1.3.2 Figure 5). We therefore addressed how activation of RhoA, using LPA, influenced the actin cytoskeleton in Swiss 3T3 cells and monitored adhesion complexes by staining cells with an anti-vinculin antibody. Treatment of cells with LPA resulted in stress fibre formation (Figure 10a), which was also associated with the localisation of vinculin at the cell periphery, in focal adhesions tethered at the ends of actin stress fibres (compare Figure 10d and Figure 8e). Both lamellipodia and filopodia contain focal complexes within their structures, which have been defined as small adhesion structures of indistinguishable protein composition from the larger focal adhesions as described above (Nobes & Hall, 1995). We therefore addressed how activation of Rac1 or Cdc42 using PDGF or bradykinin, could influence the actin cytoskeleton in Swiss 3T3 cells and monitored adhesion complex formation by staining cells with an anti-vinculin antibody. In our system cells were serum starved overnight for 16 h to reduce the heterogeneity amongst the cell population following growth factor stimulation. Cells serum starved for 16 h show only a slight reduction in stress fibre formation (compare Figure 10a and Figure10b) as opposed to 48 h serum starvation, which results in complete stress fibre loss (Figure10c). The absence of serum 16 h prior to growth factor stimulation however enhances the actin remodelling induced by PDGF and Bradykinin resulting in a reduced heterogeneity amongst cells following stimulation (see Methods Section 2.10.1). Activation of Rac1 by PDGF resulted in the remodelling of the actin cytoskeleton to produce lamellipodia and a leading edge to the cell (Figure 10e, solid arrows) with small vinculin-containing complexes forming around the base of the lamellipodia (Figure 10h, solid arrows). Similar to these observations, we found that bradykinin stimulated the formation of actin microspikes at the cell periphery (Figure 10f, solid arrows) with small focal complexes forming at the base (Figure 10i, broken arrows) and along the length of the filopodia (Figure 10i, solid arrows).

We then set out to determine whether the actin cytoskeleton could be manipulated using pharmalogical intervention. Previous reports have demonstrated that similar to the effects of serum starvation, the addition of cytochalasin D which binds to the barbed end of actin monomers, disrupts the actin cytoskeleton and thereby results in the loss of actin stress fibres (Ridley & Hall, 1992Nobes, 1995 #402). Following treatment of cells with cytochalasin D, the reformation of stress fibres in response to serum (compare Figure 11a and b) or LPA (compare Figure 11c and d) was prevented.

Figure 10

Activation of RhoGTPases using external growth factors to manipulate the actin cytoskeleton. Cells serum-starved overnight to reduce heterogeneity amongst the cell population were stimulated with LPA (200 ng ml^{-1}) for 30 min (a,d), PDGF (25 ng ml^{-1}) for 30 min (b,e) or bradykinin (100 nM) for 5 min (c,f). Actin was visualised with rhodamine-phalloidin (a-c) and vinculin using an anti-viculin antibody (d-f). Scale bars 25 μm.

Figure 10

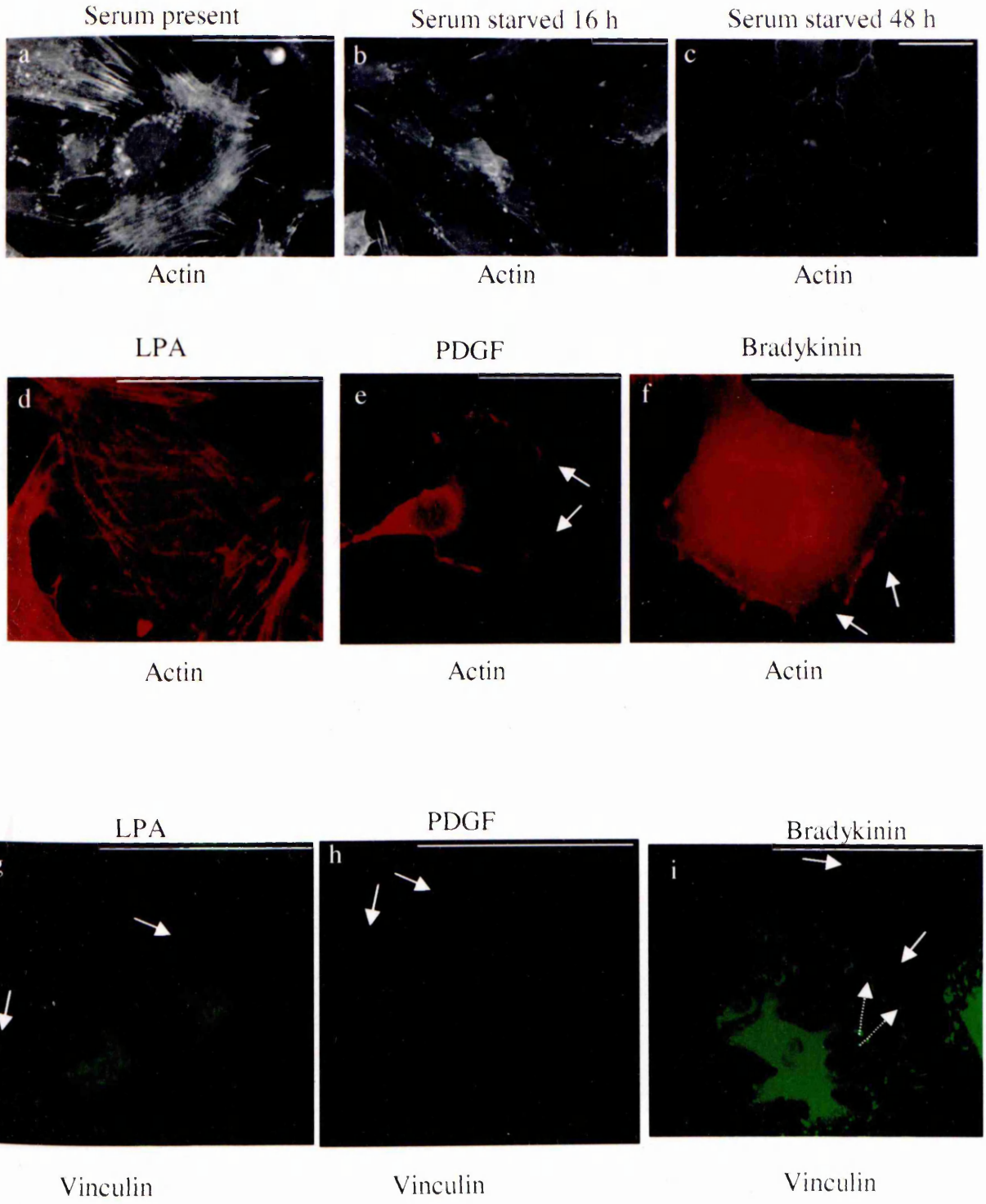
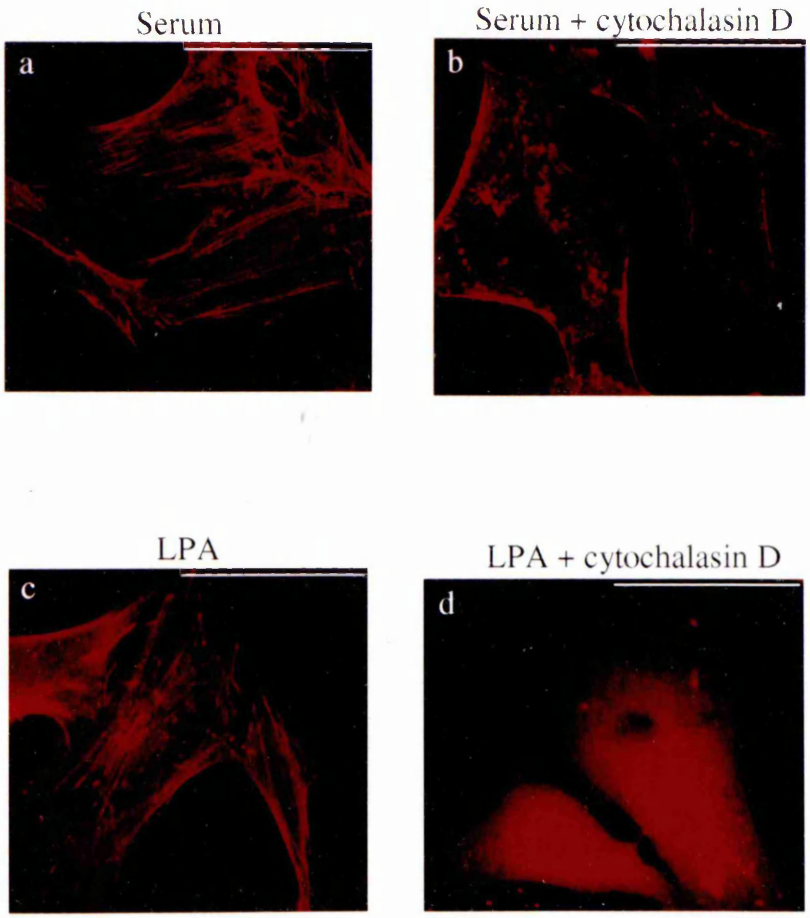
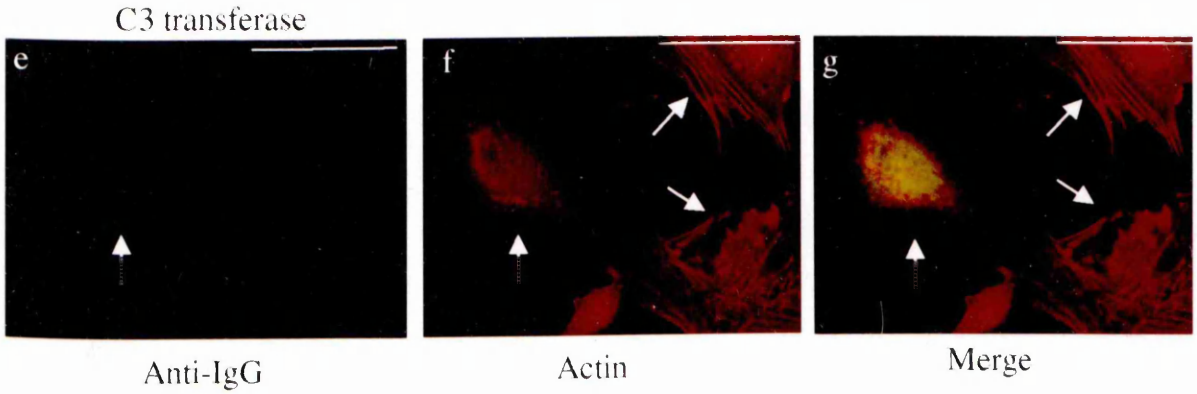

Serum present
Serum starved 16 h
Serum starved 48 h
a
b
c
Actin
Actin
Actin
LPA
PDGF
Bradykinin
d
e
f
Actin
Actin
Actin
LPA
PDGF
Bradykinin
g
h
i
Vinculin
Vinculin
Vinculin

Figure 11

Disruption and breakdown of the actin cytoskeleton by pharmacological intervention. Cells serum starved for 48 h were stimulated with 10 % serum for 30 min (a) or LPA (200 ng ml^{-1}) for 30 min (c). Serum starved cells were pre-treated with 0.1 μg ml^{-1} of cytochalasin D prior to serum (b) or LPA (200 ng ml^{-1}) (d) stimulation. (e-g) Cells in the presence of serum were co-microinjected with C3-transferase and IgG (for detection of injected cell). Broken arrows indicate injected cells, while solid arrows indicate non-injected cells. Actin was visualised with rhodamine-phalloidin (a-d,f,g), while cells microinjected with C3 transferase were detected using an anti-IgG antibody (e,g). Scale bars 25 μm.

Figure 11


Serum
a
Serum + cytochalasin D
b
LPA
c
LPA + cytochalasin D
d


C3 transferase
e
f
g
Anti-IgG
Actin
Merge

More specific methods of inhibiting RhoGTPase-induced actin rearrangements have been reported. The clostridium botulinum exoenzyme C3-transferase for example, disrupts RhoA, B, and C induced stress fibres by ADP-ribosylation of Rho (Aktories & Hall, 1989; Verschueren et al., 1997). Co-microinjection of cells with C3-transferase protein and IgG (for detection of microinjected cells) (Figure 11e) into the cytoplasm caused the loss of stress fibres in the presence of serum as previously reported (Figure 11f,g) (Aktories & Hall, 1989). A more versatile method of inhibiting RhoA induced biological effects in the cell is by using the chemical inhibitor of ROCK Y-27632, which is a downstream effector of RhoA implicated in stress fibre formation (Kimura et al., 1996; Leung et al., 1996) (see Section 1.4.1). To verify the specificity of Y-27632 as an inhibitor of RhoA induced stress fibres, we treated cells with Y-27632 prior to serum, LPA, PDGF or bradykinin stimulation. Treatment of cells with Y-27632 caused the loss of stress fibres even though serum was present (compare Figure 12a and b). Moreover, cells incubated with Y-27632 were devoid of stress fibres even after stimulation with LPA (compare Figure 12c,d). However, cells stimulated with PDGF were able to form lamellipodia even in the presence of Y-27632 (compare Figure 12e and f). Similar results were observed in cells stimulated with bradykinin where filopodia can be seen emanating from the cell body even in the presence of Y-27632 (compare Figure 12g and h). Thus the Rho-kinase inhibitor in our system demonstrates specificity towards RhoA and not the other RhoGTPases Rac1 or Cdc42.

Dominant negative inhibitory mutants of RhoGTPases have been used extensively to establish the involvement of various RhoGTPases in a number of processes (Feig, 1999). Point mutations in the nucleotide binding pocket results in dominant negative RhoGTPases which directly compete with the respective endogenous Rho family member for its nucleotide exchange factor and inhibit the GTP exchange required for activity (see Introduction Section 1.3.1). These mutations result in a higher affinity binding potential of dominant negative RhoGTPases over their endogenous counterparts, and as they cannot bind downstream effectors they block the effect of RhoGTPases within the cell. Cells were serum starved for 48 h to remove stress fibres prior to microinjection of a dominant negative RhoA protein (N19RhoA). Expression of (N19RhoA), as detected by a Myc-tag antibody (Figure 13a), prevented the reformation of stress fibres in response to serum (Figure 13b, compare transfected cell, broken arrow to non-expressing cells, solid arrows). Expression of dominant negative Rac1, as detected using a Myc-tag antibody (Figure 13c, broken arrow), blocked the formation of actin bundling and lamellipodia in response to PDGF, confirming that the dominant negative Rac1 construct was working and that PDGF is acting through Rac1 (Figure 13d, compare expressing cell broken arrow to non-

expressing cells, solid arrows). Furthermore, the expression of a dominant negative Cdc42 protein (N17Cdc42) as detected using a Myc-tag antibody (Figure 12e, broken arrow), blocked the formation of filopodia in response to bradykinin (Figure 12f, solid arrow), confirming that the dominant negative Cdc42 is working and that bradykinin is acting through Cdc42 to form filopodia (Figure 12f, compare transfected cell broken arrow to non-transfected cells solid arrows).

Thus the regulation of the actin cytoskeleton by both external stimuli and the use of molecular and pharmacological probes to modulate the respective Rho-GTPases is indistinguishable from previous reports in Swiss 3T3 cells (Hall, 1998; Nobes & Hall, 1995; Ridley & Hall, 1992). The ability to specifically manipulate the actin cytoskeleton in these cells provides a useful system in which to examine the influence of the actin cytoskeleton and in particular the role of RhoGTPases in c-Src localisation.

Figure 12

Y-27632 specifically inhibits RhoA mediated actin remodelling without effecting Rac-1 or Cdc42. Cells were placed in serum-free conditions overnight, which did not abolish stress fibres but acted to reduce the heterogeneity in the population prior to stimulation. Cells were subsequently treated with (b,d,f,h) or without (a,c,e,g) Y-27632 prior to stimulation with serum for 30 min (a,b), LPA (200 ng ml^{-1}) for 30 min (c,d), PDGF (25 ng ml^{-1}) for 30 min (e,f) or bradykinin (100 nM) for 5 min (g,h). Arrows indicate lamellipodia or filopodia respectively. Actin was visualised with rhodamine-phalloidin. Scale bars 25 μm.

Figure 12

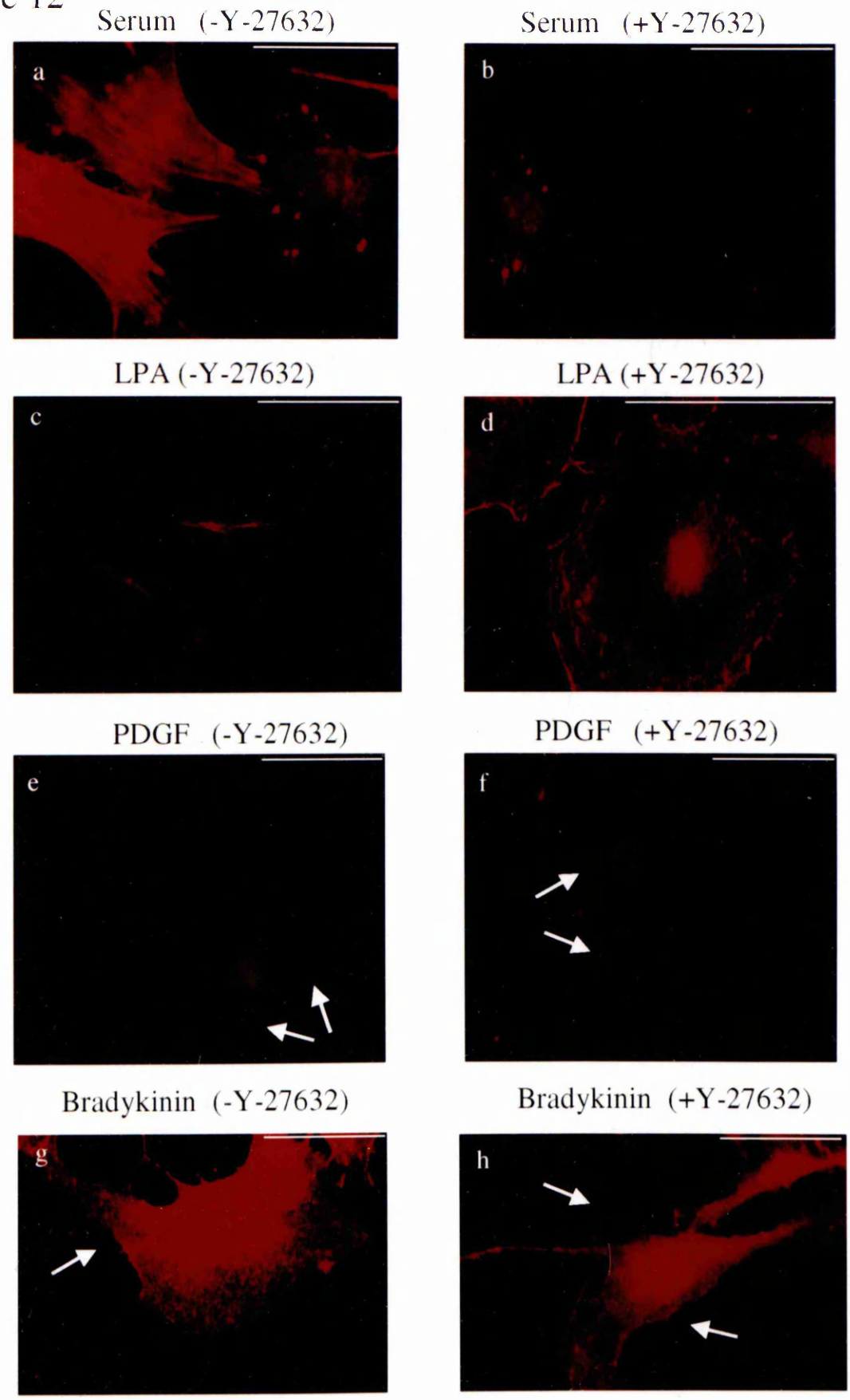
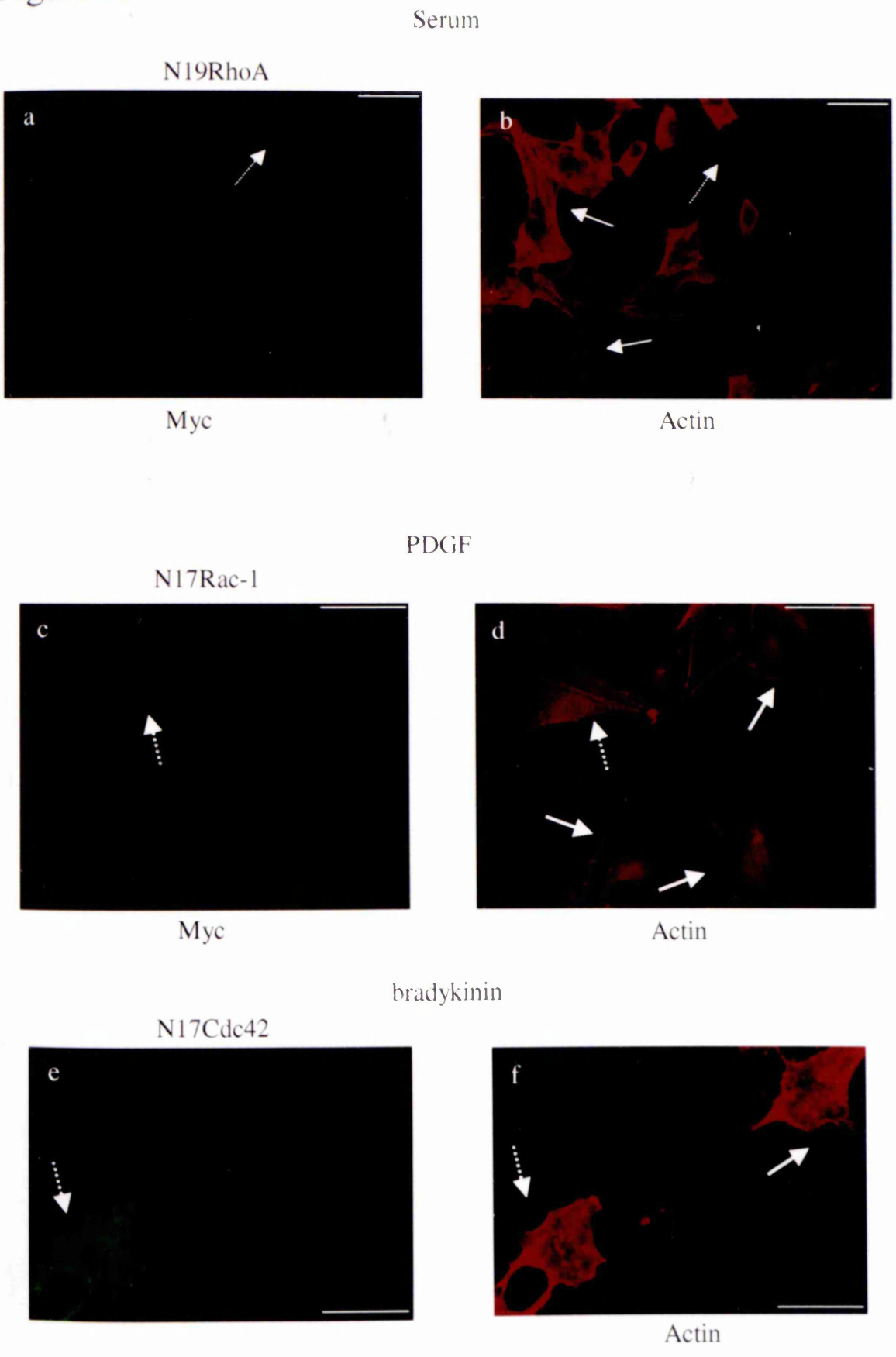

Serum (-Y-27632)
Serum (+Y-27632)
a
b
LPA (-Y-27632)
LPA (+Y-27632)
c
d
PDGF (-Y-27632)
PDGF (+Y-27632)
e
f
Bradykinin (-Y-27632)
Bradykinin (+Y-27632)
g
h

Figure 13

Expression of dominant negative RhoA, Rac1 and Cdc42 in cells inhibit the morphological actin structure induced by serum, PDGF or bradykinin stimulation respectively. Cells were serum starved for 48 h to remove stress fibres prior to microinjection of N19RhoA (a,b). Cells in the presence of serum were transfected with N17Rac (c,d) or N17Cdc42 (e,f) and stimulated with PDGF (25 ng ml^{-1}) for 30 min (c,d) or bradykinin (100 nM) for 5 min (e,f). Solid arrows indicate non-expressing cells, while broken arrows indicate expressing cell. Scale bars 25 μm.

Figure 13


Serum
N19RhoA
a
b
Myc
Actin
PDGF
N17Rac-1
c
d
Myc
Actin
bradykinin
N17Cdc42
e
f
Actin

3.2.2 Characterisation of Src251-GFP as a tool to monitor c-Src localisation in Swiss 3T3 cells.

Early observations demonstrate that c-Src is readily found in the perinuclear region of the cell where it co-localises with endosomal membrane structures (David-Pfeuty & Singer, 1980; Kaplan et al., 1992; Nigg et al., 1982). v-Src however, is abundant in both the perinuclear region of the cell and in focal adhesions (Burr et al., 1980; Nigg et al., 1982). The apparent difference in sub-cellular localisation of c-Src and v-Src led to the proposal that mutations, particularly in the C-terminal sequences of v-Src may be responsible for the altered distribution of v-Src (see Figure 1). Mutation of tyrosine-527 to phenylalainine in c-Src, which results in catalytic activation, as well as release of constraints on the SH3 and SH2 domains, led to c-Src being constitutively associated with focal adhesions (Kaplan et al., 1994). However, mutational analysis revealed that the kinase activity of c-Src was not required for its focal adhesion localisation and that domains within the first 251 residues of c-Src (Src251) were sufficient for its focal adhesion targeting. When expressed in cells, Myc-tagged Src251 was constitutively associated with focal adhesions (Kaplan et al., 1994). We have used a green fluorescent protein (GFP) fused to the first 251 amino acids of c-Src (Src251-GFP), to monitor Src localisation within the cell by direct fluorescence. This construct comprises the c-Src myristylation site, unique region and Src homology domains (SH3 and SH2), coupled to GFP, which replaces the kinase and regulatory domains at the carboxy-terminus (Figure 14). In this section we set out to characterise the behaviour of Src251-GFP in our Swiss 3T3 cells and to determine whether the localisation of Src251-GFP is regulated by the actin cytoskeleton, as has been previously described for v-Src in fibroblasts (Fincham et al., 1996). Moreover we wanted to establish whether the coupling of Src251 to GFP interferes with its targeting within the cell.

3.2.3 Translocation of Src251-GFP to focal adhesions is dependent on the actin cytoskeleton

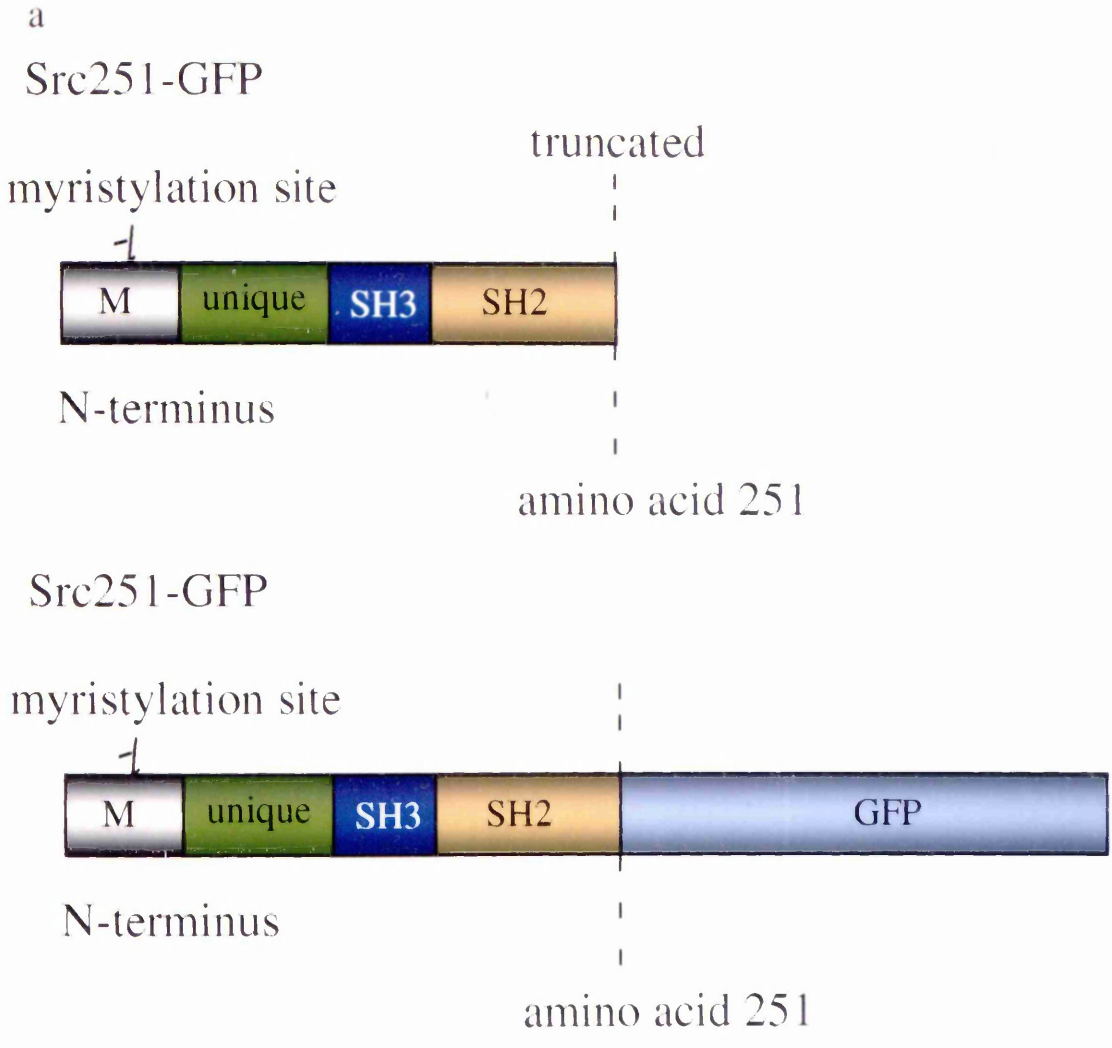
The translocation of Src251-GFP to the cell periphery is not regulated by intramolecular constraints and under culture conditions in which serum is present, Src251-GFP is therefore found at the cell periphery. As described earlier removal of serum from Swiss 3T3 fibroblasts for 48 h, results in a loss of actin stress fibres (Figure 11a and 15b). When Src251-GFP was micro-injected into serum-starved Swiss 3T3 fibroblasts, which have no stress fibres, it was located in the perinuclear region of the cell (Figure 15a,c, broken arrow). Treatment of serum starved cells with the actin disrupting drug cytochalasin D,

prevented both the reformation of the actin stress fibres in response to serum (compare Figure 15e to Figure 16a) as well as the translocation of Src251-GFP from the perinuclear region of the cell to peripheral focal adhesions (Figure 15g, broken arrow). In the presence of serum Src251-GFP was found in sites at the ends of actin stress fibres (Figure 16a,c), where it co-localised with the focal adhesion protein vinculin (Figure 16d-f) and paxillin (Figure 16g-i). This demonstrates that the targeting of Src251-GFP to focal adhesions is dependent on an intact actin cytoskeleton.

Figure 14

Src251-GFP construct. Schematic representation of the structural domains of Src251-GFP (a). Anti-Src western blot of lysates derived from Swiss 3T3 cells expressing Src251-GFP or empty vector (b).

Figure 14


a
Src251-GFP
truncated
myristylation site
M
unique
SH3
SH2
N-terminus
amino acid 251
Src251-GFP
myristylation site
M
unique
SH3
SH2
GFP
N-terminus
amino acid 251

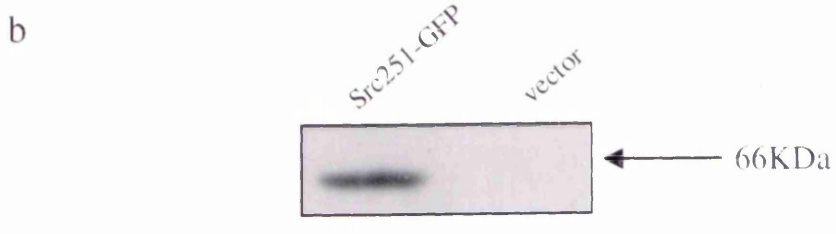

b
Src251-GFP
vector
66KDa

Figure 15

Translocation of Src251-GFP to focal adhesions is dependent on the actin cytoskeleton. Cells were serum starved for 48 h prior to microinjection of Src251-GFP (a-c). Cells serum-starved for 48h were microinjected with Src251-GFP and were pre-treated with 0.1 μg ml^{-1} of cytochalasin D for 2 h before protein expression and serum stimulation (e-g). Solid arrows indicate non-expressing cells, while broken arrows indicate expressing cells. Actin was visualised with rhodamine-phalloidin (b,c,f,g) and Src251-GFP was localised by direct fluorescence (a,c,e,g). Scale bars 25 μm.

Figure 15

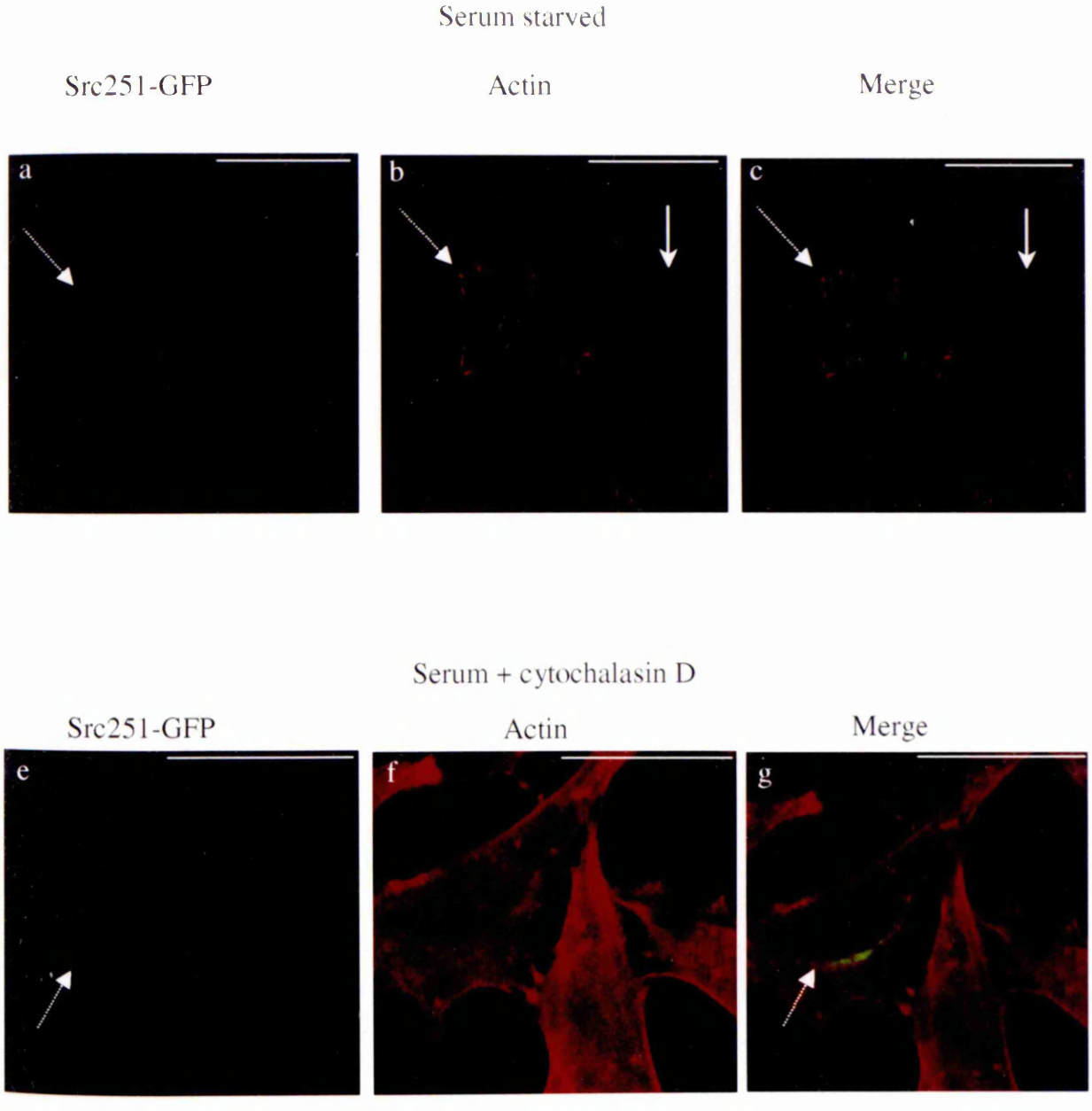
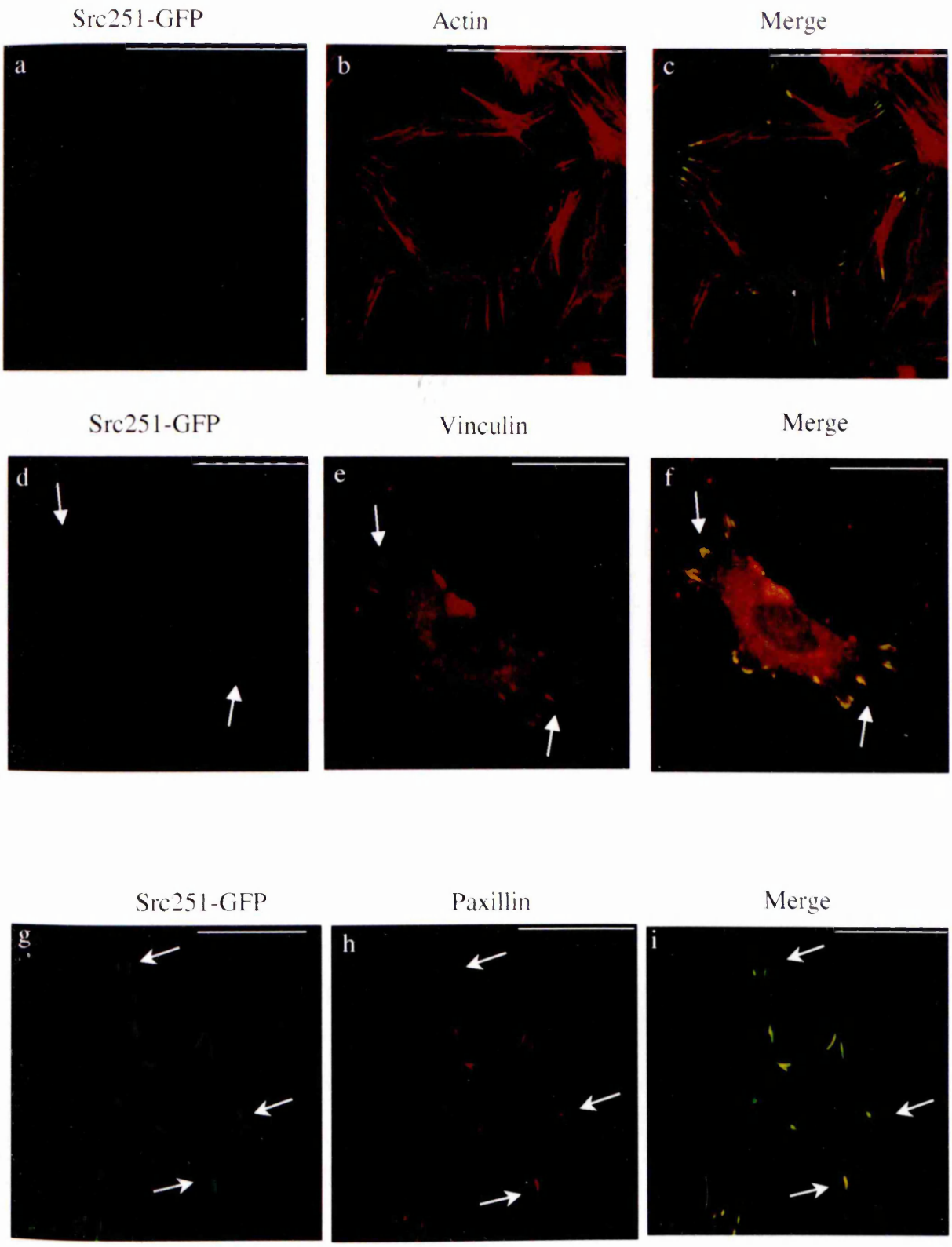

Serum starved
Src251-GFP
Actin
Merge
a
b
c
Serum + cytochalasin D
Src251-GFP
Actin
Merge
e
f
g

Figure 16

In the presence of serum Src251-GFP associates with focal adhesions. Cells were serum starved to remove stress fibres prior to microinjection of Src251-GFP. Cells serum-starved for 48 hr were microinjected with Src251-GFP and following expression of the protein were stimulated with 10% serum for 30 min (a-i), where it was found at the ends of actin fibres (a-c) or co-localising with vinculin (d-f) or paxillin (g-i). Arrows indicate Src251-GFP and vinculin staining. Actin was visualised with rhodamine-phalloidin (b,c), while vinculin (e,f) or paxillin (h,i) were visualised using anti-vinculin or anti-paxillin antibodies. Src251-GFP was detected in the same cell by direct fluorescence. Scale bars 25 μm.

Figure 16


Src251-GFP
Actin
Merge
a
b
c
Src251-GFP
Vinculin
Merge
d
e
f
Src251-GFP
Paxillin
Merge
g
h
i

3.2.4 Discussion.

The regulation of the actin cytoskeleton by both external stimuli and the use of molecular probes to modulate the respective RhoGTPases in this study was indistinguishable from previous reports in Swiss 3T3 cells (Hall, 1998; Nobes & Hall, 1995; Ridley & Hall, 1992). Different protocols were used in previous studies; cells were plated at low density and after 6-10 days, when the cell were quiescent, they were serum starved in 0.2 % serum overnight (16 h) which resulted in the loss of stress fibres. In this study, cells were grown for one day after plating and subsequently serum starved for 48 h in serum free medium to induce stress fibre disassembly. Both methods appear to induce similar morphological changes in the actin cytoskeleton.

The disruption and breakdown of the actin cytoskeleton by serum starvation or pharmocological intervention by cytochalasin D have been validated in our system (Ridley & Hall, 1992). Treatment of cells with the Rho-kinase inhibitor blocked both LPA and serum induced stress fibres as previously described by others (Kimura et al., 1996; Leung et al., 1996). Y-27632, however, has also been reported to inhibit protein kinase C related kinase 2 (PRK2) in vitro as effectively as ROCK (Kimura et al., 1996; Leung et al., 1996; Uehata et al., 1997). PRK2 is also a RhoA effector (see Figure 6 Section 1.3.3) but with different substrate specificity to ROCK. We cannot therefore rule out the possibility that Y-27632 is inhibiting other kinases than ROCK. However, in the context of these experiments we have shown that treatment with Y-27632 results in the loss of stress fibres and does not effect Rac1 or Cdc42-induced actin remodelling, implying that it is a suitable and versatile tool to block RhoA activity without altering Rac1 or Cdc42-induced actin rearrangements. Dominant negative inhibitory mutants of RhoGTPases have been used extensively to elucidate the involvement of various RhoGTPases in a number of processes (Feig, 1999). The use of dominant negative RhoGTPases and interpretation of results using these mutants however has to be met with some caution. Dominant negative RhoGTPases directly compete with the respective endogenous Rho family member for its nucleotide exchange factor (see Introduction section 1.3.1). Due to the high affinity binding potential of dominant negative RhoGTPases over their endogenous counterparts, they block the effect of RhoGTPases in the cell, by inhibiting GTP exchange required for activity. One concern therefore, is that these mutants inhibit the GEF activity towards RhoGTPases rather than the RhoGTPases themselves. As discussed in the introduction (see Section 1.3.1) many of the nucleotide exchange factors for RhoGTPases activate multiple Rho family members. Therefore, expression of one dominant negative Rho-

family member may be expected to block the activation of other members. Other methods of RhoGTPase activation have been reported to bypass this GEF-dependent activation pathway (Feig, 1999) and therefore using dominant negative mutants to block this pathway may lead to a misinterpretation of results, without caution. RhoA for example, has been shown to be activated by the RhoGEF, p115 RhoGEF however recent evidence has shown RhoA can also be activated directly by Gâã or PYK2 thereby bypassing the GEF-dependent activation pathway leading to an ineffective inhibition of RhoA (see Section 1.3.3) (Shi et al., 2000; Ueda et al., 2000). In the context of our experiments, however, expression of individual inhibitory mutants such as N19RhoA, N17Rac1 and N17Cdc42 has resulted in a remarkably specific phenotype, a property which has been frequently reported by others (Feig, 1999; Hall, 1998).

The external stimuli LPA, PDGF and bradykinin are well documented to specifically induce the activity of the respective RhoGTPases RhoA, Rac1, and Cdc42 by modulating the activity of GEFs (see Section 1.3.1), which have specific GDP-GTP exchange activity towards the specific Rho family members. The specificity of these external stimuli towards their respective RhoGTPase has previously been illustrated, both by the distinct actin structures produced upon stimulation and by directly measuring the GTP-bound (active) state of each family member after stimulation (Hall, 1998; Nobes & Hall, 1995; Ren et al., 1999; Ridley & Hall, 1992; Ridley et al., 1992). The external stimuli used here are therefore suitable to modulate the activity of RhoGTPases within Swiss 3T3 cells and thereby serve as versatile tools to examine their influence on c-Src localisation. To compliment this, a more specific method of altering the individual RhoGTPase activity was also employed by expressing constitutively active mutants of the respective RhoGTPases. These mutants mimic the permanently active GTP-bound state of the proteins, as a result of insensitivity to GAP activity, (Van Aelst & D'Souza-Schorey, 1997). As described above the morphological rearrangements of the actin cytoskeleton were indistinguishable from earlier reports in which constitutively activated RhoGTPase proteins were microinjected into the cytoplasm of serum starved, quiescent cells.

We can therefore conclude that manipulation of the cytoskeletal network in the Swiss 3T3 fibroblast cells in our hands is comparable to previous reports used to assess and analyse the role of RhoGTPases and is therefore a suitable model in which to examine the influence of the actin cytoskeleton and in particular the role of RhoGTPases in c-Src localisation.

The use of GFP to detect proteins by direct fluorescence has provided unprecedented insight into the localisation of proteins, that may otherwise, not have been observed by more conventional methods. The fusion of GFP to proteins has been shown in most cases not to interfere with the behaviour of the proteins in question. GFP tagged vinculin or actin for example, have frequently been used to monitor adhesion and actin dynamics in real time during cell migration (Small et al., 1995; Small et al., 1999; Small et al., 2002; Westphal et al., 1997). The potential for examining Src251-GFP localisation in real time during cell migration and stimuli-induced actin remodelling will be discussed later (Chapter 7, Future work). Here we conclude, firstly, that the addition of GFP to the C-terminal end of Src251, like the addition of a Myc tag (Kaplan et al., 1994), did not interfere with its translocation to focal adhesions. Moreover, a recent study monitoring integrin-dependent traction forces important for cell migration, utilised Src251-GFP and did not report any alterations in the known activity and behaviour of this protein compared to Src251 alone (Felsenfeld et al., 1999).

The localisation of v-Src and c-Src to the cell periphery in fibroblasts and the localisation of c-Src to cell-cell junctions in epithelial cells requires an intact actin cytoskeletal network (Fincham et al., 1996; Owens et al., 2000). Moreover, other ubiquitously expressed family members such as Yes and Fyn have also been shown to translocate to cell-cell adhesions in an actin dependent manner (Owens et al., 2000). In this study we have shown that the peripheral localisation to focal adhesions of Src251-GFP, a fusion protein that contains the Src SH3 and SH2 domains but has no kinase domain, is also dependent on an intact actin cytoskeleton. These results imply that Src251-GFP is a useful tool to monitor the control of c-Src intracellular targeting by direct fluorescence.

3.3 Summary

In this chapter we have demonstrated that the Swiss 3T3 fibroblasts can be used to examine the influence of the actin cytoskeleton and in particular the role RhoGTPases play in c-Src localisation. Furthermore, we have characterised Src251-GFP in these cells and demonstrated it to be a valuable tool to monitor c-Src within the cell. We therefore set out to assess whether RhoGTPases play a key role in the spatial regulation of c-Src.

Chapter 4

4 Co-ordinated regulation of the actin cytoskeleton by RhoGTPases spatially regulates Src targeting to the cell periphery.

4.1 Purpose

We have shown in the previous chapter, that the Rho family GTPases Cdc42, Rac1 and RhoA can be manipulated in Swiss 3T3 fibroblasts, by stimulation of transmembrane receptors or by molecular intervention to produce morphologically distinct actin structures. Co-ordination of this actin remodelling and these structures has been implicated in cell motility by providing both the protrusive and contractile forces required for cell migration (Chrzanowska-Wodnicka & Burridge, 1996; Hall, 1998). The role however of RhoGTPases in controlling the temporal and spatial localisation of proteins has not been fully elucidated. The purpose of this chapter was to test the hypothesis that RhoGTPases serve a central role in the spatial regulation of proteins, such as Src, thereby serving to regulate their action within the cell.

4.2 Results

4.2.1 Localisation of Src251-GFP to specific peripheral sites is under the control of Rho family GTPases.

Previously we demonstrated that the translocation of v-Src to focal adhesions is under the control of RhoA (Fincham et al., 1996). Furthermore, as treatment with serum, which activates RhoA, resulted in translocation of Src251-GFP from a perinuclear location to focal adhesions (Figure 16), we used molecular and pharmacological intervention to determine whether this was a RhoA-dependent event. Expression of a constitutively active RhoA protein (V14RhoA) in cells resulted in the formation of stress fibres in the absence of serum (Figure 17a). In Figure 17, the broken arrow indicates a cell expressing V14RhoA where stress fibres are present whereas the non-expressing cell (solid arrow) had no visible stress fibres. Cells expressing Src251-GFP and V14RhoA where detected using direct fluorescence by Src251-GFP. Co-expression of V14RhoA with Src251-GFP was sufficient to localise Src to the cell periphery (Figure 17b, broken arrow), were it targeted to the ends of actin stress fibres (Figure 17c, broken arrow). Expression of V14RhoA in

serum starved cells also induced the formation of prominent focal adhesions (Figure 17d). The broken arrow indicates a cell expressing V14RhoA where the focal adhesion protein vinculin was located, whereas the non-expressing cells (solid arrows) had weak focal adhesion staining. Co-expression of Src251-GFP with V14RhoA (Figure 17e) resulted in the co-localisation of Src with the focal adhesion protein vinculin (Figure 17f).

As described in Chapter 3 (see Section 3.1.1), Rac1 activation leads to localised actin polymerisation at the cell periphery resulting in the formation of lamellipodia (Figure 9b,e) (Nobes & Hall, 1995; Ridley et al., 1992). We therefore addressed how activation of Rac1 influenced the localisation of Src251-GFP within the cell. Cells in the presence of serum (i.e. a RhoA stimuli) were co-transfected with L61Rac1 and Src251-GFP. Expression of an activated Rac1 protein (L61Rac1) resulted in the formation of lamellipodia (Figure 18a, broken arrow) and co-expression of Src251-GFP with L61Rac1 resulted in the localisation of Src251-GFP to the Rac1-induced peripheral lamellipodia (Figure 18b,c, broken arrows). Both lamellipodia and filopodia contain small focal complexes within their structures, which have been defined as small focal adhesion structures (see Chapter 3) (Nobes & Hall, 1995). In conjuction with the actin rearrangement described for Rac1 and Cdc42 (see Chapter 3 Figure 9b,e and c,f) there is also thought to be a dynamic remodelling and breakdown of focal adhesions to focal complexes during lamellipodia or filopodia formation (Laukaitis et al., 2001). In cells expressing activated Rac1 protein, vinculin localised to small focal complexes at the leading edge of the cell (Figure 18d, broken arrows) (Nobes & Hall, 1995) where it co-localised with Src251-GFP (Figure 18e,f, broken arrows). However, in cells not expressing activated Rac1 (Figure18d, solid arrow) vinculin was located in larger discrete structures representing focal adhesions due to the presence of serum, which as described above induces RhoA activity causing both stress fibres formation and focal adhesion assembly. This therefore demonstrates that in parallel with the remodelling of the actin cytoskeleton, lamellipodia formation is associated with the loss of focal adhesions and the formation of smaller focal complexes to which Src251-GFP is located.

Similar to these observations we found that expression of a constitutively active Cdc42 protein (V12Cdc42) induced the formation of filopodia (Figure 19a, broken arrows show expressing cell) and co-expression of Src251-GFP with activated Cdc42 induced some of the expressed Src251-GFP to move from the perinuclear region of the cell to membrane regions at the base of filopodia (Figure 19b). At enhanced resolution (Figure 19c, inset) it can be seen in distinct complexes spaced along the actin filopodia (solid arrows, inset).

Similar to the observations with the expression of activated Rac1, we found that activated Cdc42 induced focal adhesion re-modelling to produce focal complexes along the base and length of the filopodia (Figure 19 d,e and see Chapter 3 Figure 10). Src251-GFP co-localised with vinculin (Figure 19f), in both focal adhesion complexes at the base of filopodia (Figure 19f, broken arrows, inset) and in complexes spaced along filopodia (Figure 19f, solid arrow).

Figure 17

Src localisation to focal adhesions is under the control of RhoA mediated stress fibres. Cells were serum starved for 48 h to remove stress fibres prior to co-microinjection of V14RhoA and Src251-GFP (broken arrows). Solid arrow indicates non-injected cells. Actin was visualised with rhodamine-phalloidin (a), while vinculin (d) was visualised using an anti-vinculin antibody. Src251-GFP was detected in the same cells by direct fluorescence (b,e) with merged images shown in (c, f). Scale bars 25 μm.

Figure 17

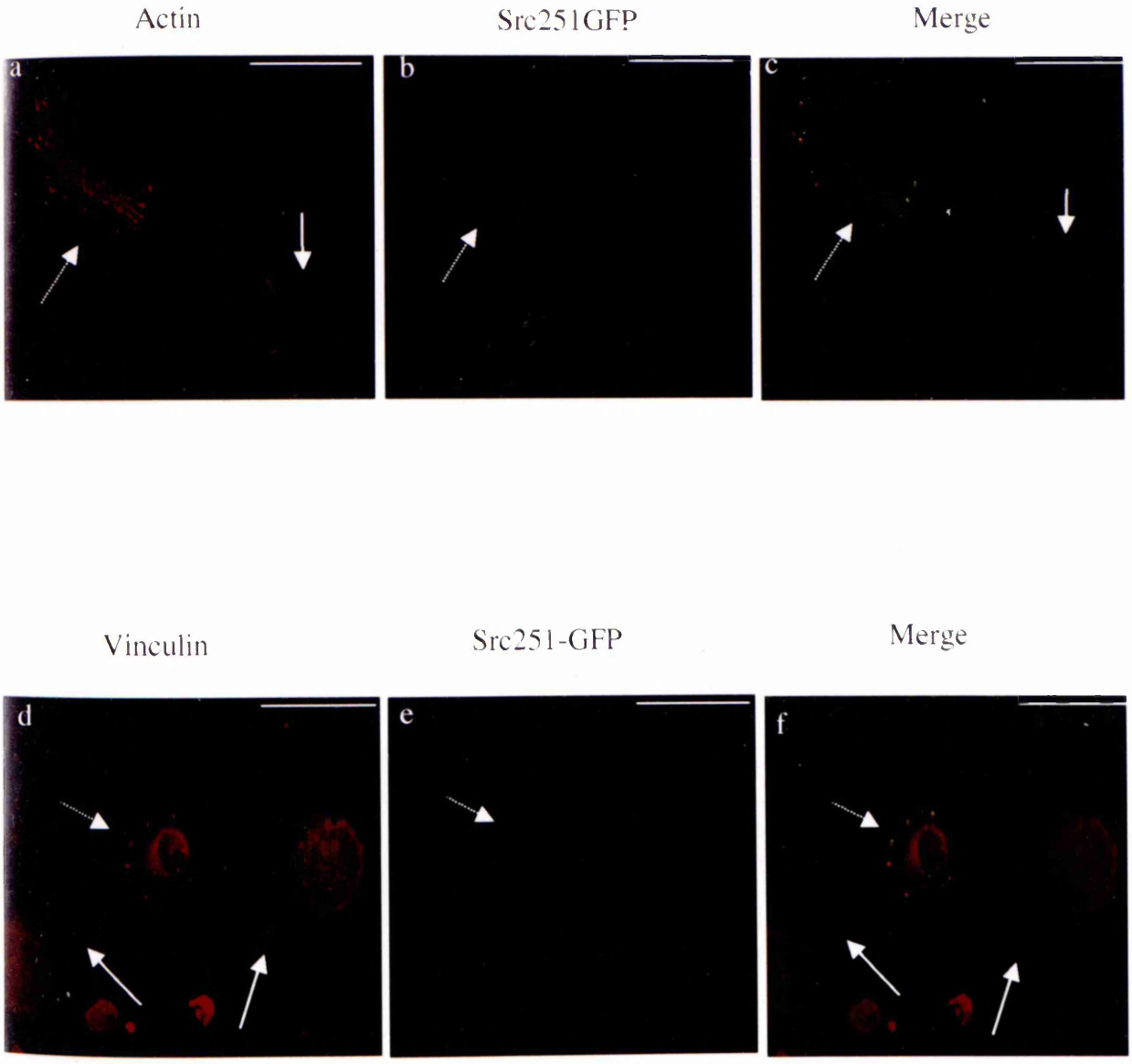


Figure 18

Src localisation to lamellipodia is under the control of Rac mediated actin remodelling. Cells in the presence of serum were co-transfected with L61Rac and Src251-GFP. Broken arrows indicate transfected cells while solid arrows indicate non-transfected cells. Actin was visualised with rhodamine-phalloidin (a,c), while vinculin (d,f) was visualised using an anti-vinculin antibody. Src251-GFP was detected in the same cells by direct fluorescence (b,e,c,f). Scale bars 25 μm.

Figure 18

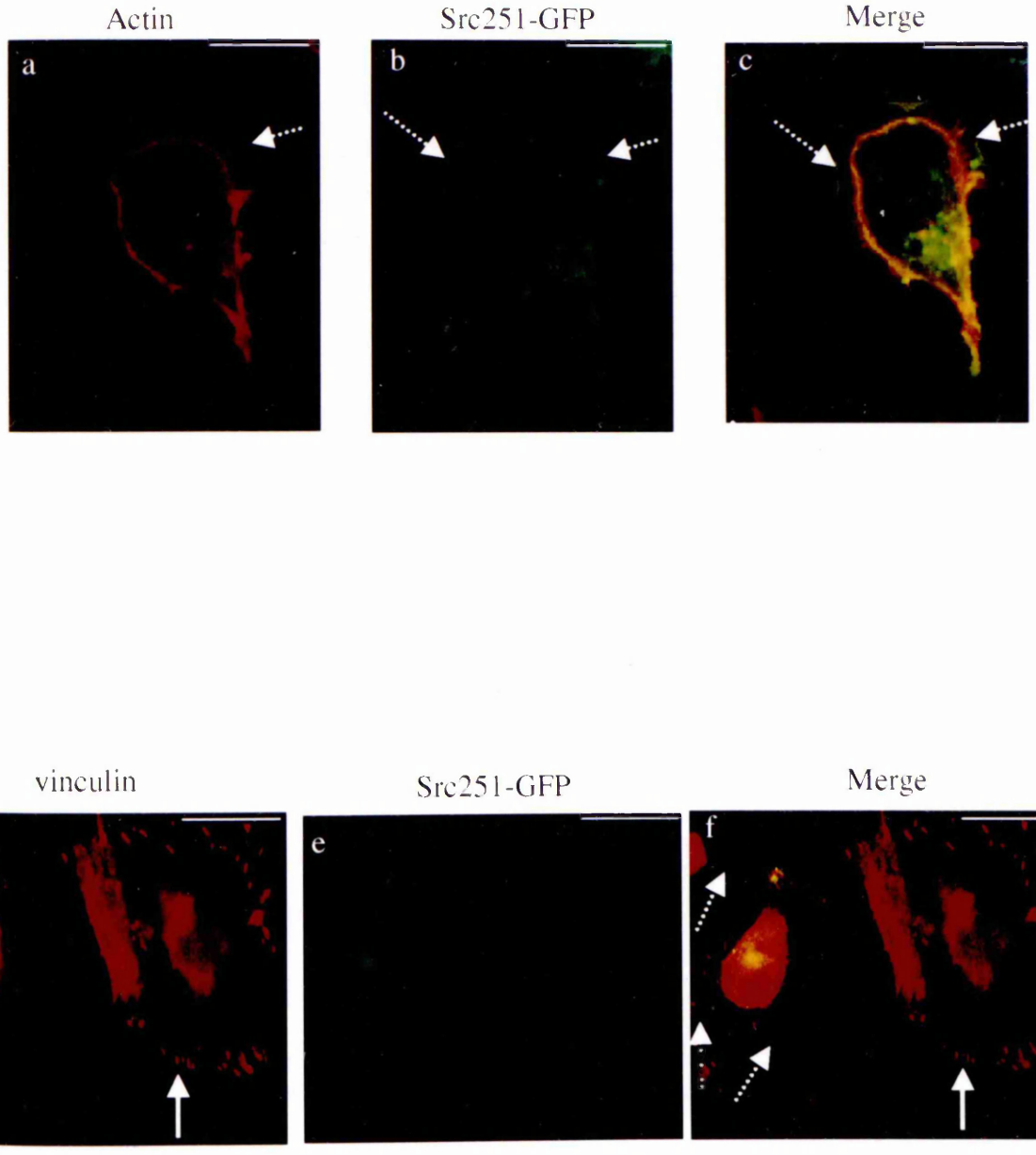
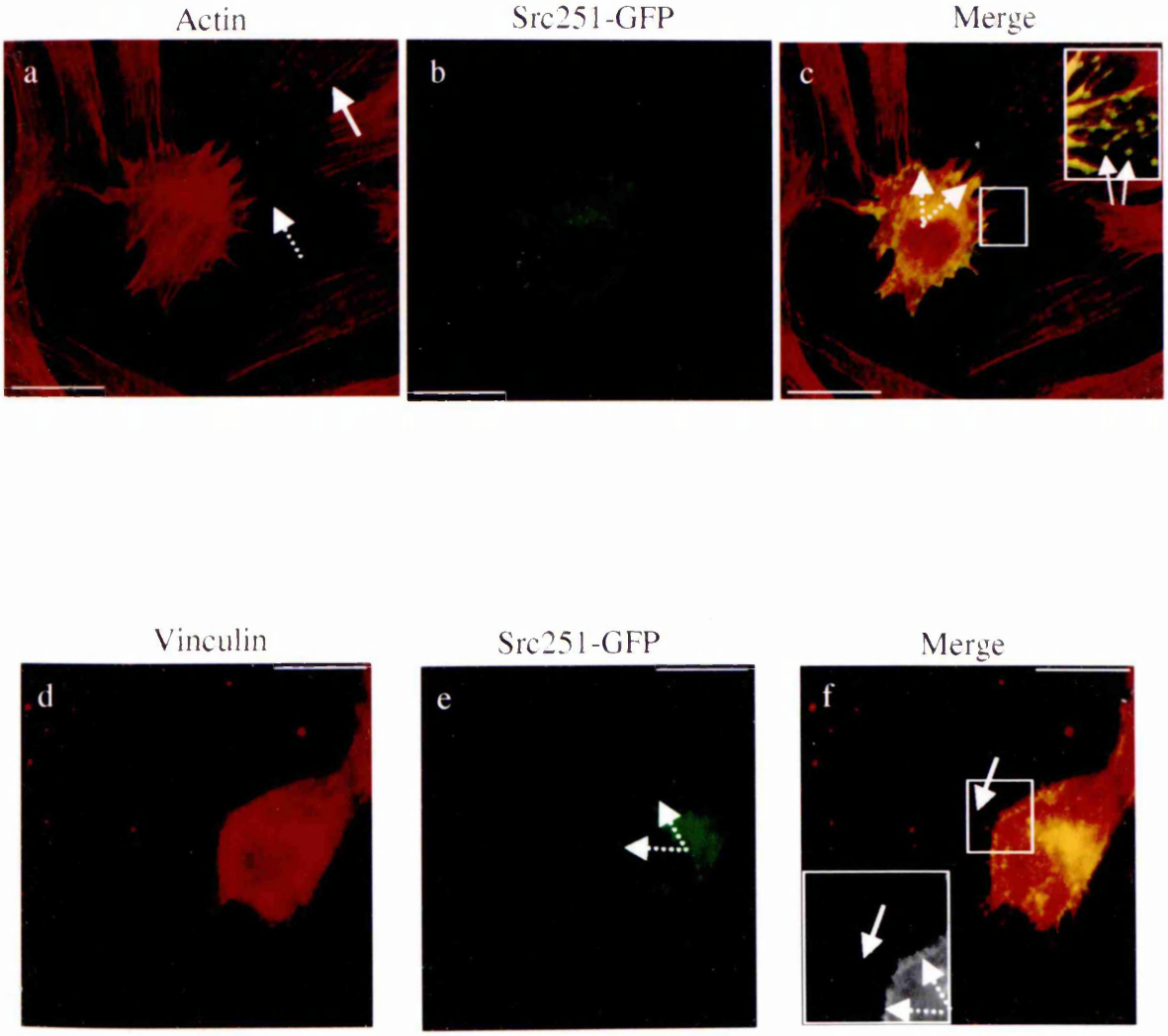


Figure 19

Src localisation to filopodia is under the control of Cdc42 mediated actin remodelling. Cells in the presence of serum were co-transfected with V12Cdc42 and Src251-GFP. Broken arrows indicate transfected cells while solid arrows indicate filopodia. Actin was visualised with rhodamine-phalloidin (a,c), while vinculin (d,f) was visualised using an anti-vinculin antibody. Src251-GFP was detected in the same cell by direct fluorescence (b,e,c,f). Scale bars 25 μm.

Figure 19



4.2.2 Localisation of endogenous or wt-Src by RhoGTPases is indistinguishable from the targeting of Src251-GFP.

To eliminate the possibility that the localisation of Src251-GFP to the peripheral sites described above was a consequence of over-expression of a mutated c-Src protein, we set out to relate the observed targeting of Src251-GFP to the endogenous protein. We were able to demonstrate the presence of endogenous Src in focal adhesions in response to LPA (Figure 20a), where it was found at the ends of actin stress fibres (Figure 20b). Stimulation of cells with PDGF, which activates Rac1, was sufficient to induce re-localisation of endogenous Src to lamellipodia (Figure 20c), where it co-localised with actin bundles (Figure 20d). It was difficult to visualise endogenous Src in filopodia following stimulation with bradykinin which is most likely due to problems in detecting low levels of endogenous proteins in such small structures. However, this could be over come by expressing full length wild type Src protein (wt-Src) in the cells (Figure 21a). Treatment of cells over-expressing wt-Src with bradykinin resulted in Src being readily seen at the base of filopodia (Figure 21b) and at enhanced resolution along the length of the filopodia (Figure 21b, inset). Treatment with LPA also resulted in the localisation of wt-Src at the cell periphery (Figure 21d), at the end of actin stress fibres (Figure 21e), while PDGF stimulation of cells expressing wt-Src resulted in its localisation to lamellipodia (Figure 21f), where it co-localised with actin bundles (Figure 21g).

In this section we have shown that the peripheral localisation of Src251-GFP, a fusion protein that contains Src's SH3 and SH2 domains but has no kinase domain, to focal adhesions, is dependent on RhoA-mediated assembly of actin stress fibres. Furthermore the Rho family proteins Rac1 and Cdc42 direct the precise intracellular targeting of Src to other functional structures such as lamellipodia and filopodia respectively. The data presented here on the targeting of endogenous and wild type Src implies that the control of Src251-GFP targeting is the same as for endogenous Src or the expression of wild type Src. This validates the use of Src251-GFP as a tool for following c-Src targeting within the cell.

4.2.3 Molecular or pharmacological inhibition of RhoA blocks Src targeting to focal adhesions.

Experiments in which cells expressing the Myc-tagged N19RhoA, proteins were detected using a Myc-tag antibody revealed that in all cases co-injection or co-transfection of Src251-GFP and the Rho family proteins resulted in the expression of both proteins, thus

allowing the use of the GFP tag to visualise the expressing cells by direct fluorescence. Cells were serum starved for 48 h to remove stress fibres (see Chapter 3 Section 3.2.1), prior to microinjection of a dominant negative RhoA protein (N19RhoA) and Src251-GFP. As described in Chapter 3, expression of N19RhoA prevented the reformation of stress fibres in response to serum. Co-expression of Src251-GFP with N19RhoA resulted in the inhibition of stress fibre assembly in response to serum as a result of N19RhoA expression (Figure 22b). The broken arrow shows a cell expressing N19RhoA, which is consequently devoid of actin stress fibres and in which Src251-GFP remained perinuclear (Figure 22a,c), while non-expressing cells have reformed stress fibres in response to serum (Figure 22b,c solid arrows). This was in contrast to Src's location at peripheral focal adhesions in cells stimulated with serum in the absence of N19RhoA (Figure 22d,f), where it was found at the ends of stress fibres (Figure 22e,f broken arrows).

Figure 20

Localisation of endogenous Src by RhoGTPases is indistinguishable from the targeting of Src251-GFP. Cells were placed in serum-free conditions overnight, which did not abolish stress fibres but acted to reduce the heterogeneity in the population following growth factor stimulation. These cells were then stimulated with LPA (200 ng ml^{-1}) for 30 min (a,b) or PDGF (25 ng ml^{-1}) for 30 min (c,d) and endogenous Src visualised using the N2-17 Src antibody (a,c) and actin was visualised with rhodamine-phalloidin (b,d). Scale bars 25 μm.

Figure 20

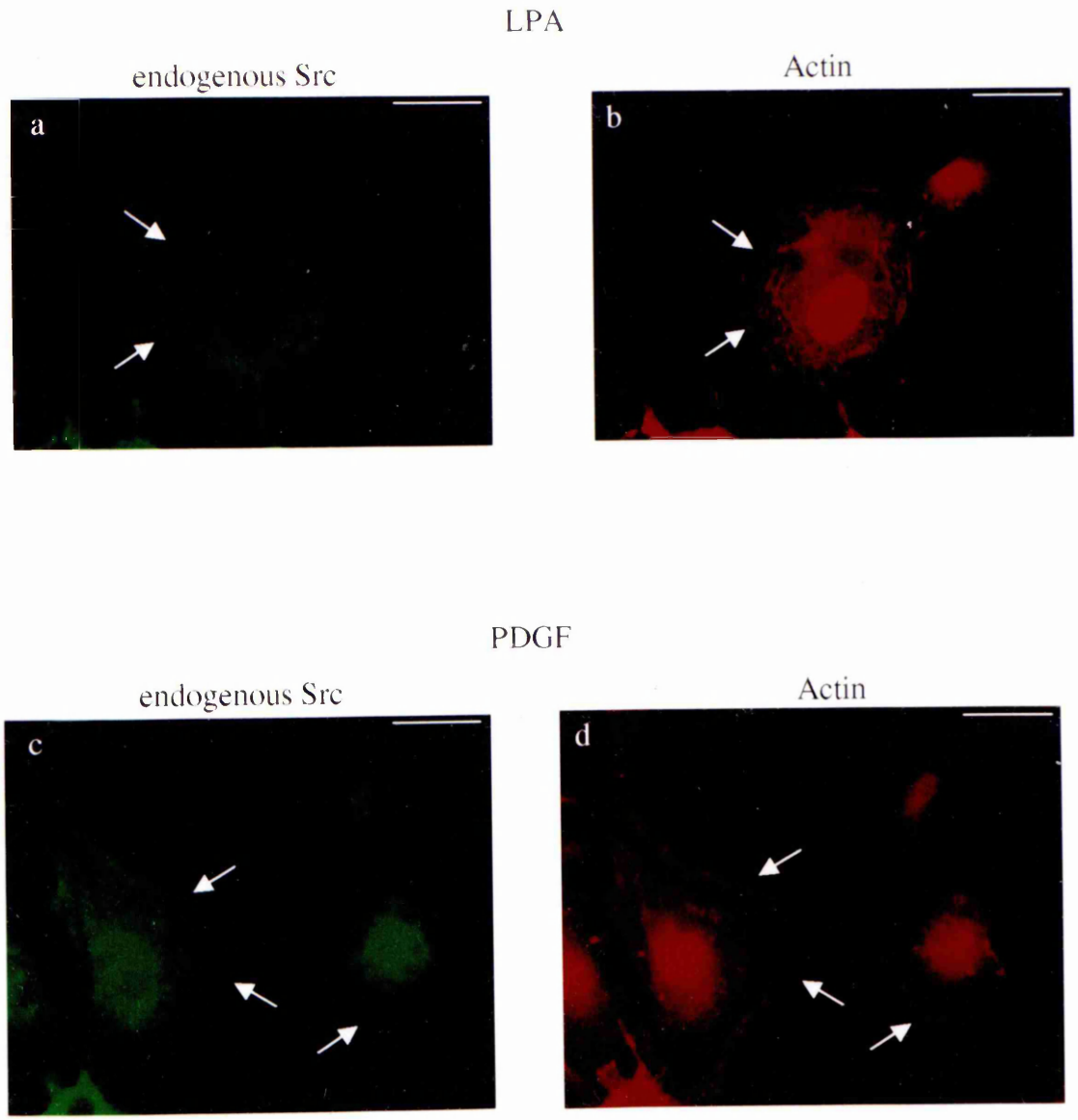


Figure 21

Localisation of wt-Src by RhoGTPases is indistinguishable from the targeting of Src251-GFP. Anti-Src western blot of lysates derived from Swiss 3T3 cells expressing wt-Src and vector alone (a). Cells were transfected with wt-Src and following expression of the protein were placed in serum-free conditions overnight (b-g). Cells were then stimulated with bradykinin (100 nM) for 5 min (b,c), LPA (200 ng ml^{-1}) for 30 min (d,e) or PDGF (25 ng ml^{-1}) for 30 min (f,g). Exogenously expressed Src was visualised using the chicken specific Src EC10 antibody (b,d,f) and actin was visualised with rhodamine-phalloidin (c,e,g). Scale bars 25 μm.

Figure 21

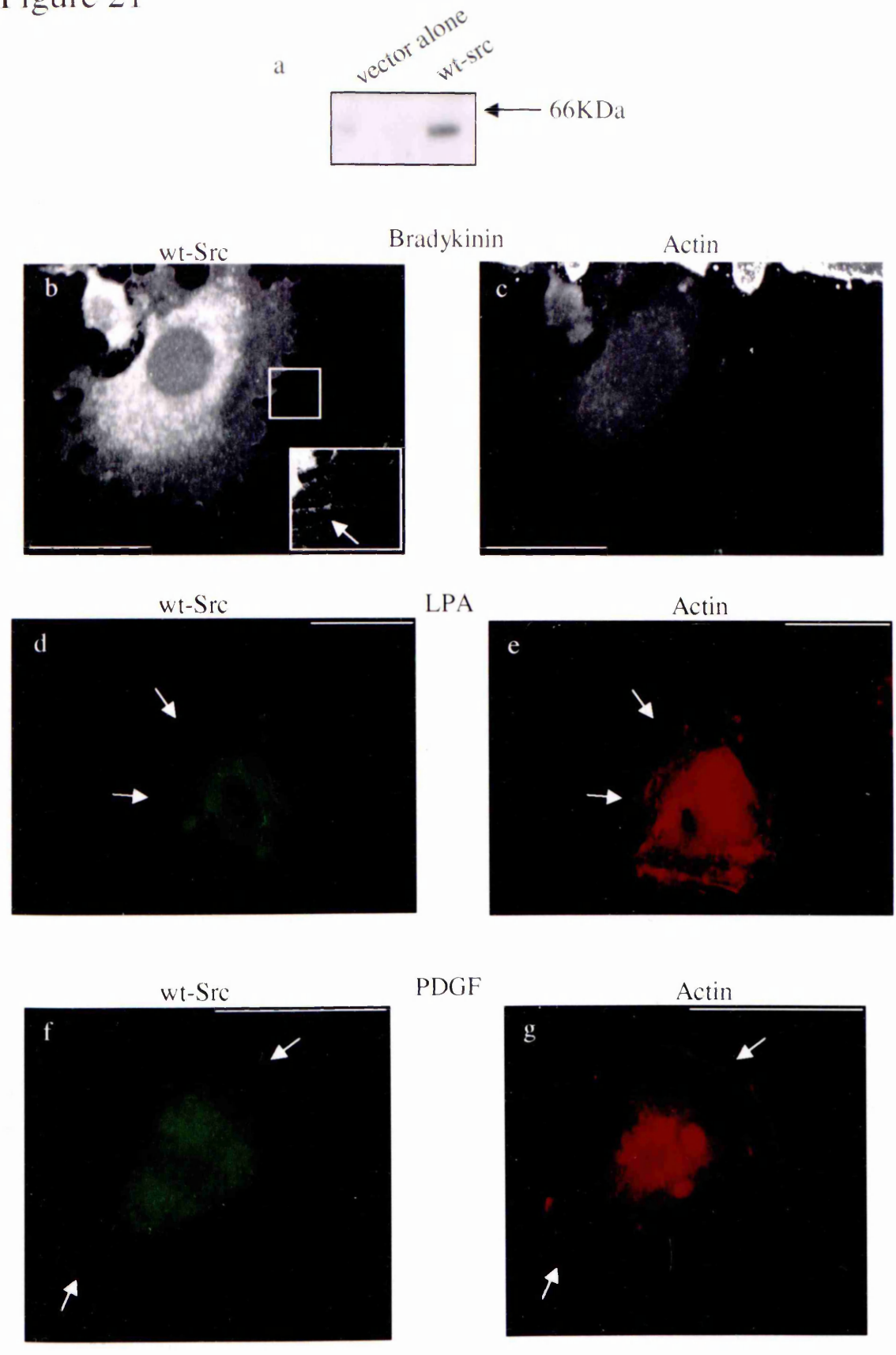
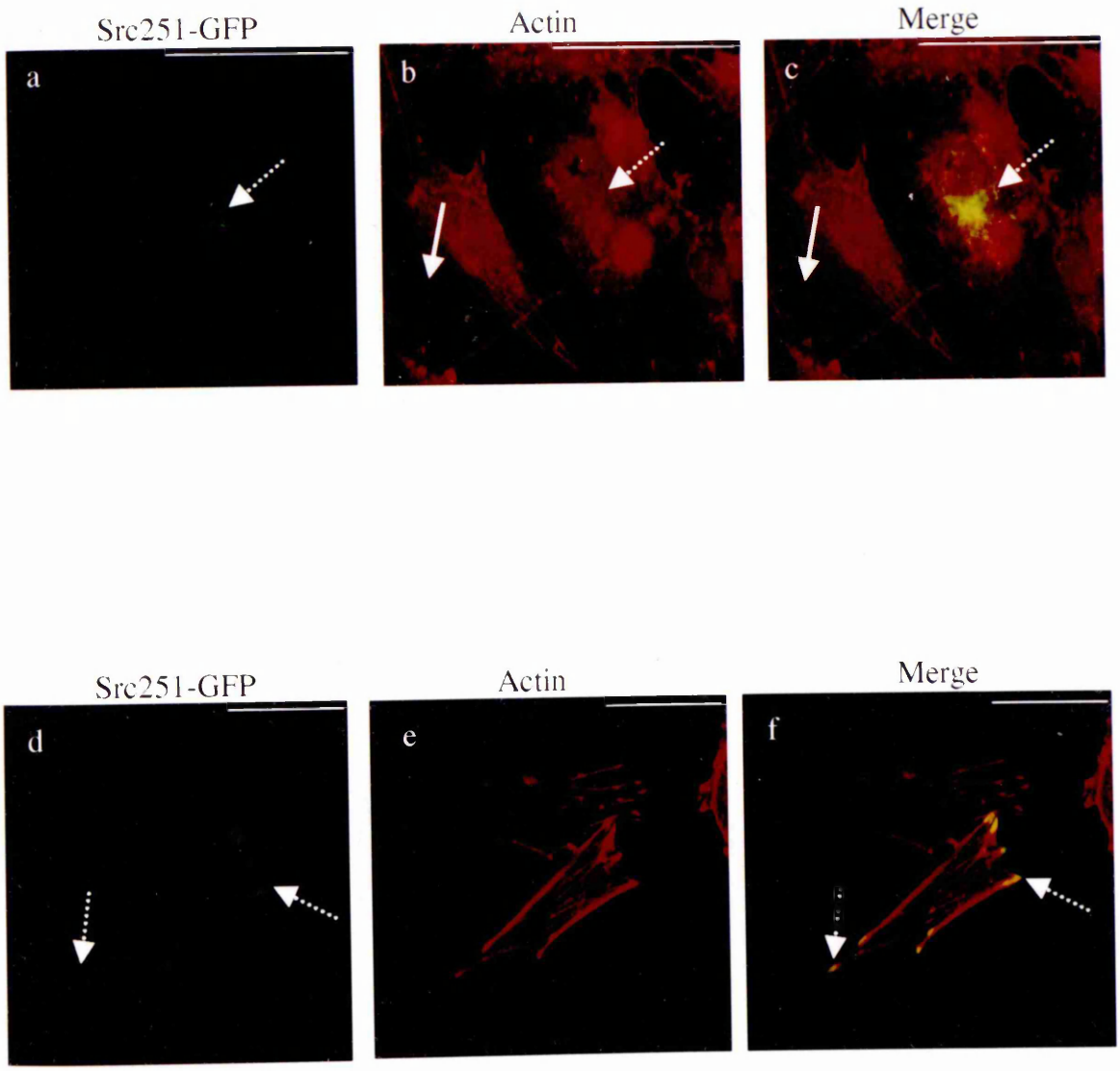

a
vector alone
wt-src
66KDa
wt-Src
Bradykinin
Actin
b
c
wt-Src
LPA
Actin
d
e
wt-Src
PDGF
Actin
f
g

Figure 22

Expression of dominant negative RhoA in cells blocks Src targeting to focal adhesions. Cells were serum starved for 48 h to remove stress fibres prior to microinjection of Src251-GFP and N19RhoA (a-c) or microinjection of Src251-GFP alone (d-f). Following expression of the proteins, cells were then stimulated with 10% serum for 30 min (a-f). Broken arrows indicate microinjected cells while solid arrows are non-expressing cells. Actin was visualised with rhodamine-phalloidin (b,c,e,f). Src251-GFP was detected by direct fluorescence (a,c,d,f). Scale bars 25 μm.

Figure 22

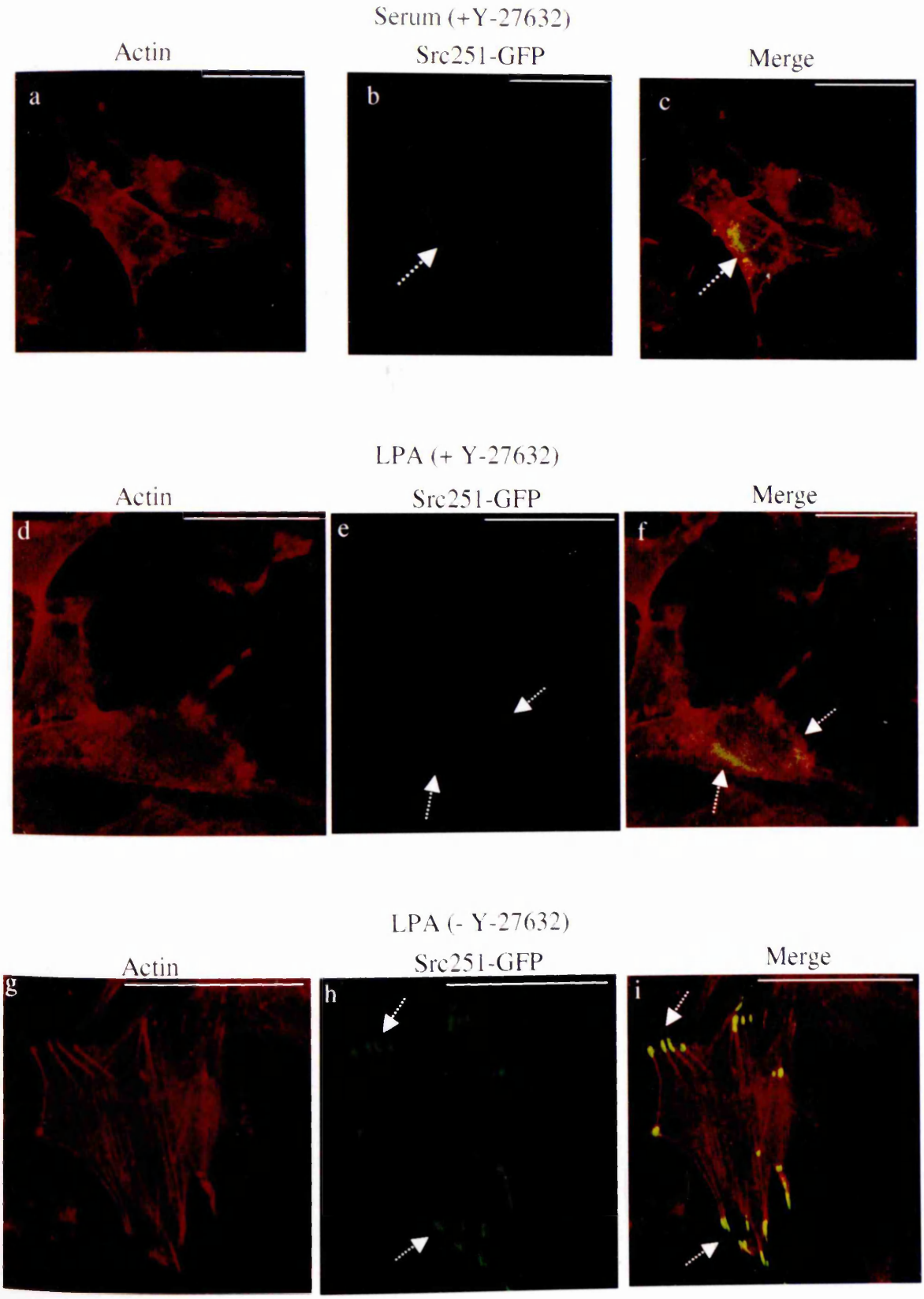


As described in Chapter 3 (see section 3.2.1), Y-27632 is a chemical inhibitor of Rho-kinase (Uehata et al., 1997), which is a downstream effector of RhoA implicated in stress fibre formation (Kimura et al., 1996; Leung et al., 1996). We have described in this study that treatment of cells with Y-27632 specifically block RhoA-induced stress fibre formation, while Rac1 and Cdc42-induced actin rearrangements were unaffected in the presence of the inhibitor. We therefore used Y-27632 to further establish whether Src's peripheral targeting was a RhoA dependent process. Following transfection, cells were treated with Y-27632 prior to expression of Src251-GFP. Under these conditions the cells were devoid of stress fibres even though serum was present (Figure 23a) and translocation of Src251-GFP to peripheral focal adhesions in response to serum was blocked (Figure 23b,c, broken arrow). As in cells expressing N19RhoA, Src251-GFP remained in the perinuclear region of the cell. Furthermore treatment of cells with Y-27632 prior to expression of Src251-GFP and LPA stimulation resulted in the loss of stress fibres (Figure 23d) and consequently blocked the translocation of Src251-GFP to peripheral focal adhesions in response to LPA stimulation (Figure 23e,f). This was in direct contrast to its localisation at peripheral focal adhesions in cells stimulated with LPA alone (Figure 23g), where it is found at the tips of actin stress fibres (Figure 23h,i). In Chapter 3 (see Section 3.3) it was suggested that Y-27632 may inhibit other kinases than ROCK. However, in the context of these experiments we have shown that treatment with Y-27632 blocks RhoA-induced stress fibres without effecting Rac1 or Cdc42 induced actin remodelling (see Chapter 3 Section 3.2.1). Moreover, this taken together with the ability of N19RhoA to block the translocation of Src251-GFP to focal adhesions leads to the conclusion that the actin-dependent localisation of Src251-GFP to focal adhesions is controlled by RhoA.

Figure 23

The Rho-kinase inhibitor Y-27632 blocks RhoA-dependent targeting of Src to focal adhesions. Cells were transfected with Src251-GFP and prior to expression were incubated with Y-27632 (10 μM). Cells were then stimulated with 10% serum for 30 min (a-c) or LPA (200 ng ml^{-1}) for 30 min (d-f). Cells serum starved for 48 h were microinjected with Src251-GFP and were stimulated with LPA (200 ng ml^{-1}) for 30 min in the absence of Y-27632 (g-i). Broken arrows indicate expressing cells. Actin was visualised with rhodamine-phalloidin (a,c,d,f,g,i) and Src251-GFP was detected by direct fluorescence (b,c,e,f,h,i). Scale bars 25 μm.

Figure 23


Serum (+Y-27632)
Actin
Src251-GFP
Merge
a
b
c
LPA (+ Y-27632)
Actin
Src251-GFP
Merge
d
e
f
LPA (- Y-27632)
Actin
Src251-GFP
Merge
g
h
i

4.2.4 *Co-ordinated regulation of the actin cytoskeleton by RhoGTPases controls the intracellular targeting of Src to lamellipodia.*

There has previously been a great deal of evidence demonstrating cross-talk between Rho family GTPases (see Introduction Section 1.4.3). Work in Swiss 3T3 cells in particular has suggested that a hierarchical cascade exists between these proteins such that Cdc42 can activate Rac1 which in turn can activate RhoA (Hall, 1998; Nobes & Hall, 1995), while others have reported antagonistic behaviour between Rho-GTPases (Zondag et al., 2000) (Leeuwen et al., 1997; Rottner et al., 1999; Sander et al., 1998). The role of the individual RhoGTPases in the sub-cellular targeting of c-Src was therefore investigated.

We and others have previously shown that PDGF stimulates the formation of lamellipodia at the cell periphery (Fincham et al., 1996; Hall, 1998) via a Rac1 dependent mechanism (Nobes & Hall, 1995; Nobes et al., 1995; Ridley et al., 1992). We have also demonstrated that both endogenous Src, wt-Src and Src251-GFP localise to lamellipodia following treatment with PDGF (see Chapter 3). To address the relationship between RhoA and Rac1 signalling in the localisation of c-Src to lamellopodia, we stimulated cells with PDGF to activate Rac1, while inhibiting the formation of stress fibres and RhoA activity, by treating the cells with Y-27632. Y-27632 was added to the cells prior to expression of Src251-GFP resulting in loss of stress fibres (Figure 24a). After stimulation with PDGF, Src251-GFP remained perinuclear (Figure 24b,c, broken arrows) and did not translocate to lamellipodia at the cell periphery, although the shape of the cell did change in response to PDGF to form lamellipodia (Figure 24a,c, solid arrows). Consistent with this, when we co-expressed constitutively active Rac1 (V12Rac1) with Src251-GFP, in the presence of Y-27632 there was no relocalisation of Src from the perinuclear region (Figure 24e,f, broken arrows), although the transfected cells were still able to form lamellipodia (Figure 25d,f solid arrows). However, if Y-27632 was washed out prior to stimulation with PDGF, hence allowing RhoA-induced stress fibres to re-form, Src251-GFP localised to lamellipodia (Figure 25a, solid arrows), where it co-localised with actin bundles at the leading edge of the cell (Figure 25b,c). This implies that RhoA activity is partially involved in targeting of Src from a perinuclear location to lamellipodia. To verify that the Rho kinase inhibitor Y-27632 had been removed after the washing procedure, cells were incubated with Y-27632, fixed and incubated with conjugated phalloidin before (Figure 25d) or after washing cells with serum (Figure 25e). Cells after the washing procedure had reformed stress fibres in response to serum (Figure 25e) in contrast to cells in the presence of Y-27632 that remained devoid of stress fibres (Figure 25d).

To further investigate the relationship between RhoA and Rac1 in c-Src localisation, we stimulated cells with PDGF while inhibiting Rac1 activity in the presence of serum (ie RhoA stimuli). As demonstrated in the previous chapter (see Section 3.2.1) expression of dominant negative Rac1 blocked the formation of actin bundling and lamellipodia in response to PDGF. Co-expression of Src251-GFP with dominant negative N17Rac1 therefore blocked the formation of lamellipodia in response to PDGF (Figure 26a,b). No lamellipodia were formed in cells expressing N17Rac1, (Figure 26a,b broken arrow as compared to the non-transfected cells, solid arrows). Although cells expressing N17Rac1 did not form lamellipodia, these cells still contained actin stress fibres (due to the presence of serum) and Src251-GFP was consequently located at the ends of these stress fibres in peripheral focal adhesions (Figure 26c, broken arrow). Thus Rac1 drives the targeting of c-Src to lamellipodia, but is not involved in the initial movement of c-Src from the cell interior to the cell periphery, which is mediated by RhoA-dependent formation of stress fibres.

Figure 24

Addition of the Rho-kinase inhibitor Y-27632, prior to PDGF stimulation or Racl **expression impairs stress fibre formation and retains Src251-GFP in a perinuclear location during lamellipodia formation**. Cells were transfected with Src251-GFP and prior to expression of the protein were treated overnight with Y-27632 to abolish stress fibres. The cells were then stimulated with PDGF (25 ng ml^{-1}) for 30 min (a-c). Cells were co-transfected with Src251-GFP and L61Rac and prior to expression of the proteins were treated overnight with Y-27632 to abolish stress fibres (d-f). Solid arrows indicate the localisation of lamellipodia while the broken arrows show the perinuclear localisation of Src251-GFP. Actin was visualised with rhodamine-phalloidin (a,c,d,f) and Src251-GFP was detected by direct fluorescence (b,c,e,f). Scale bars 25 μm.

Figure 24

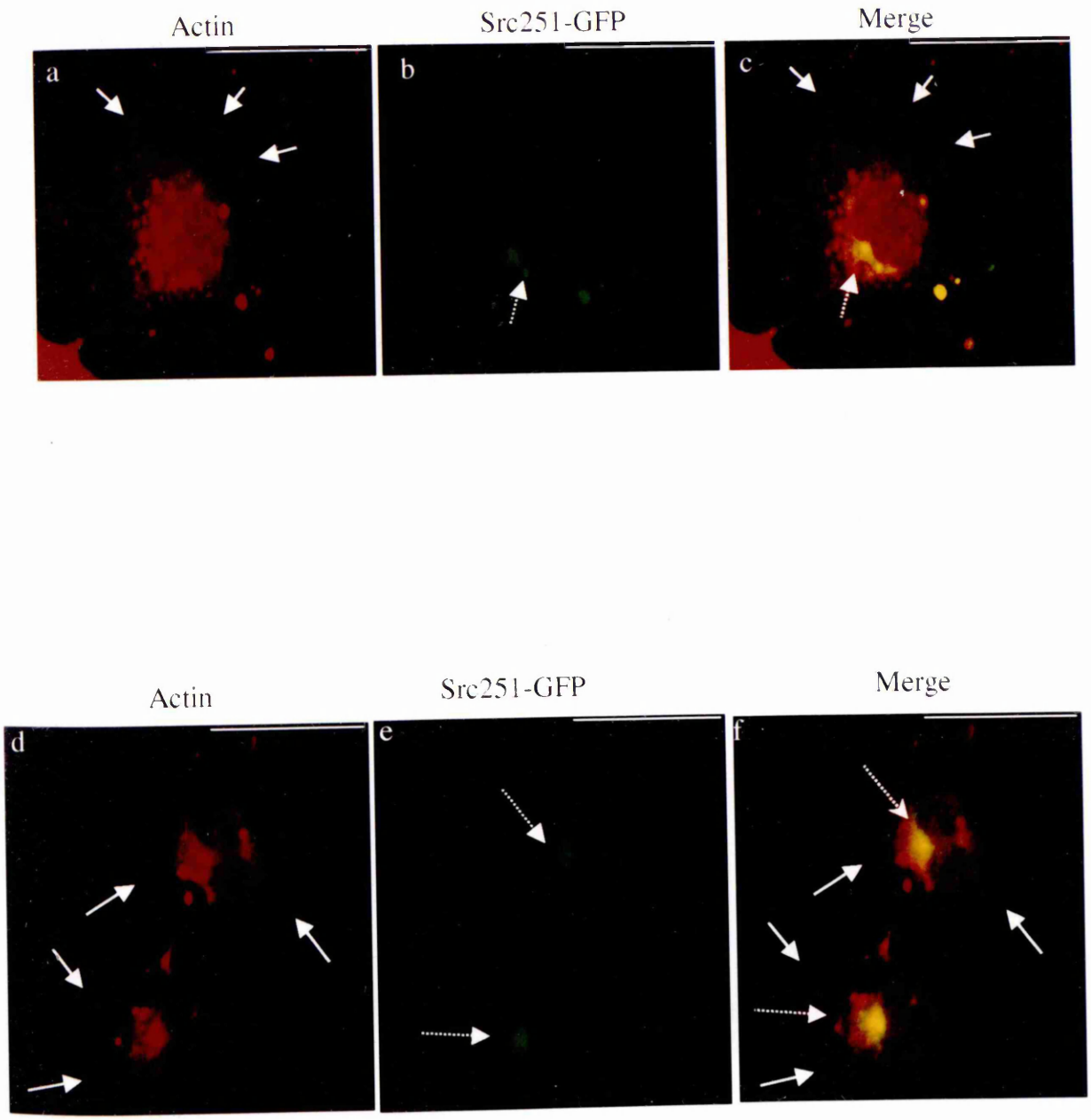


Figure 25

Removal of the inhibitory effect of Y-27632 allows stress fibre re-formation and PDGF induced targeting of Src to lamellipodia. Cells were transfected with Src251-GFP and prior to expression of the protein were treated overnight with Y-27632 to abolish stress fibres. The cells were then stimulated with PDGF (25 ng ml^{-1}) for 30 min following wash out of the Rho-kinase inhibitor Y-27632 (a-c). Cells were treated with Y-27632 fixed and incubated with conjugated phalloidin before (d) or after (e) removal of Y-27632 by washing the cells with serum containing medium. Arrows indicate Src251-GFP co-localising with actin in lamellipodia. Actin was visualised with rhodamine-phalloidin (b-e) and Src251-GFP was detected by direct fluorescence (a,c). Scale bars 25 μm.

Figure 25

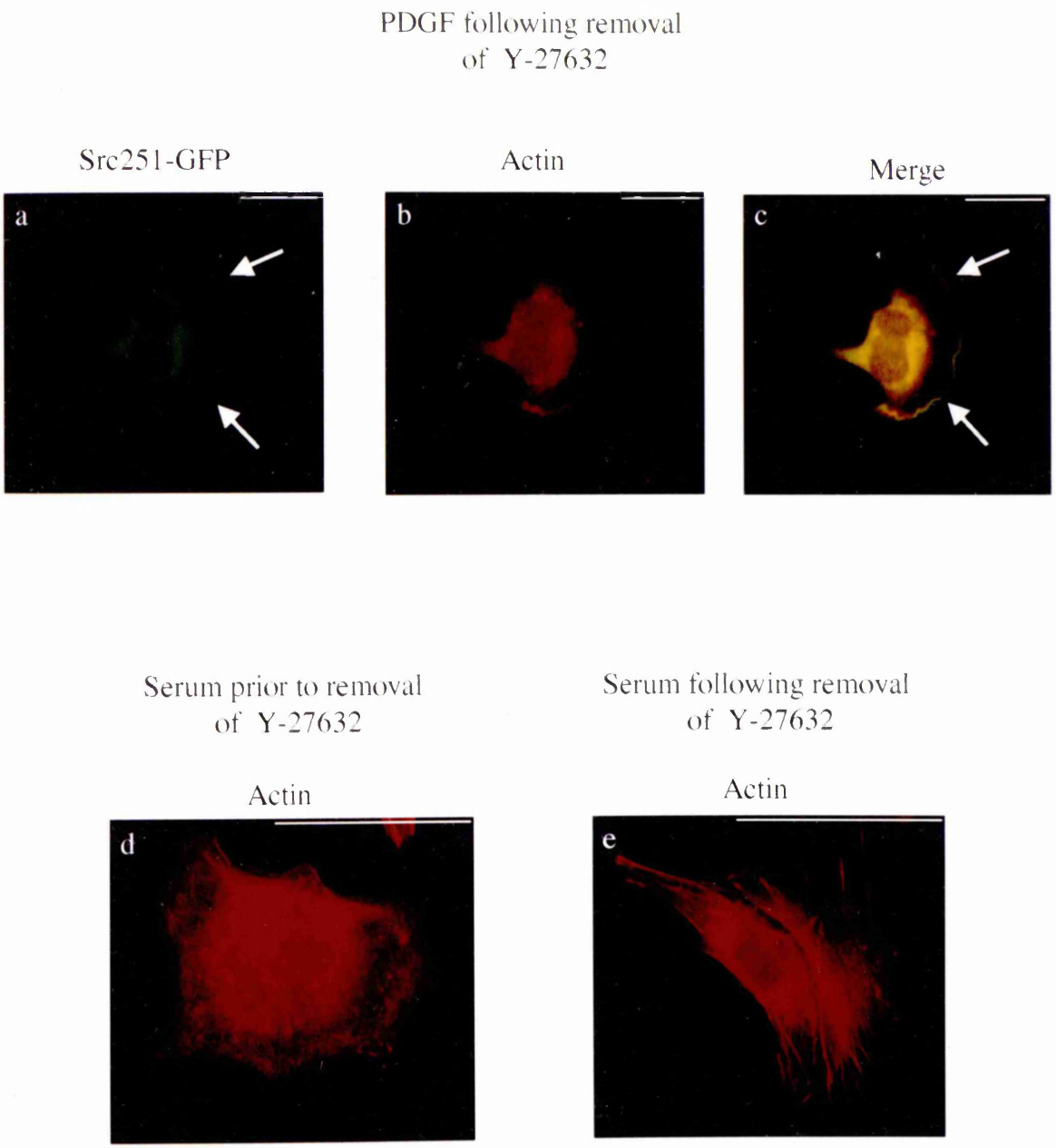
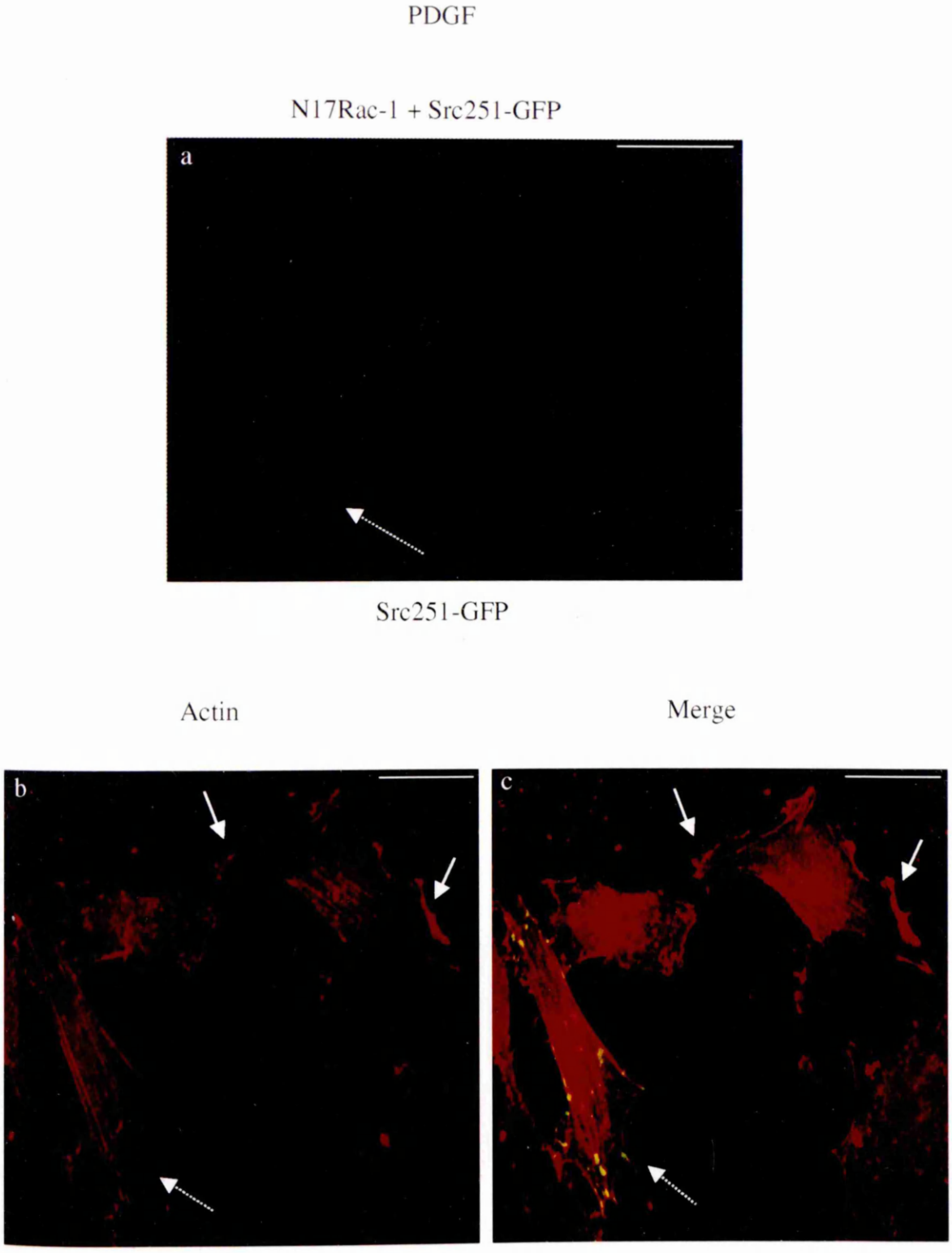

PDGF following removal
of Y-27632
Src251-GFP
Actin
Merge
a
b
c
Serum prior to removal
of Y-27632
Serum following removal
of Y-27632
Actin
Actin
d
e

Figure 26

Inhibition of Rac1 blocks the formation and targeting of Src to lamellipodia but has no effect on Src's peripheral targeting from a perinuclear location to focal adhesions. Cells in the presence of serum were co-transfected with Src251-GFP and N17Rac and then stimulated with PDGF (25 ng ml^{-1}) for 30 min. Solid arrows show the localisation of lamellipodia in non-transfected cells, while the broken arrow shows a cell expressing N17Rac and Src251-GFP. Actin was visualised with rhodamine-phalloidin (b,c). Src251-GFP was detected by direct fluorescence (a,c). Scale bars 25 μm.

Figure 26



4.2.5 *Co-ordinated regulation of actin cytoskeleton by RhoGTPases controls the intracellular targeting of Src to filopodia.*

Based on work from the previous section we carried out similar experiments to define the relationship between RhoA and Cdc42. As discussed earlier bradykinin stimulates the formation of actin microspikes, known as filopodia, at the cell periphery, by a Cdc42-mediated pathway (Kozma et al., 1995; Nobes & Hall, 1995). Addition of bradykinin to Y-27632 treated cells resulted in the formation of filopodia seen as small actin microspikes (Figure 27a, solid arrows) or vinculin containing focal complexes protruding from the cell body (Figure 27d, solid arrows). Under these conditions Src251-GFP remained perinuclear (Figure 27b,c and e,f, broken arrows). Similarly, when we co-expressed constitutively active Cdc42 (V12Cdc42) with Src251-GFP, in the presence of Y-27632 there was no relocalisation of Src from the perinuclear region (Figure 28a,c and d,f, broken arrows), although the transfected cells were still able to form filopodia (Figure 28b,e). Broken arrows show perinuclear Src localisation and solid arrows show actin containing filopodia. However, as shown previously for PDGF, removal of Y-27632 prior to addition of bradykinin, which allows stress fibres to re-form (Figure 25d,e), resulted in the translocation of Src251-GFP to filopodia in response to bradykinin (Figure 29, solid arrows). This implies that RhoA activity, at least in part is also involved in the targeting of Src from a perinuclear location to filopodia.

To further investigate the role of RhoA and Cdc42 in c-Src localisation we stimulated cells with bradykinin while inhibiting Cdc42 activity in the presence of serum (ie. RhoA stimuli). Again as described in Chapter 3 (Section 3.2.1) the expression of a dominant negative Cdc42 protein (N17Cdc42) blocked the formation of filopodia in response to bradykinin. The co-expression of Src251-GFP with dominant negative Cdc42 protein (N17Cdc42) therefore blocked bradykinin induced filopodia formation (Figure 30a-c, for actin co-staining and Figure 30d-f, for vinculin co-staining). Compare expressing cells in both cases (broken arrows) to non-expressing cells (solid arrows) that were able to induce filopodia in response to bradykinin. Furthermore, co-expression of Src251-GFP with N17Cdc42, blocked bradykinin-induced translocation of Src to filopodia, however Src251-GFP was not retained in the perinuclear region but was present in peripheral focal adhesions under these conditions (due to the presence of serum) (Figure 30c,f broken arrows). Thus, the RhoA pathway is also required to target Src from the perinuclear region of the cell periphery, prior to its localisation to filopodia induced by Cdc42.

It is interesting that inhibition of Src251-GFP targeting to PDGF-induced lamellipodia or bradykinin-induced filopodia by N17Rac1 or N17Cdc42 respectively, did not result in Src being retained or 'returned' to the perinuclear region; instead Src251-GFP remained able to target to the cell periphery, specifically residing in focal adhesions, presumably because RhoA is active in the presence of serum. Thus, although dominant-negative Rac1 or Cdc42 proteins inhibit the translocation of Src to lamellipodia or filopodia respectively, these do not interfere with the RhoA-dependent transit from the cell interior to the cell periphery.

Figure 27

Addition of the Rho-kinase inhibitor Y-27632, prior to bradykinin stimulation impairs stress fibre formation and retains Src251-GFP in a perinuclear location during filopodia formation. Cells were transfected with Src251-GFP and prior to expression of the protein were treated overnight with Y-27632 to abolish stress fibres. The cells were then stimulated with bradykinin (100 nM) for 5 min. Solid arrows indicate the localisation of filopodia while the broken arrows show the perinuclear localisation of Src251-GFP. Actin was visualised with rhodamine-phalloidin (a,c) and vinculin was visualised using an anti-viculin antibody (d,f). Src251-GFP was detected by direct fluorescence (b,c,e,f). Scale bars 25 μm.

Figure 27

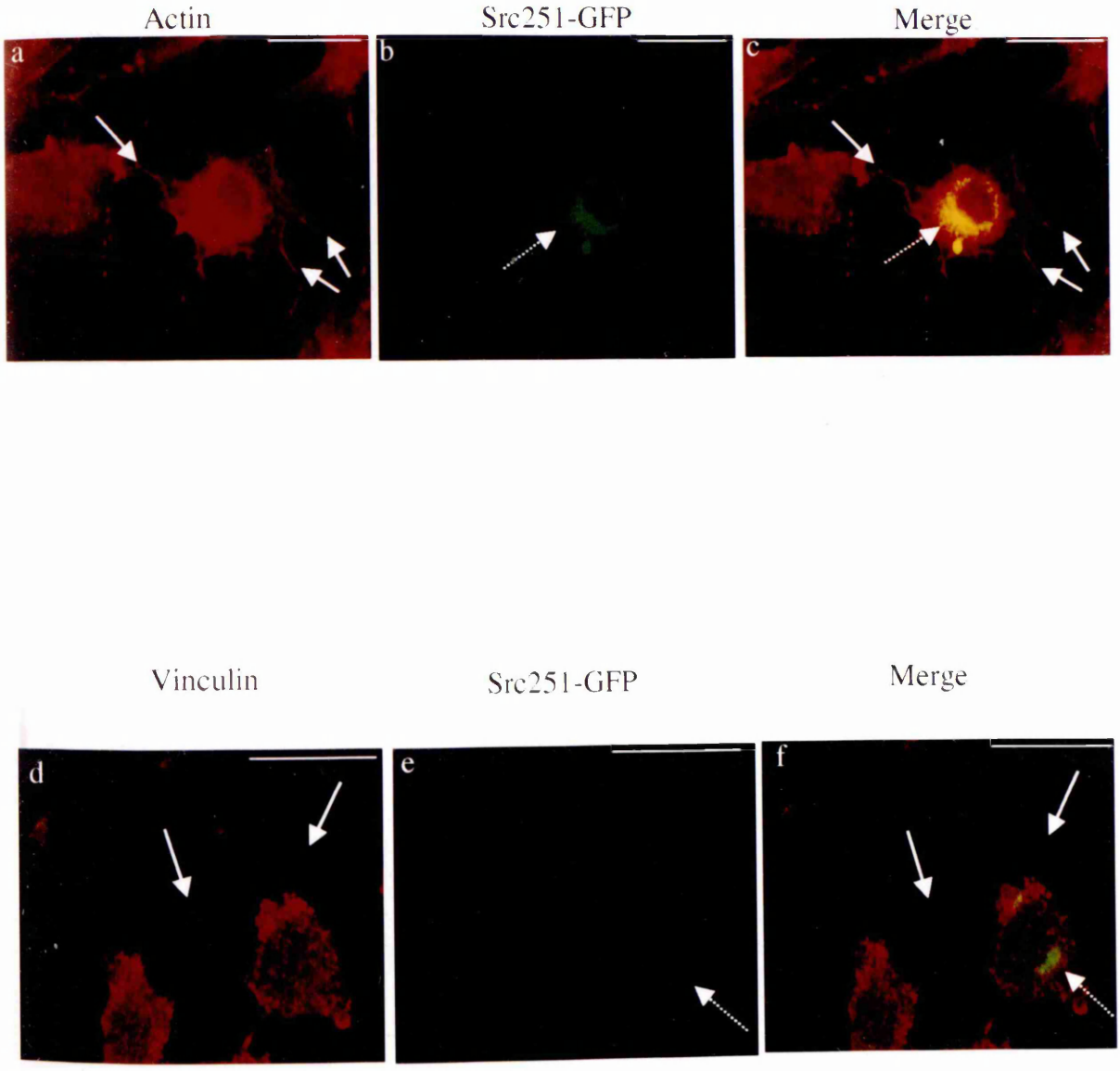
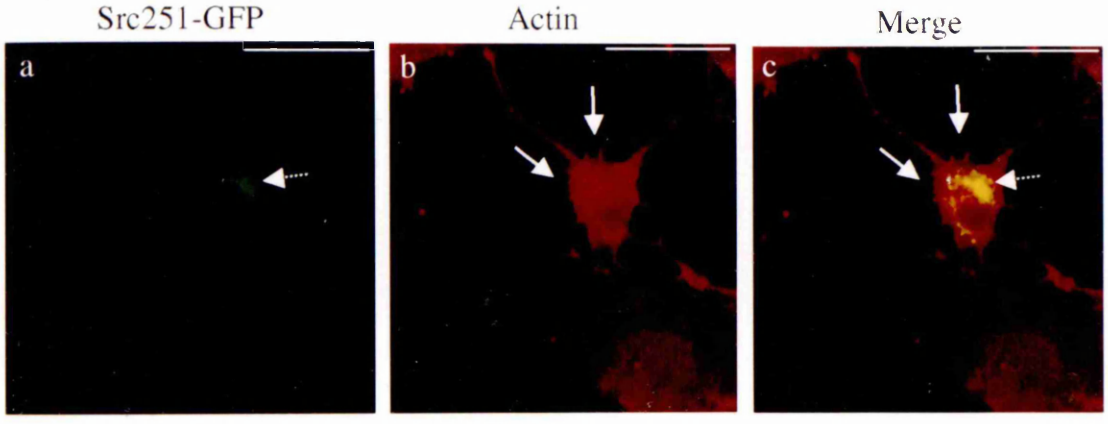


Figure 28

Addition of the Rho-kinase inhibitor Y-27632, prior to Cdc42 expression impairs stress fibre formation and retains Src251-GFP in a perinuclear location during filopodia formation. Cells were co-transfected with Src251-GFP and V12Cdc42 and prior to expression of the proteins were treated overnight with Y-27632 to abolish stress fibres. Solid arrows indicate the localisation of filopodia while the broken arrows show the perinuclear localisation of Src251-GFP. Actin was visualised with rhodamine-phalloidin (b,c) and vinculin was visualised using an anti-vinculin antibody (d,f). Src251-GFP was detected by direct fluorescence (a,c,d,f). Scale bars 25 μm.

Figure 28



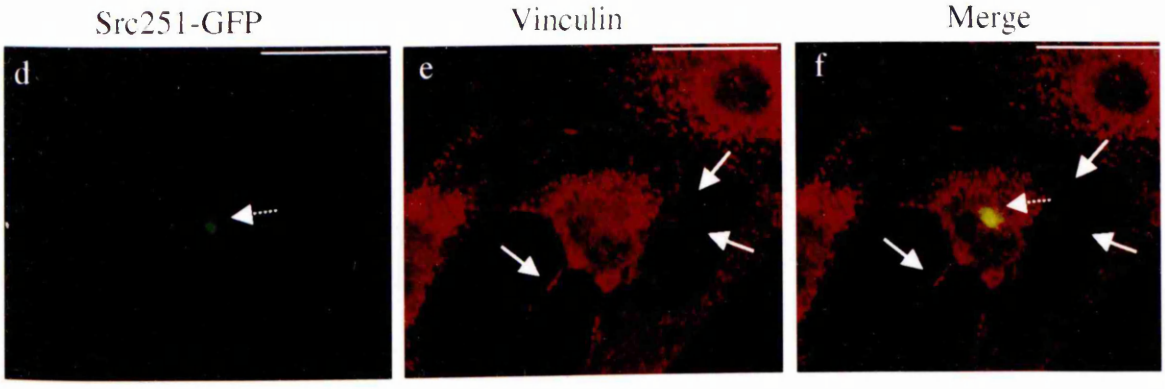


Figure 29

Removal of the inhibitory effects of Y-27632 allows stress fibre re-formation and bradykinin induced targeting of Src to filopodia. Cells were transfected with Src251-GFP and prior to expression of the protein were treated overnight with Y-27632 to abolish stress fibres. The cells were then stimulated with bradykinin (100 nM) for 5 min following wash out of the Rho-kinase inhibitor Y-27632. Arrows indicate filopodia and Src251-GFP localisation. Actin was visualised with rhodamine-phalloidin (b,c). Src251-GFP was detected by direct fluorescence (a,c). Scale bars 25 μm.

Figure 29

Bradykinin following removal of Y-27632

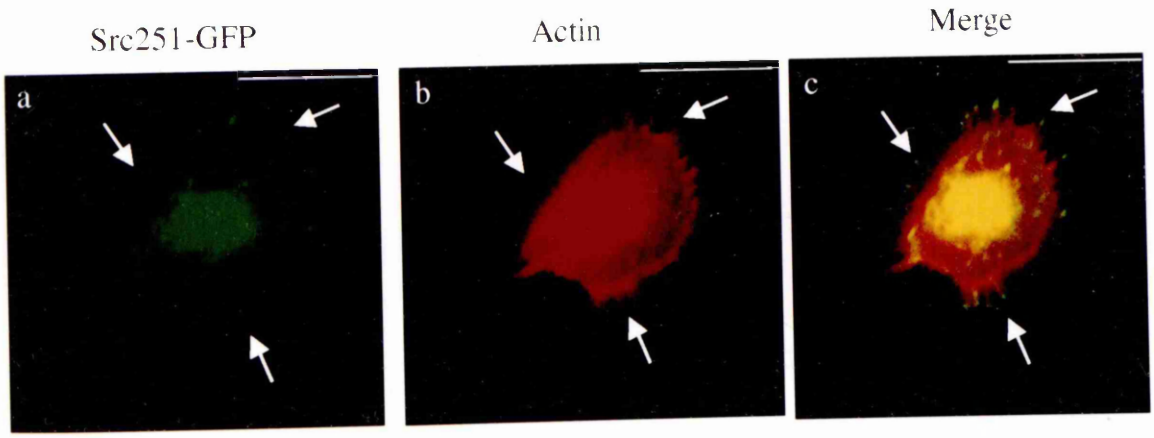
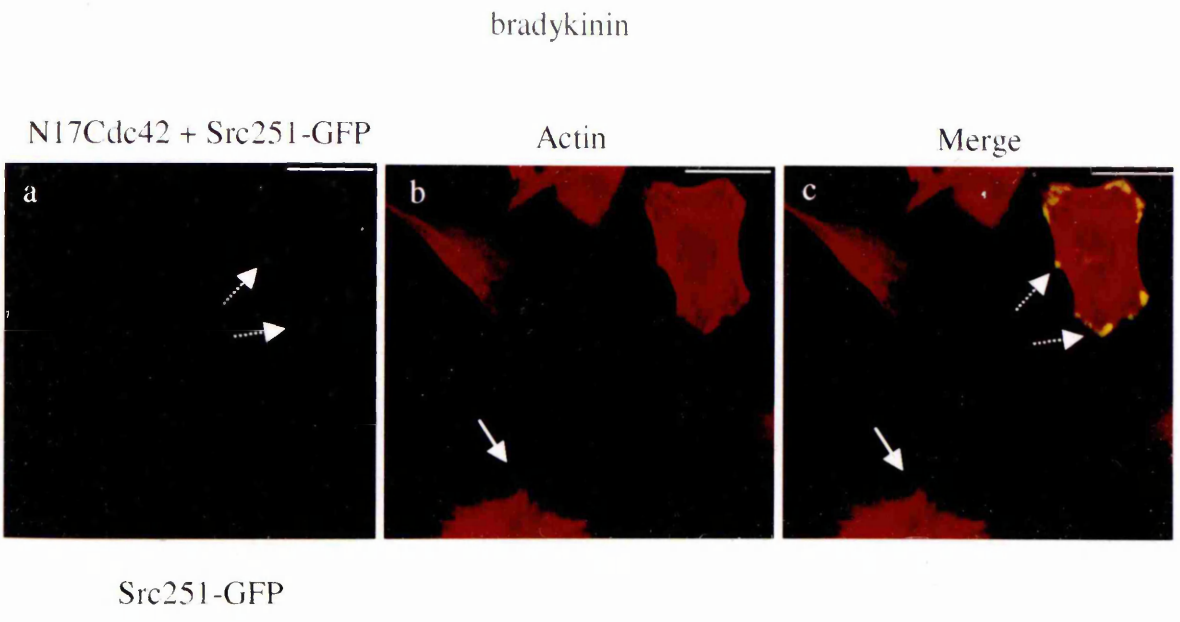


Figure 30

Inhibition of Cdc42 blocks the formation and targeting of Src to filopodia but has no effect on Src's peripheral targeting from a perinuclear location to focal adhesions. Cells in the presence of serum were co-transfected with Src251-GFP and N17Cdc42 and then stimulated with bradykinin (100 nM) for 5 min. The broken arrows indicate transfected cells, while filopodia in non-expressing cells are shown by the solid arrows. Vinculin was visualised using an anti-vinculin antibody (e,f). Actin was visualised with rhodamine-phalloidin (b,c) and Src251-GFP was detected by direct fluorescence (a,c,d,f). Scale bars 25 μm.

Figure 30


bradykinin
N17Cdc42 + Src251-GFP
Actin
Merge
a
b
c
Src251-GFP
bradykinin
N17Cdc42 + Src251-GFP
Vinculin
Merge
d
e
f
Src251-GFP

4.3 Discussion.

We have demonstrated that an inhibitor of Rho-kinase, as well as N19RhoA, inhibit the re-localisation of Src251-GFP to focal adhesions, indicating the requirement for RhoA, its effector Rho-kinase and actin filaments in the peripheral targeting of c-Src. Moreover v-Src targeting to focal adhesions in Swiss 3T3 cells has been shown to require RhoA activity (Fincham et al., 1996). In keeping with the requirement for bundled actin stress fibres, the translocation of v-Src to focal adhesions has also been shown to require myosin activity, at least when the actin cytoskeleton is being dynamically regulated (Fincham et al., 2000). As well as RhoA-induced targeting of Src to focal adhesions, the Rho family proteins Rac1 and Cdc42 direct the precise intracellular targeting of Src to other functional structures such as lamellipodia and filopodia respectively. It is clear then that RhoGTPases play a pivotal role in the spatial regulation of Src and it is therefore possible that they regulate Src's activity within the cell. As stated earlier (see Introduction Section 1.5.3 and 1.6.1), both RhoGTPases and Src have been implicated to be involved in regulating cell migration. RhoGTPases in particular have been thought to contribute to cell motility and polarisation by controlling cell contraction and protrusion, while Src is thought to be involved in focal adhesion turnover. In this study we highlight an additional method of how RhoGTPases can contribute to directional cell migration by spatially controlling Src activity during this process. RhoGTPase-induced actin structures could serve as tracks, directing Src, and for that matter other proteins reported to be involved in cell migration, towards adhesion sites, providing both, scaffolding proteins for the formation of adhesions as well as inducing adhesion turnover. A similar hypothesis has been proposed for the involvement of the microtubule network during cell migration (Kaverina et al., 1998; Nabi, 1999). The co-ordination of Src activity and RhoGTPase-induced targeting of Src during cell migration and polarisation will be addressed and discussed in more detail in the next chapter.

One potential problem of over-expressing Src251-GFP to monitor c-Src localisation within the cell is that access to the SH3/SH2 domains of Src, which are conformationally unrestrained in Src251-GFP, may be acting as an adaptor protein non-specifically sequestering various binding partners and allowing its recruitment into the respective structures described. The data presented in this study however on the targeting of endogenous and wild type Src implies that the control of Src251-GFP targeting is the same as for endogenous Src or the expression of wild type Src, helping to verify the use of Src251-GFP to monitor c-Src targeting within the cell. As yet little is known of the

mechanism involved in the transport of Src from the perinuclear region to the cell periphery. We, and others, have demonstrated that it is not dependent on the catalytic activity of c-Src but does require the Src SH3 domain (Fincham et al., 2000; Fincham & Frame, 1998; Kaplan et al., 1994). In *vitro* experiments have demonstrated that the Src SH3 domain binds to a number of cellular partners and we have shown that the regulatory sub-unit of PI3-kinase is amongst the cellular targets of the v-Src SH3 domain that is important for its peripheral targeting (Fincham et al., 2000). Although we have shown that the catalytic activity of Src is not required for its localisation to peripheral adhesion sites, a number of stimuli which result in translocation of Src to these sites have also been reported to activate Src kinase activity (eg. LPA, PDGF and bradykinin) (Jalink et al., 1993; Rodriguez-Fernandez & Rozengurt, 1996). It seems likely therefore that these stimuli release constraints on both the kinase domain and the SH3 domain of c-Src thus ensuring that the activated protein is not aberrantly localised within the cell.

Although Src251-GFP was found in lamellipodia or filopodia following activation of Rac1 or Cdc42 respectively, data presented here using dominant negative RhoGTPases, surprisingly, indicate that the precise localisation of Src to these structures is not simply regulated by the individual RhoGTPase responsible for these actin based structures, but that co-ordination between Rho family members is required for this precise targeting of c-Src to these sites.

Firstly, PDGF- and Rac1-induced targeting of Src251-GFP to lamellipodia, is inhibited by treatment with the Rho-kinase inhibitor, Y-27632. In this case, Src251-GFP is held in the perinuclear region of the cell and is unable to move to the cell periphery. Moreover, although formation of PDGF-induced lamellipodia, as well as the targeting of Src251-GFP to these sites, is blocked by dominant inhibitory Rac1, Src251-GFP is still able to translocate from the perinuclear region of the cell to focal adhesions, presumably mediated by RhoA that is unaffected by N17Rac1. This suggests that Src251-GFP translocates from the perinuclear region of the cell to the cell periphery via stress fibres to focal adhesions, and when Rac1 is stimulated, subsequently moves to much smaller focal complexes in lamellipodia (see Figure 31). Similarly, bradykinin- and Cdc42-induced targeting of Src251-GFP to filopodia, are also inhibited by treatment with the Rho-kinase inhibitor. Again, Src251-GFP is held in the perinuclear region of the cell and is unable to move to the cell periphery. Furthermore, although formation of bradykinin-induced filopodia, as well as the targeting of Src251-GFP to these sites, is blocked by dominant inhibitory Cdc42, Src251-GFP is still able to translocate from the perinuclear region of the cell to focal adhesions, which is mediated by RhoA and unaffected by N17Cdc42. Together these

data suggest that Src251-GFP translocates from the perinuclear region of the cell to focal adhesions via stress fibres, and when appropriate stimuli is received, subsequently moves to much smaller focal complexes such as filopodia (see Figure 32). The activation of Rac1 has been reported to initiate the turnover and remodelling of existing focal adhesions into smaller focal complexes during lamellipodia formation (Laukaitis et al., 2001). This process has been shown for example, using GFP tagged paxillin to visualise the process in real time. In particular, when new lamellipodia protrusions form paxillin appears to remodel from older focal adhesions to new focal complexes at the leading edge of the cell. (Laukaitis et al., 2001; Small et al., 1995; Small et al., 1999). It is therefore possible that upon localisation of Src in focal adhesions, Rac1 can subsequently induce focal adhesion remodelling to form small focal complexes allowing Src to then relocate from focal adhesions to uniformly distributed focal complexes along the base of lamellipodia (Figure 33). A similar mechanism can therefore be proposed for Src targeting to filopodia (Figure 33). The involvement of Src kinase activity in the focal adhesions to focal complex transition is examined and discussed in detail in the next chapter.

Figure 31

Schematic representation of the co-ordinated actin remodelling proposed to be involved in Src's sequential targeting from the perinuclear region of the cell to lamellipodia. Src initially translocates form a perinuclear location to the cell periphery at focal adhesions via RhoA and following Rac1 stimulation subsequently moves to focal complexes in lamellipodia.

Figure 31

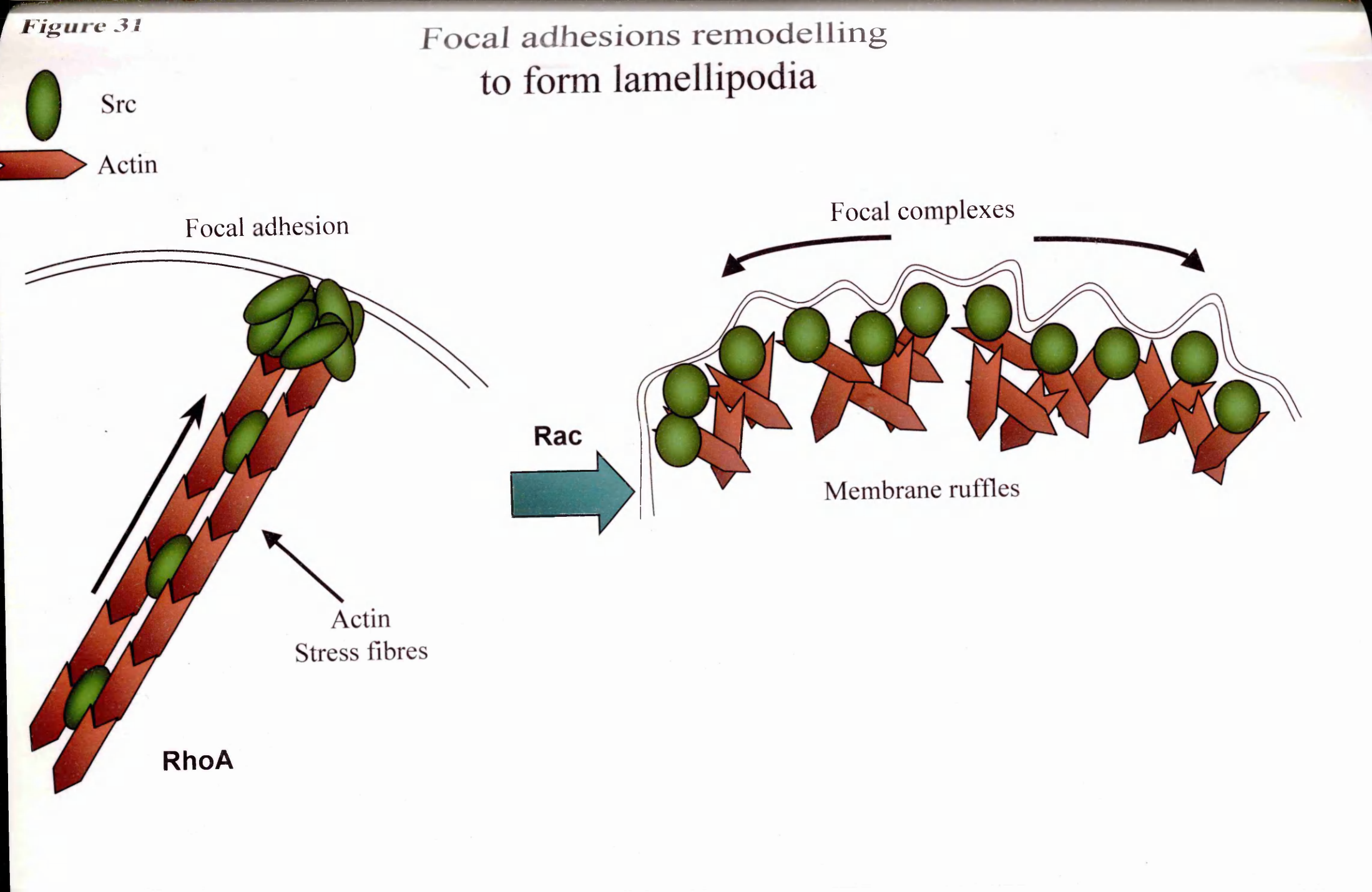

Focal adhesions remodelling
to form lamellipodia
Src
Actin
Focal adhesion
Focal complexes
Rac
Membrane ruffles
Actin
Stress fibres
RhoA

Figure 32

Schematic representation of the co-ordinated actin remodelling proposed to be involved in Src's sequential targeting from the perinuclear region of the cell to filopodia. Src initially translocates form a perinuclear location to the cell periphery at focal adhesions via RhoA and following Cdc42 stimulation subsequently moves to focal complexes in filopodia.

Figure 32

Focal adhesions remodelling to form filopodia

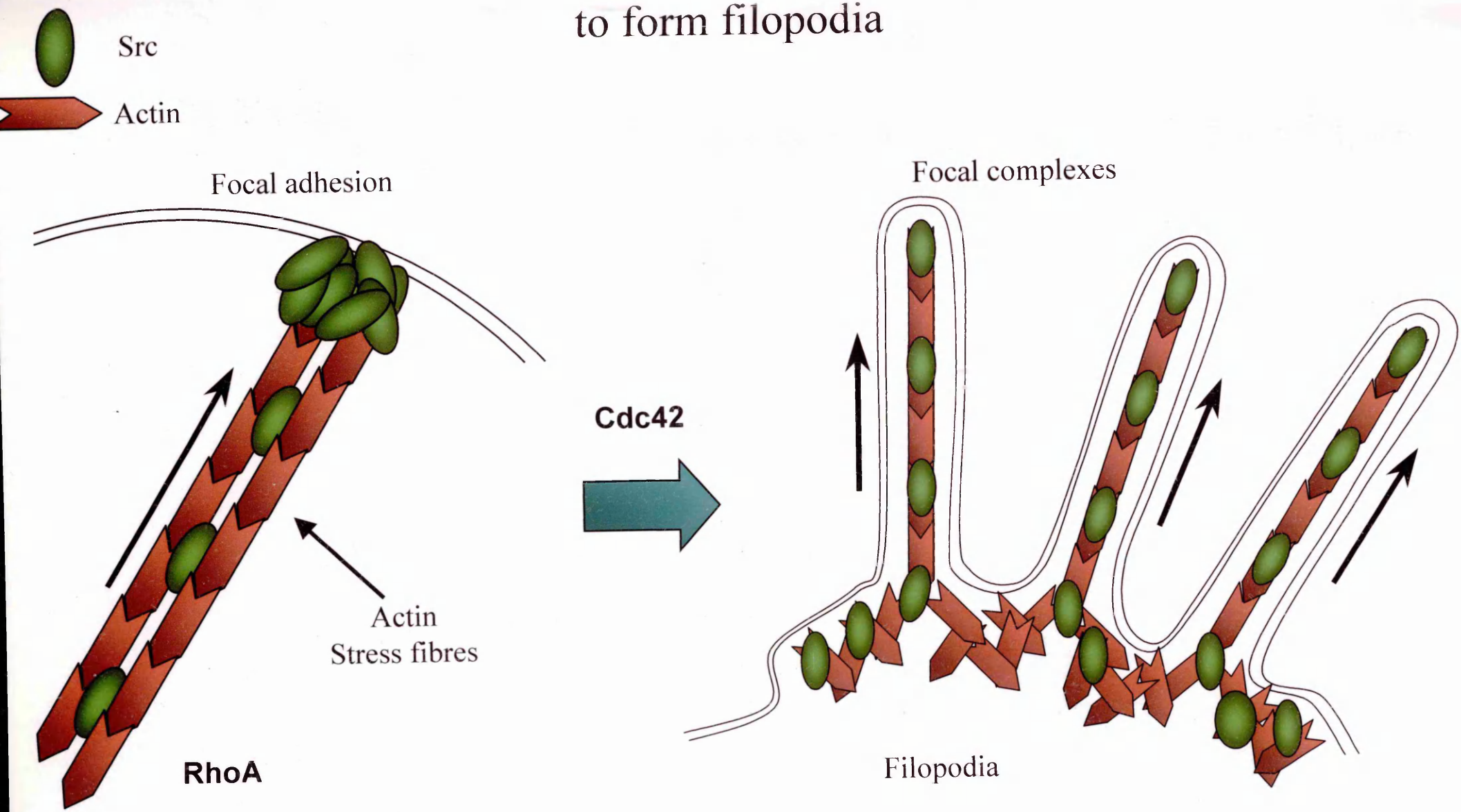
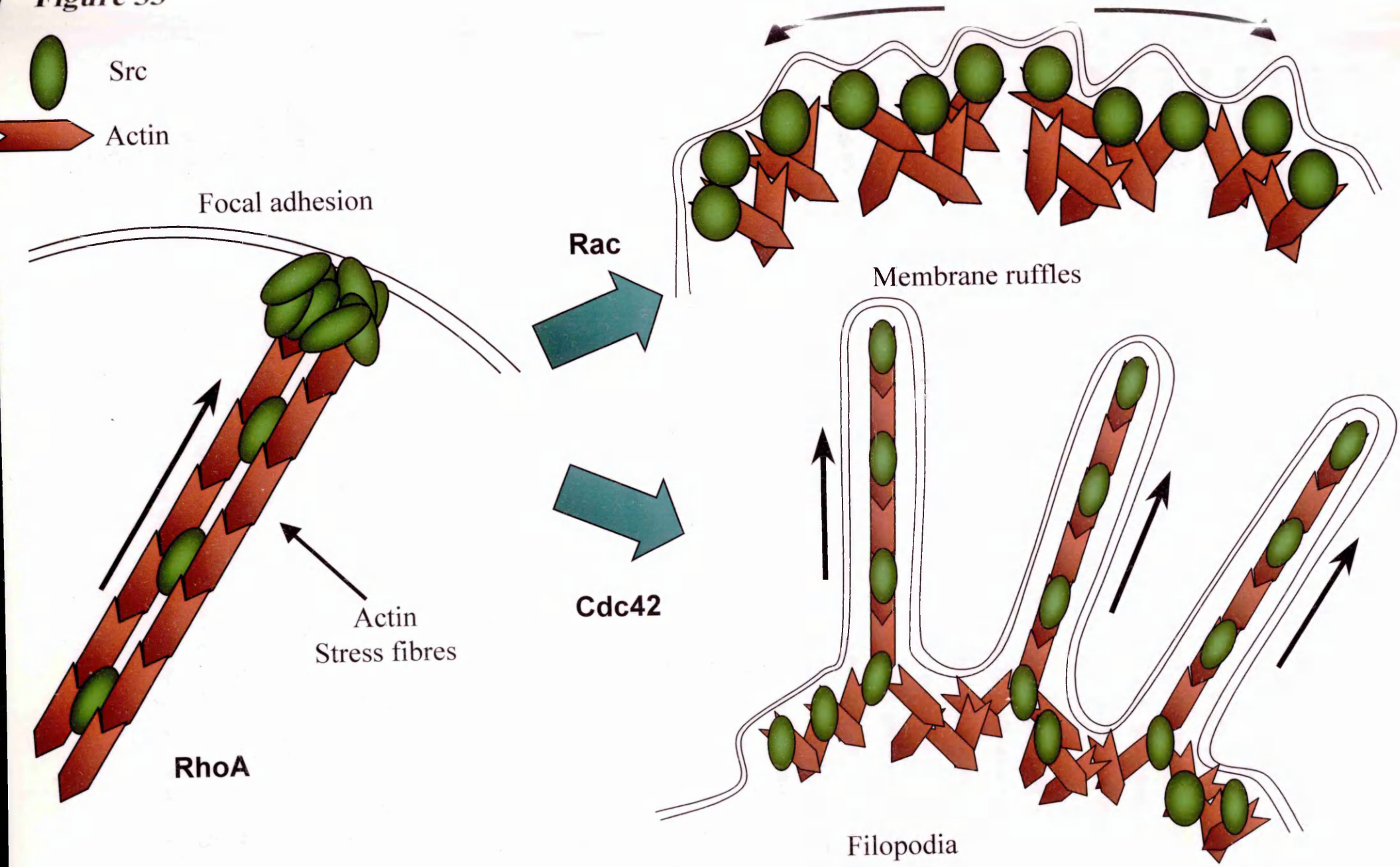


Figure 33

Schematic representation of Src's sequential targeting from the perinuclear region of the cell to lamellipodia or filopodia.

Figure 33


Src
Actin
Focal adhesion
Rac
Membrane ruffles
Actin
Stress fibres
Cdc42
RhoA
Filopodia

It should be noted that in these sequential stimuli experiments, RhoA activity was blocked to examine whether RhoA was involved in this process. Serum starvation of cells for 48 h to cause the loss of RhoA activity, and thereby stress fibres, was not a viable approach because expression of Rac1 or Cdc42 could potentially lead to RhoA activation via the hierarchical cascade described earlier (Hall, 1998; Nobes & Hall, 1995; Ridley & Hall, 1992; Ridley et al., 1992). This could have led to a misinterpretation of the role RhoA activity plays in this process. Thus, the reversible Rho-kinase inhibitor Y-27632, was used here to block RhoA-induced stress fibres, which allows rapid inactivation of RhoA, while leaving the option to relieve this inhibitory effect using the washing out procedure outlined earlier.

Various reports have demonstrated cross-talk to exist between Rho family members in a number of processes. In particular, the hierarchical cascade in Swiss 3T3 cells along with the proposed involvement of this cascade in macrophage migration, provides one example for potential cross-talk between RhoGTPases during cell migration such that Cdc42 is required for cells to polarise and migrate in the direction of a chemotatic gradient, while RhoA and Rac1 are required for cell migration (Allen et al., 1997). Moreover, antagonistic behaviour between RhoGTPases has frequently been reported and is thought to be responsible for neurite retraction or collapse as well as protrusion in neuroblastomas cell (Kozma et al., 1997). Co-operation or convergence of RhoGTPase activity has also been shown during cadherin mediated cell-cell junction formation (Braga et al., 1997), where both RhoA and Rac1 are required for the establishment of cadherin mediated cell-cell junctions, illustrating the importance of Rho family cross-talk in a number of celiular processes. Here we illustrate a new role of RhoGTPases in directing Src to morphologically functional structures and also report a novel co-operative role for RhoGTPases in this targeting process.

4.4 Summary

We have demonstrated that Src251-GFP is found in lamellipodia or filopodia following activation of the RhoGTPases Rac1 and Cdc42 respectively. Moreover data presented in this chapter indicates that the precise localisation of Src to these sites is not regulated by individual RhoGTPases, but that co-ordination between Rho family members is required for this targeting process. Both lamellipodia and filopodia have been documented to be transient structures in which the actin cytoskeleton and adhesion contacts are rapidly remodelling and turning over. In the next chapter we assess whether Src plays a passive or active role in this remodelling process.

Chapter 5

5 Src kinase activity participates in the efficient remodelling of focal adhesions to focal complexes during lamellipodia and filopodia formation.

5.1 Purpose

We have shown that c-Src can localise to focal adhesions, lamellipodia and filopodia at the cell periphery. Lamellipodia and filopodia contain focal complexes within their structures, which have been defined as small adhesion structures of indistinguishable protein composition from larger focal adhesions as previously discussed (Nobes et al., 1995). It is the dynamic remodelling and interchange between focal adhesions and focal complexes that regulate cell motility. In this chapter, we therefore addressed whether the activity of c-Src at these cellular adhesions altered their remodelling and establish the influence this has on cellular processes such as cell migration and chemotaxis. Src kinase activity has frequently been linked to focal adhesions turnover (Fincham & Frame, 1998) and cell motility (Hall et al., 1996). We therefore tested the hypothesis that Src kinase activity is required for the dynamic remodelling of focal adhesions and focal complexes during cell migration.

5.2 Results

In this study, we introduced constitutively active- and inactive-Src mutants into cells to establish the importance of Src kinase activity during adhesion remodelling. Cells were transfected with the respective Src mutants and maintained in serum (ie. a RhoA stimuli) to localise Src to focal adhesions. Cells were subsequently incubated with the protein synthesis inhibitor emetine prior to PDGF or bradykinin treatment. This protocol allowed us to focus on 'focal adhesions to focal complex' remodelling events during lamellipodia or filopodia formation and address the role Src kinase activity plays in this process.

5.2.1 Src251-GFP interferes with the efficient formation of uniform lamellipodia

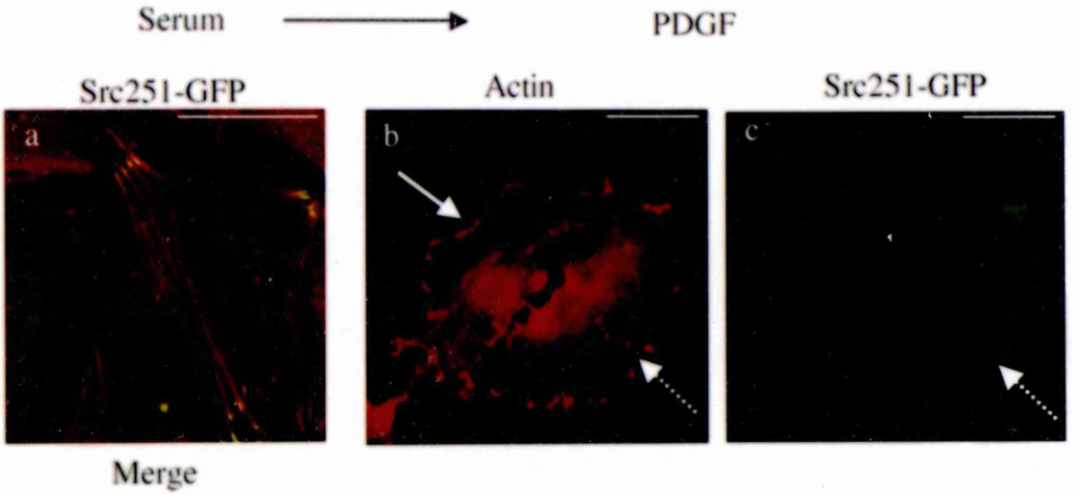
To address whether Src kinase activity plays a role in adhesion remodelling, Swiss 3T3 cells were transfected with Src251-GFP in the presence of serum (Figure 34a). Under

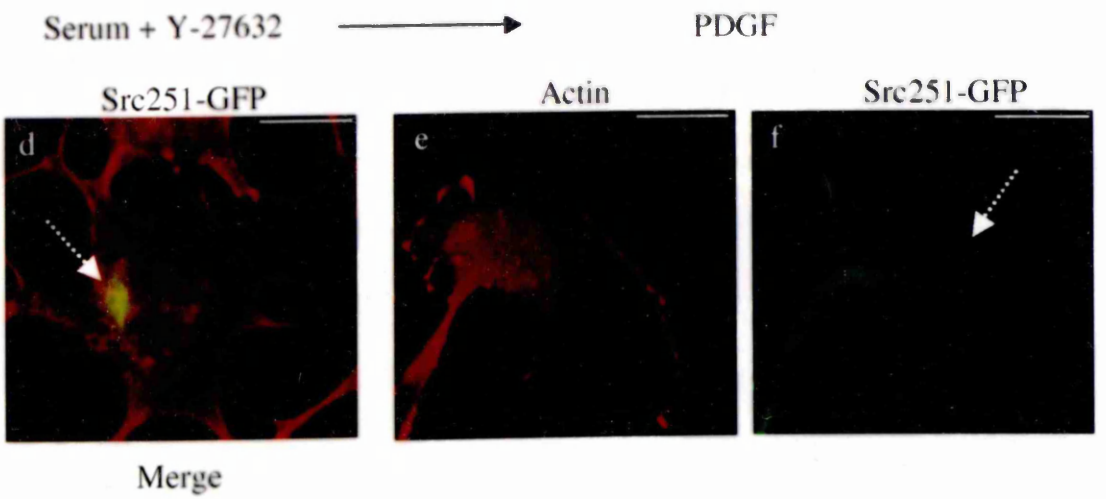
these conditions the kinase null Src251-GFP was localised to focal adhesions (Figure 34a) Subsequent treatment of these cells with PDGF, which leads to Rac1 activation, resulted in PDGF-induced shape changes and formation of lamellipodia. Although expression of Src251-GFP at focal adhesions did not significantly alter the number of cells able to form lamellipodia (around 80% of cells expressing Src251-GFP underwent shape changes associated with the formation of lamellipodia), we found that under these conditions the actin localised to discrete patches around the forming lamellopodia (Figure 34b, broken arrow). Src251-GFP also re-located to the forming lamellipodia, but remained in the same discrete patches or adhesion-like structures around the edge of the lamellipodia (Figure 34c, broken arrow). Furthermore, the discontinuous actin staining in cells expressing Src251-GFP indicated that the lamellipodia were less uniform than in cells not expressing Src251-GFP (compare the solid arrow, which indicates a cell not expressing Src251-GFP, with the Src251-GFP expressing cell shown by the broken arrow in Figure 34b). This is in contrast to the uniform lamellipodia formed when Src251-GFP is held in the perinuclear region of the cell following treatment with Y-27632 (Figure 34d), prior to stimulation with PDGF (Figure 34e,f), where Src251-GFP is found in small structures continuous along the lamellipodia. As described above, cells were incubated with the protein synthesis inhibitor emetine prior to PDGF treatment in these experiments, implying that Rac1 activation led to re-localisation of Src that pre-existed in focal adhesions into complexes that are continuous along lamellipodia. This suggests that it is the presence of Src251-GFP at focal adhesions prior to addition of PDGF that interferes with the efficient formation of uniform lamellipodia.

Figure 34

Src kinase activity at focal adhesions is required for remodelling of the actin cytoskeleton during lamellipodia formation. Cells were transfected with Src251-GFP and maintained in 10% serum containing medium (a). Cells were then placed in serum-free medium overnight and treated with the protein synthesis inhibitor emetine (55 μg ml^{-1}) for 30 min prior to stimulation with PDGF (25 ng ml^{-1}) for 30 min (b,c). This protocol allowed us to reduce the heterogeneity in the population following PDGF stimulation by removing the serum, while maintaining Src251-GFP in the peripheral focal adhesions due to the incomplete loss of stress fibres after this short time in serum-free conditions. Cells were transfected with Src251-GFP and prior to expression of the protein were treated overnight with Y-27632 to abolish stress fibres (d). The cells were then stimulated with PDGF (25 ng ml^{-1}) for 30 min following wash out of the Rho-kinase inhibitor Y-27632 (e,f). Broken arrows indicate Src251-GFP expressing cells while solid arrow indicate non-expressing cell. Actin was visualised with rhodamine-phalloidin (a,b,d,e). Src251-GFP was detected by direct fluorescence (a,c,d,f). Scale bars 25 μm.

Figure 34


Serum
PDGF
Src251-GFP
Actin
Src251-GFP
a
b
c
Merge


Serum + Y-27632
PDGF
Src251-GFP
Actin
Src251-GFP
d
e
f
Merge

5.2.2 Src mutants used as tools to determine the role Src kinase activity plays in adhesion remodelling.

Since Src251-GFP lacks a c-Src kinase domain (see Figure 35a), we examined whether the presence of an activated c-Src kinase at focal adhesions facilitated the Rac1-dependent formation of uniform, continuous lamellipodia. For this, we expressed a c-Src protein (Figure 35b), which has an activating tyrosine to phenylalanine mutation at position 527 in the c-Src coding sequence (SrcY527F) (Thomas & Brugge, 1997). We also expressed a kinase-defective Src mutant, SrcMF in cells, and examined the ability of these cells to form lamellipodia in response to PDGF. SrcMF has a point mutation in the kinase domain (K295M) (Figure 35c), rendering it kinase inactive. SrcMF also has the Y527F mutation in the regulatory carboxy-terminal region that results in an open confirmation with the SH2 and SH3 domains available for protein-protein interactions (Figure 35c). As described in Section 1.1.3 autophopsphorylation on tyrosine-416 is required for full activation of Src kinase family members (Gonfloni et al., 2000). In these experiments we therefore used autophosphorylation on tyrosine-416 as a surrogate marker of Src kinase activity. Direct comparisons between phosphorylation of tyrosine-416 and Src kinase activity have verified the use of this site to reflect the biological changes in Src kinase activity within the cell (Chen et al., 2000; Hanke et al., 1996; Yoshizumi et al., 2000). Cells lysates expressing the respective Src constructs SrcY527F, SrcMF or Src251-GFP were probed with a phospho-Src-416 antibody (Figure 35d). Cells expressing SrcY527F demonstrated an enhanced autophosphorylation on tyrosine-416 compared to kinase null (Src251-GFP) or kinase defective (SrcMF) expressing cells, thereby confirming, as expected, their relative activity within the cell (Figure 35d). Initially, we characterised the behaviour of constitutively active SrcY527F in cells as previously described for Src251-GFP (see Chapter 3). When SrcY527F was microinjected into serum-starved Swiss 3T3 fibroblasts it was located in the perinuclear region of the cell (Figure 36a,c broken arrows). However, upon addition of serum, which resulted in reformation of stress fibres (Figure 36d-f), SrcY527F translocated to sites at the end of the actin cables (Figure 36d,f), where it co-localised with the focal adhesion protein paxillin (Figure 36g-i). SrcMFs intracellular targeting to focal adhesions was also characterised. SrcMF, like Src251-GFP and SrcY527F remained perinuclear when microinjected into serum starved Swiss 3T3 cells (Figure 37a,c, broken arrow). Upon serum stimulation it localised to focal adhesions (Figure 37d-f), where it co-localised with the focal adhesion protein paxillin (Figure 37g-i). Anti-paxillin staining was used for co-localisation studies with anti-SrcY527F or SrcMF because anti-vinculin and anti-Src sera used were from the same species, restricting co-

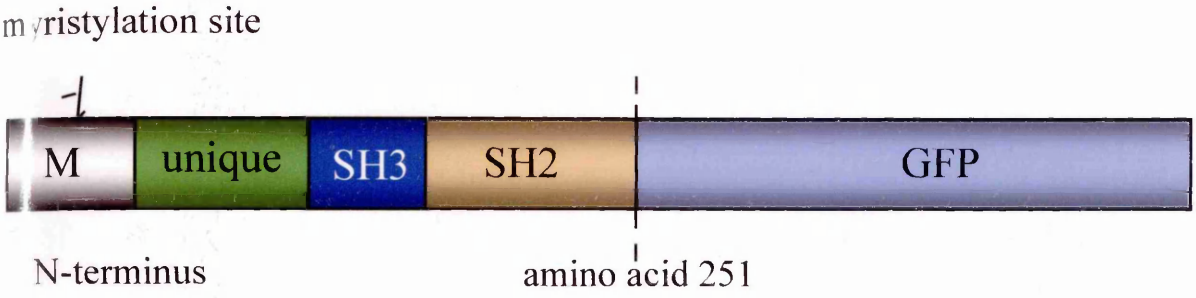
localisation studies. Thus the actin-dependent translocation of SrcY527F or SrcMF is the same as for Src251-GFP (see Chapter 3).

Figure 35

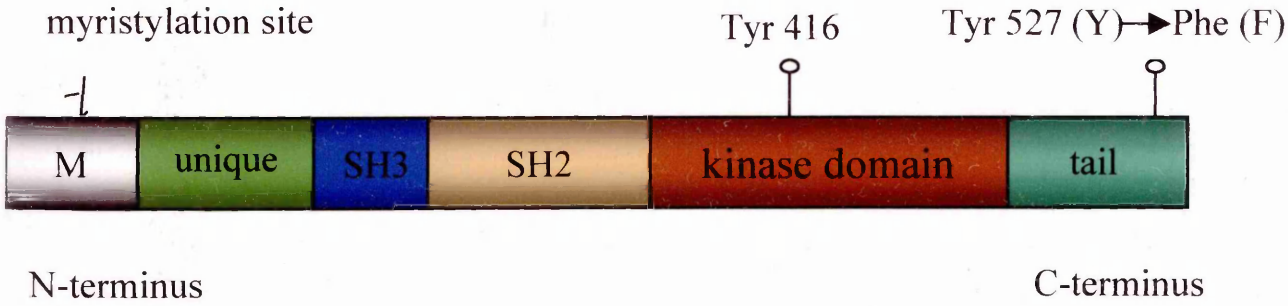
c-Src constructs. Schematic representation of the structural domains of Src251-GFP (a), SrcY527F and SrcMF (b). Anti-phospho-Src-Y416 western blot of cell extracts from non-transfected cells (lane 1), Src251-GFP (lane 2), SrcMF (lane 3) or SrcY527F (lane 4). The same blot was re-probed with anti-Src antibody (bottom panel).

Figure 35

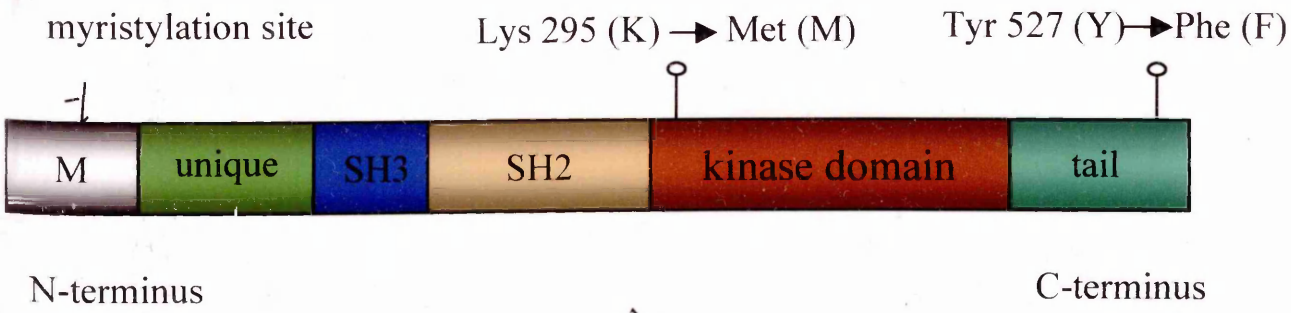
a. **Src251-GFP**



b. **SrcY527F (constitutively active)**



c. **SrcMF (kinase defective; K295M, Y527F)**



d.

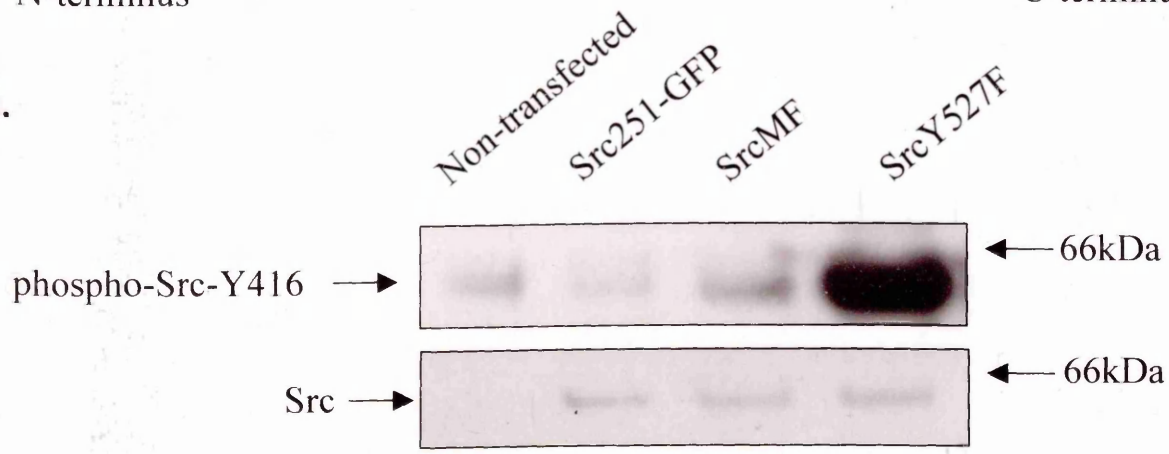


Figure 36

Translocation of SrcY527F to focal adhesions is indistinguishable from Src251-GFP. Cells were serum starved for 48 h to remove stress fibres prior to microinjection of SrcY527F (a-c). Serum-starved cells were microinjected with SrcY527F and following expression of the protein were stimulated with 10% serum for 30 min (d-i). SrcY527F was visualised using an anti-Src antibody (a,c,d,f,g,i). Arrows indicate perinuclear SrcY527F. Actin was visualised with rhodamine-phalloidin (b,c,e,f). Paxillin was visualised with rhodamine-conjugated anti-paxillin (h,i). Scale bars 25 μm.

Figure 36

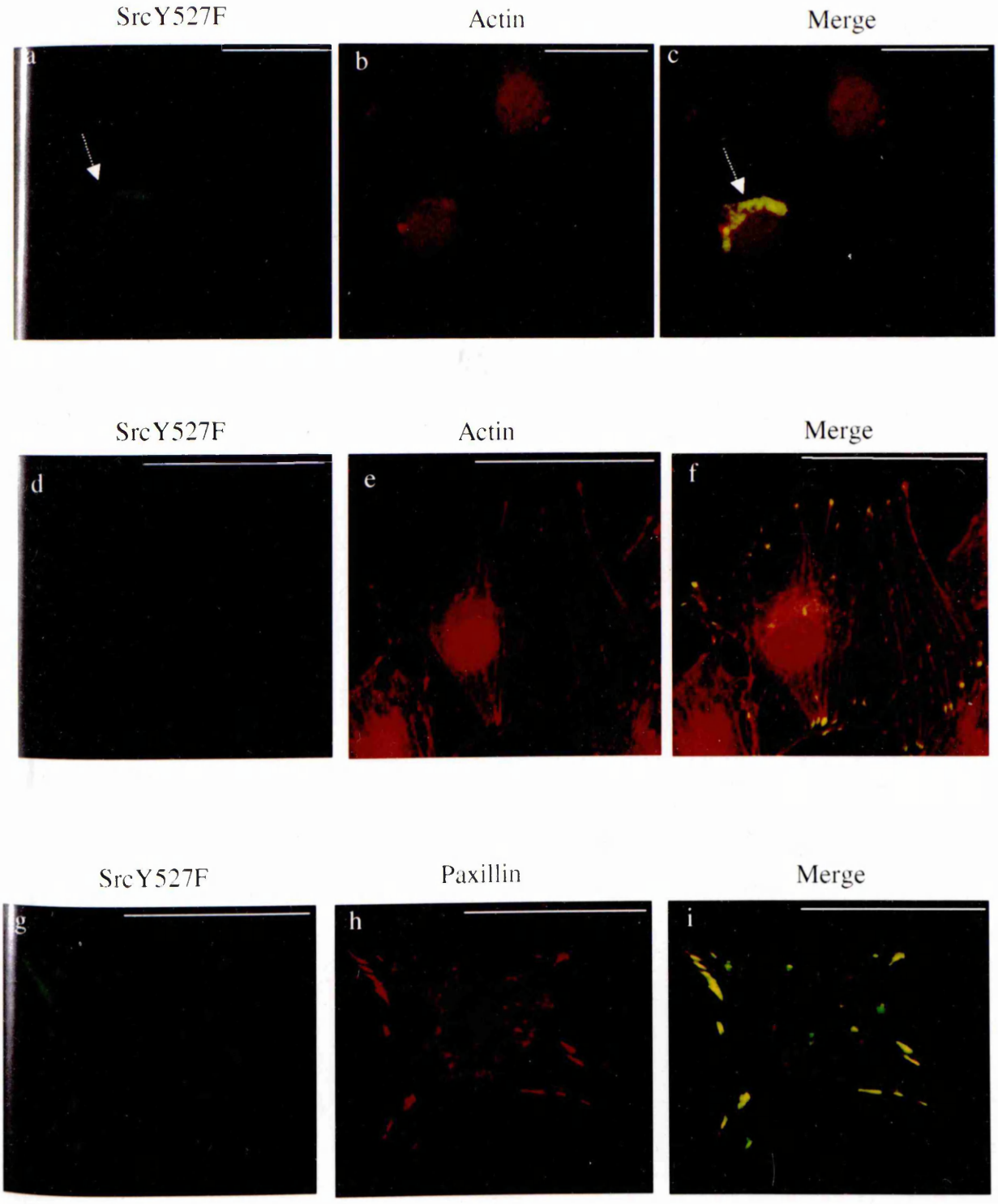
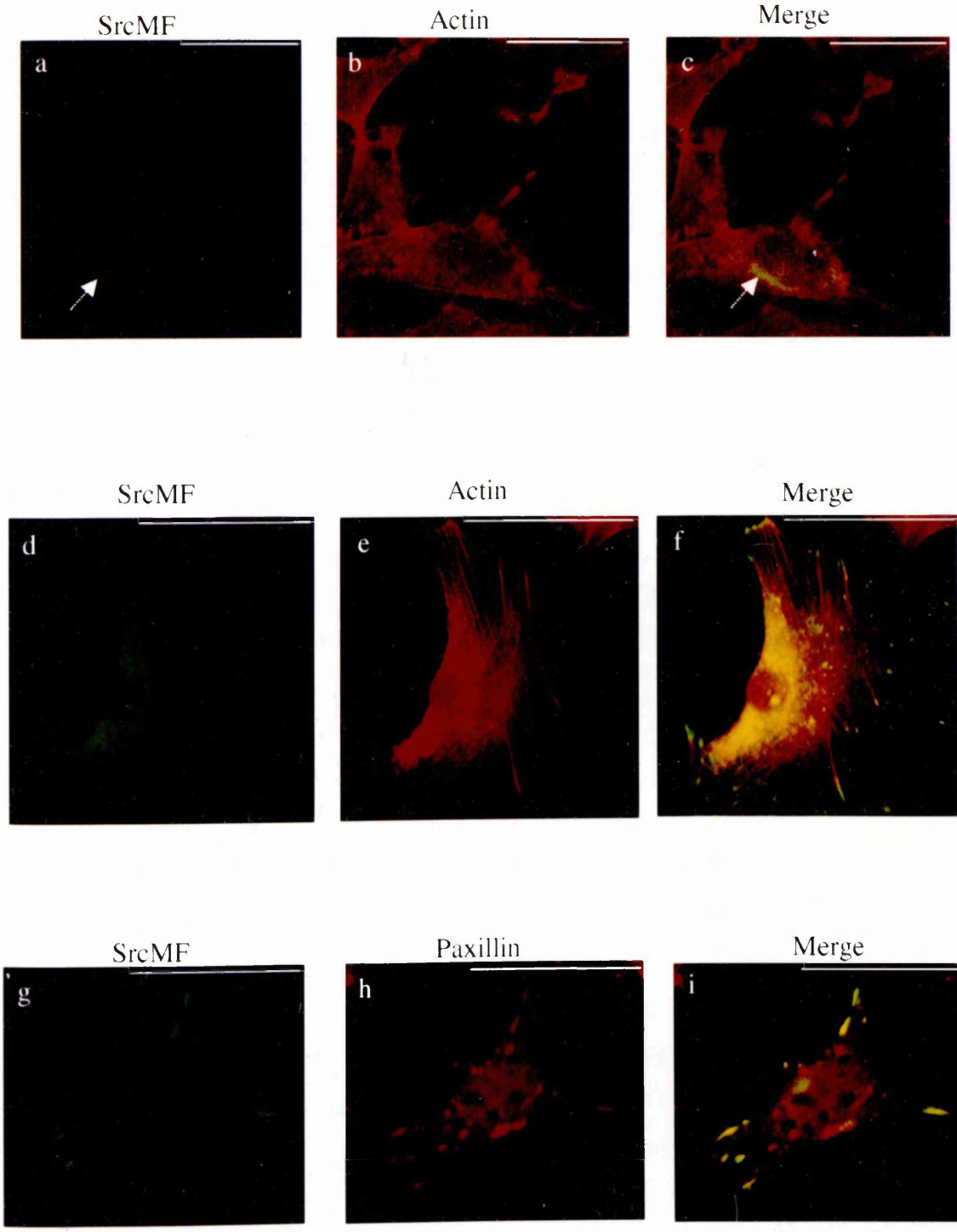

SrcY527F
Actin
Merge
a
b
c
SrcY527F
Actin
Merge
d
e
f
SrcY527F
Paxillin
Merge
g
h
i

Figure 37

Translocation of SrcMF to focal adhesions is indistinguishable from Src251-GFP. Cells were serum starved to remove stress fibres prior to microinjection of SrcMF (a-c). Serum-starved cells were microinjected with SrcMF and following expression of the protein were stimulated with 10% serum for 30 min (d-i). SrcMF was visualised using an anti-Src antibody (a,c,d,f,g,i). Arrows indicate perinuclear SrcMF. Actin was visualised with rhodamine-phalloidin (b,c,e,f). Paxillin was visualised with rhodamine-conjugated anti-paxillin (h,i). Scale bars 25 μm.

Figure 37


SrcMF
Actin
Merge
a
b
c
SrcMF
Actin
Merge
d
e
f
SrcMF
Paxillin
Merge
g
h
i

5.2.3 c-Src kinase activity is required for complete remodelling of the actin cytoskeleton during lamellipodia formation.

SrcY527F was localised to focal adhesions in the presence of serum (Figure 38a), as for Src251-GFP (Figure 34a). Following treatment with PDGF, SrcY527F was re-located from focal adhesions to lamellipodia that appeared continuous and relatively uniform (Figure 38c, broken arrows), in contrast to the discontinuous structures formed in cells expressing Src251-GFP (Figure 34c, broken arrow). This implies that the efficient re-location of c-Src from RhoA-induced focal adhesions into Rac1-induced structures along lamellipodia requires Src's catalytic activity. To test whether a chemical inhibitor of c-Src kinase activity had a similar effect to Src251-GFP expression on PDGF/Rac1-induced lamellipodia formation, we treated cells expressing SrcY527F with the Src kinase inhibitor PP2 (Hanke et al., 1996) at a concentration that inhibits autophosphorylation of c-Src on tyrosine-416 (Figure 38g). Under these conditions, SrcY527F was found in focal adhesions in the presence of serum (Figure 38d). However, upon PDGF stimulation, lamellipodia formation was fragmented (Figure 38e) and the re-localisation of c-Src from focal adhesions was to discrete larger complexes along the cell periphery (Figure 38f, broken arrow). At an enhanced resolution (Figure 38h), the edge of lamellipodia in cells expressing Src251-GFP, SrcY527F + PP2 or SrcY527F alone emphasises the requirement of Src kinase activity to induce uniform lamellipodia. This effect of a kinase inhibitor, which blocks c-Src activity, supports the interpretation that expression of Src251-GFP inhibited complete Rac1-induced actin and adhesion remodelling due to its lack of kinase activity.

However, as PP2 is known to inhibit other kinases (see Discussion Section 5.3), we also expressed a kinase-defective Src mutant, SrcMF in the cells and examined the ability of these cells to form lamellipodia in response to PDGF. As described for Src251-GFP and SrcY527F, SrcMF was targeted to focal adhesions in the presence of serum (Figure 39a). Subsequent stimulation of these cells with PDGF resulted in the relocalisation of SrcMF to discrete patches around the edge of the forming lamellipodia (Figure 39b) rather than to continuous lamellipodia as seen in cells expressing the activated Src protein (Figure 38c). These discrete structures along the lamellipodia were similar to those found in cells treated with the kinase inhibitor PP2 or in cells expressing the Src251-GFP protein which lacks the kinase domain (Figure 38f and Figure 34c respectively). Magnification of the edge of the lamellipodia in SrcMF and activated Src expressing cells (Figure 39c) shows more clearly the impaired lamellipodia formation in cells expressing kinase defective SrcMF. This data

implies that, like the presence of kinase null Src251-GFP in focal adhesions, it is the presence of kinase defective Src at focal adhesions prior to PDGF-induced lamellipodia formation that interferes with the efficient formation of uniform lamellipodia.

5.2.4 c-Src kinase activity is required for remodelling of focal adhesions during lamellipodia formation.

To demonstrate that the discontinuous lamellipodia found in Src251-GFP expressing cells was associated with impaired focal adhesion re-modelling into focal complexes we compared Rac1-induced peripheral adhesion re-modelling in cells expressing Src251-GFP with cells expressing the activated c-Src mutant, SrcY527F, monitoring adhesion complexes by staining with anti-vinculin or anti-paxillin, where appropriate (see Section 5.2.2). Anti-vinculin and anti-paxillin staining were indistinguishable, and in cells treated with PDGF we observed small vinculin- and paxillin-containing complexes around the base of lamellipodia (Figure 40a and d respectively). In cells expressing Src251-GFP, treatment with PDGF induced the co-localisation of Src251-GFP with vinculin at the cell periphery but was located in large discrete structures along the length of the lamellipodia (Figure 40b,c). In contrast, in activated SrcY527F-expressing cells, c-Src co-localised with paxillin along the base of uniform lamellipodia following lamellipodia stimulation (Figure 40e,f).

Figure 38

Src kinase activity is required for remodelling of the actin cytoskeleton during lamellipodia formation. Cells were transfected with SrcY527F and maintained in 10% serum containing medium (a). Cells were then placed in serum-free medium overnight and treated with the protein synthesis inhibitor emetine (55 μg ml^{-1}) for 30 min prior to stimulation with PDGF (25 ng ml^{-1}) for 30 min (b,c). This protocol allowed us to reduce the heterogeneity in the population following PDGF stimulation by removing the serum, while maintaining SrcY527F in the peripheral focal adhesions due to the incomplete loss of stress fibres after this short time in serum-free conditions. Cells expressing SrcY527F were pre-treated with the Src kinase inhibitor PP2 (5 μM) for 3 h prior to stimulation with PDGF using the same protocol as outlined above (e,f). Anti- phospho-Src-Y416 western blot of cell extracts from SrcY527F expressing cells in the absence (lane 1) or presence (lane 2) of PP2 (5 μM) for 3 h (top panel). The same blot was re-probed with anti-Src antibody (bottom panel) (g). High resolution image of cells expressing Src251-GFP, SrcY527F and SrcY527F + PP2 following stimulation with PDGF (h). Actin was visualised with rhodamine-phalloidin (a,b,d,e) and SrcY527F using an anti-Src antibody (a,c,d,f). Scale bars 25 μm.

Figure 38

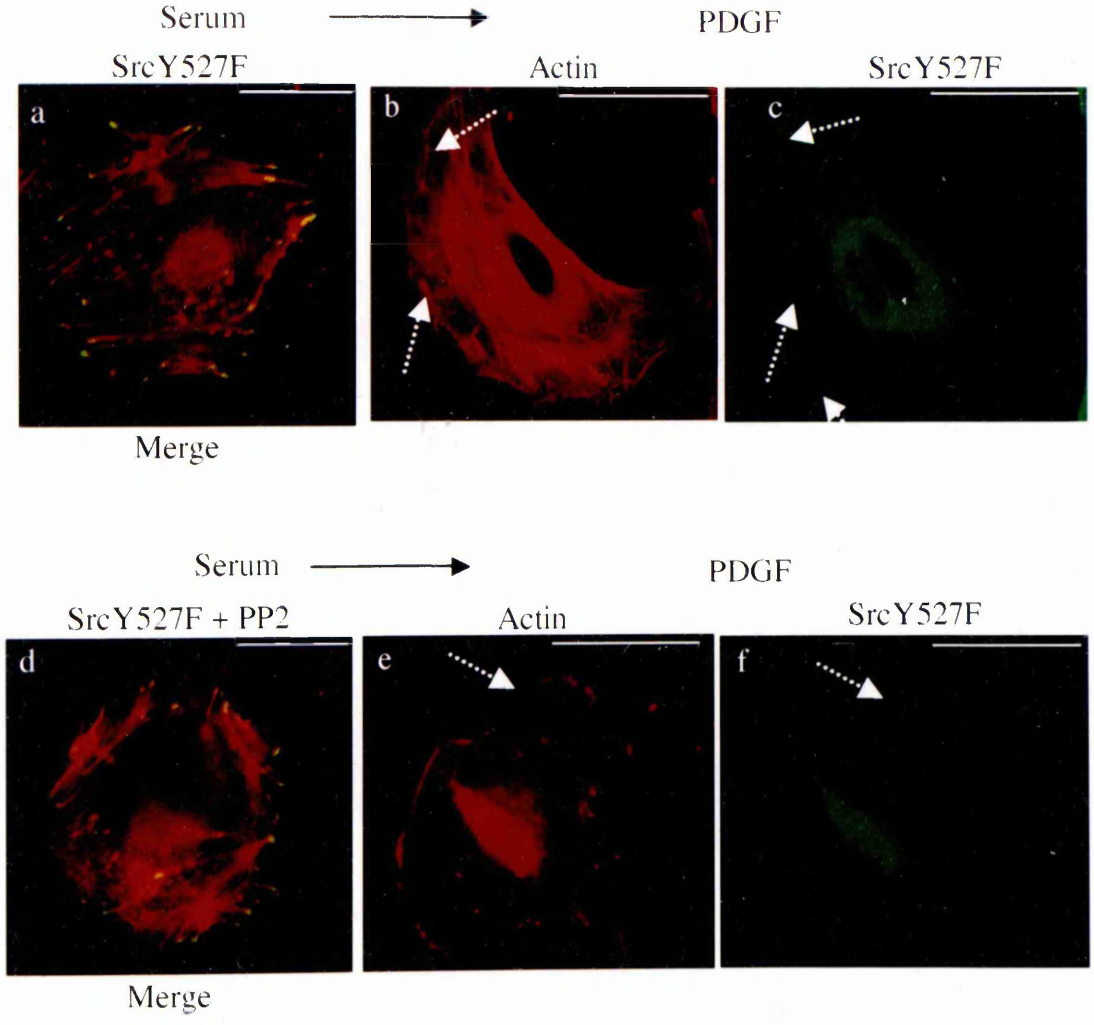
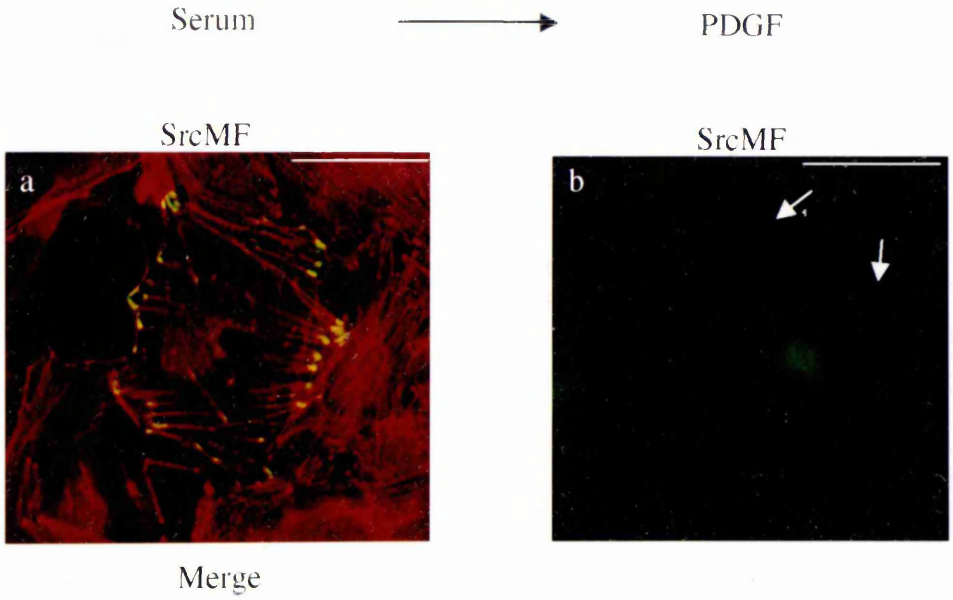

Serum
PDGF
SrcY527F
Actin
SrcY527F
a
b
c
Merge
Serum
PDGF
SrcY527F + PP2
Actin
SrcY527F
d
e
f
Merge
(g)
SrcY527F
SrcY527F+ PP2
phospho-Src-Y416
Src
(h)
Src251-GFP
(kinase-null)
SrcY527F
(+ PP2)
SrcY527F
(kinase-active)

Figure 39

SrcMF expression in focal adhesion prior to PDGF stimulation inhibits actin cytoskeletal remodelling during lamellipodia formation. Cells were transfected with SrcMF and maintained in 10% serum containing medium (a). Cells were then or placed in serum-free medium overnight and treated with the protein synthesis inhibitor emetine (55 μg ml^{-1}) for 30 min prior to stimulation with PDGF (25 ng ml^{-1}) for 30 min (b). High resolution image of the edge of the lamellipodia in cells expressing SrcMF and SrcY527F (c). SrcMF was visualised using an anti-Src antibody (a,b). Arows indicate SrcMF or SrcY527F localisation. Actin was visualised with rhodamine-phalloidin (a,c). Scale bars 25 μm.

Figure 39


Serum
PDGF
SrcMF
a
SrcMF
b
Merge

(c)

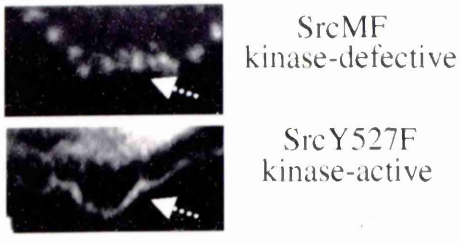
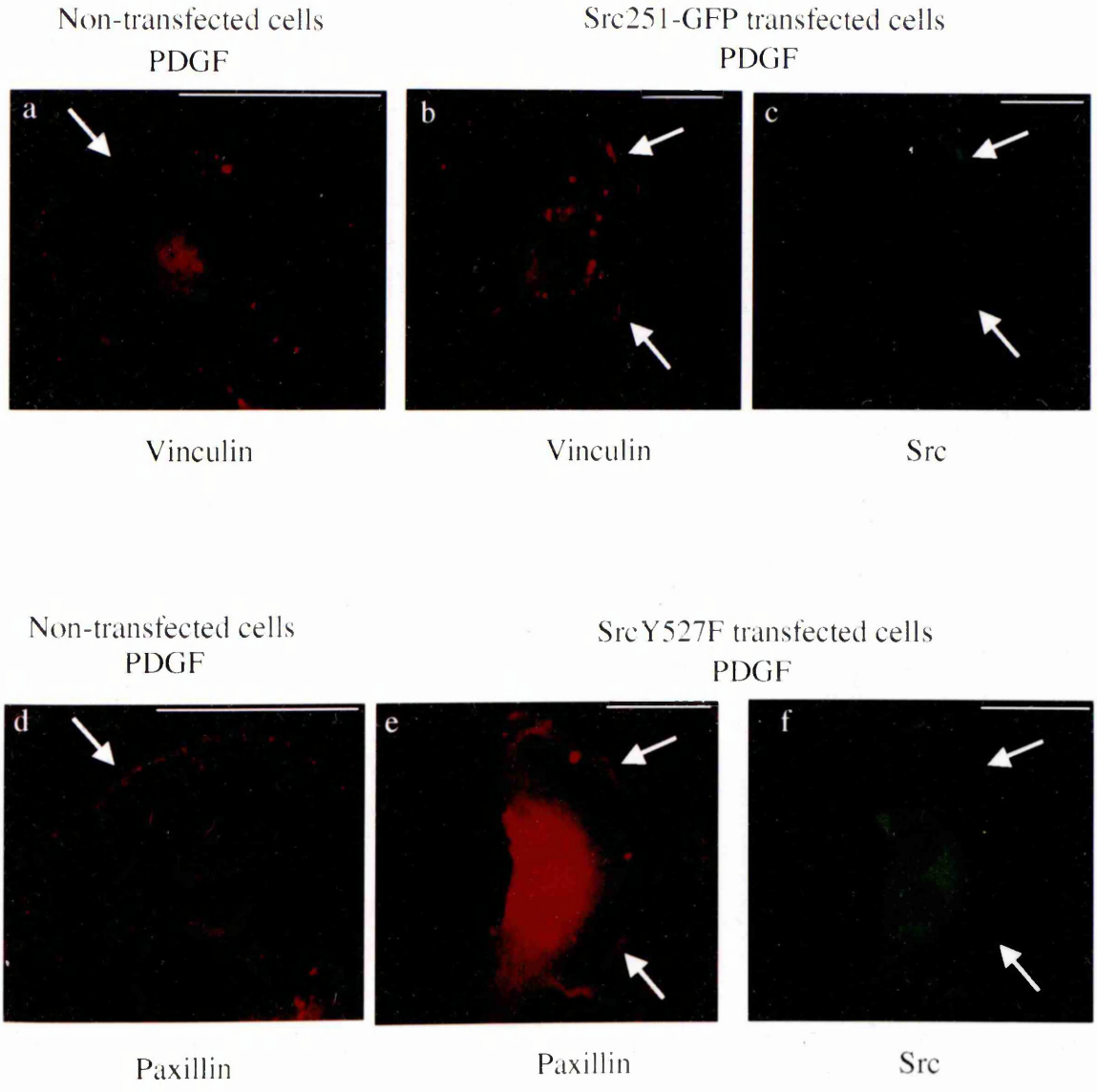

SrcMF
kinase-defective
SrcY527F
kinase-active

Figure 40

Src kinase activity is required for the disassembly of focal adhesions during lamellipodia formation. Cells were grown in serum-free conditions overnight prior to stimulation with PDGF (25 ng ml^{-1}) for 30 min and stained with anti-vinculin (a) or rhodamine conjugated anti-paxillin antibodies (d). Cells transfected with Src251-GFP (b,c), or SrcY527F (e,f) were placed in serum-free medium overnight and then treated with the protein synthesis inhibitor emetine (55 μg ml^{-1}) for 30 min prior to stimulation with PDGF (25 ng ml^{-1}) for 30 min (b,c,e,f). This protocol allowed us to reduce the heterogeneity in the cells following growth factor stimulation while maintaining the Src proteins in the focal adhesions prior to stimulation. SrcY527F and vinculin were visualised by anti-Src (f) and anti-vinculin (b) antibodies respectively. Paxillin was visualised with rhodamine-conjugated anti-paxillin (e) and Src251-GFP by direct immunofluoresence (c). Scale bars 25 μm.

Figure 40


Non-transfected cells
PDGF
Src251-GFP transfected cells
PDGF
a
b
c
Vinculin
Vinculin
Src
Non-transfected cells
PDGF
SrcY527F transfected cells
PDGF
d
e
f
Paxillin
Paxillin
Src

5.2.5 c-Src kinase activity is also required for complete remodelling of the actin cytoskeleton and focal adhesions during filopodia formation.

Similar to the observations with PDGF, we found that bradykinin-induced focal adhesion remodelling to produce filopodia was impaired in Src251-GFP-expressing cells. If cells expressing SrcY527F or Src251-GFP were grown in serum, thus allowing their localisation to focal adhesions and subsequently treated with bradykinin, SrcY527F localised to small actin containing filopodia that were uniformly distributed around the cell body (Figure 41a-c, soild arrows). In contrast, in Src251-GFP expressing cells, Src251-GFP was found in large discrete complexes (Figure 41d-f, broken arrows) at the base of filopodia (Figure 41d-f, solid arrows). At high resolution (Figure 41g) the edge of filopodia in cells expressing kinase-null Src251-GFP and kinase active SrcY527F emphasises the requirement of Src kinase activity for efficient filopodia formation and Src targeting to these structures. This effect of Src251-GFP supports the interpretation, as previously discussed for lamellipodia, that the kinase activity of Src is required for the complete remodelling of the actin cytoskeleton and focal adhesions to efficiently produce filopodia.

By monitoring adhesion complexes during this process we found that as described for PDGF, bradykinin-induced focal adhesion remodelling to produce focal complexes at filopodia was impaired in Src251-GFP expressing cells. Anti-vinculin and anti-paxillin staining was indistinguishable in cell treated with bradykinin, in which we observed small vinculin and paxillin-containing complexes at the ends of filopodia (Figure 42a,d). As described above, SrcY527F and Src251-GFP were located to focal adhesions in the presence of serum and upon treatment with bradykinin, SrcY527F and paxillin co-localised to small structures at the ends of filopodia (Figure 42b,c), while Src251-GFP and vinculin co-localised mainly to large discrete complexes at the cell periphery from which filopodia were emanating (Figure 42e,f).

Consistent with the effect of Src251-GFP on filopodia formation, expression of SrcMF and subsequent stimulation of these cells with bradykinin led to the remodelling of the actin cytoskeleton to form discrete patches of SrcMF at the base of forming filopodia (Figure 43a,b broken arrows), rather than the continuous and more uniformed distribution of filopodia in cells expressing SrcY527F (Figure 41b,c). Although cells expressing SrcMF were able to assemble actin containing microspikes (Figure 43b, solid arrows), these were not uniformly spread around the cell body and the filopodia appeared to originate from

large clusters of paxillin containing complexes (Figure 43c,d broken arrows) in contrast to the more diffuse and smaller complexes in SrcY527F expressing cells (Figure 42b,c).

Thus, our data supports a critical role for the catalytic activity of c-Src in the Rac1- and Cdc42-induced peripheral adhesion re-modelling that results in the breakdown of larger focal adhesions and the generation of smaller focal complex adhesion structures at lamellipodia and filopodia, respectively.

Figure 41

Src kinase activity is required for re-modelling of the actin cytoskeleton during filopodia formation. Cells transfected with SrcY527F (a-c), or Src251-GFP (d-f) were placed in serum-free medium overnight and then treated with the protein synthesis inhibitor emetine (5 μg ml^{-1}) for 30 min prior to stimulation with bradykinin (100 nM) for 5 min (a-f). This protocol allowed us to reduce the heterogeneity in the cells following growth factor stimulation while maintaining the Src proteins in the focal adhesions prior to stimulation. Actin was visualised with rhodamine-phalloidin (b,c,e,f). SrcY527F was visualised using an anti-Src antibody (a,c) and Src251-GFP was detected by direct fluorescence (d,f). Solid arrows indicate Src in filopodia, while broken arrows indicate Src at the base of filopdia. High resolution image of filopodia in cells expressing Src251-GFP (upper panel) and SrcY527F (lower panel) (g). Scale bars 25 μm.

Figure 41

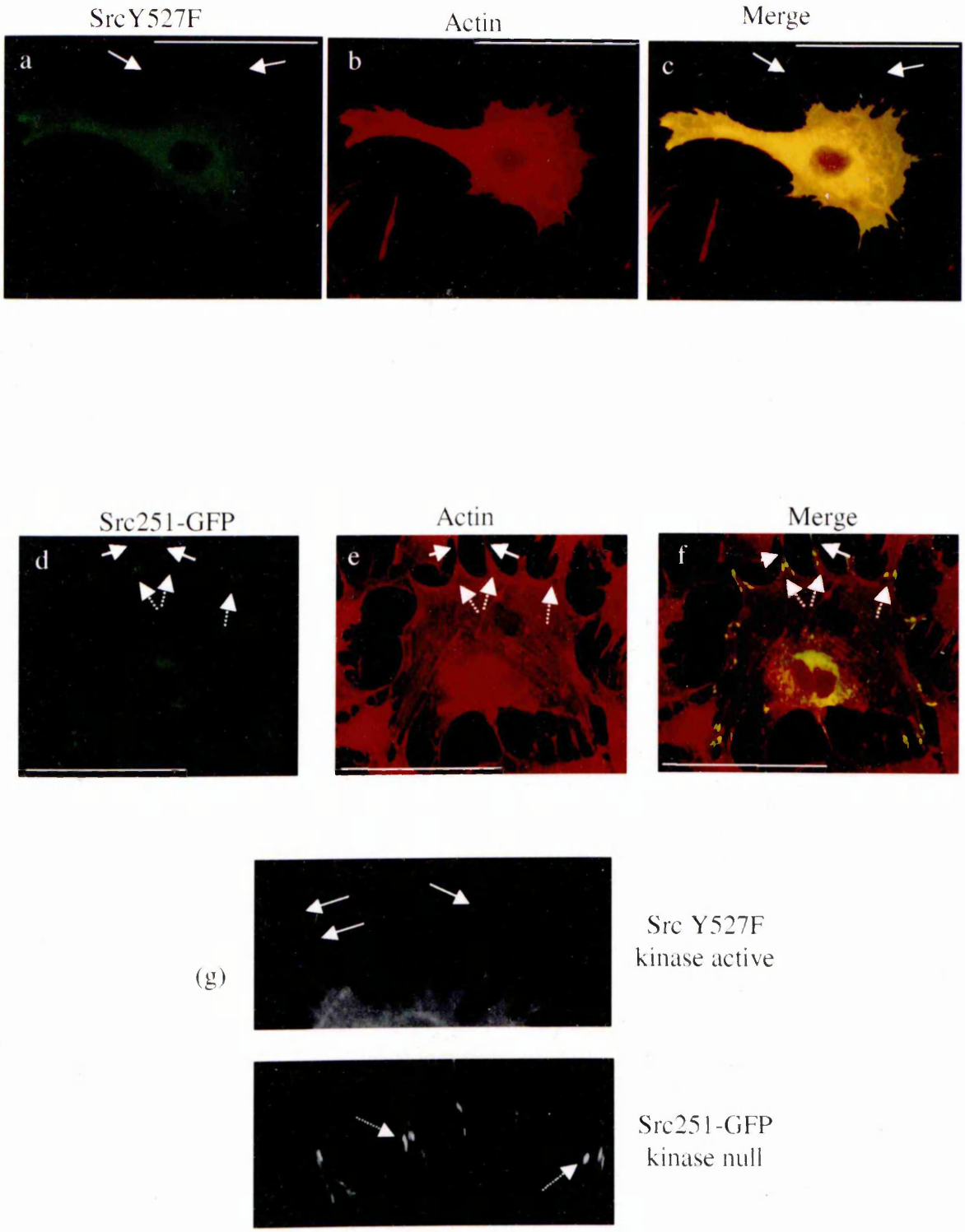

SrcY527F
Actin
Merge
a
b
c
Src251-GFP
Actin
Merge
d
e
f
(g)
Src Y527F
kinase active
Src251-GFP
kinase null

Figure 42

Src kinase activity is required for the disassembly of focal adhesions during filopodia formation. Cells were grown in serum-free conditions overnight prior to stimulation with bradykinin (100 nM) for 5 min and stained with anti-vinculin (a) or anti-paxillin antibodies (d). Cells transfected with SrcY527F (b,c), or Src251-GFP (e,f) were placed in serum-free medium overnight and treated with the protein synthesis inhibitor emetine (55 μg ml^{-1}) for 30 min prior to stimulation with bradykinin (100 nM) for 5 min (b,c,e,f). This protocol allowed us to reduce the heterogeneity in the cells following growth factor stimulation while maintaining the Src proteins in the focal adhesions prior to stimulation. SrcY527F (c) and vinculin (e) were visualised by anti-Src and anti-vinculin antibodies respectively. Paxillin was visualised with rhodamine-conjugated anti-paxillin (b) and Src251-GFP by direct immunofluoresence (f). Scale bars 25 μm.

Figure 42

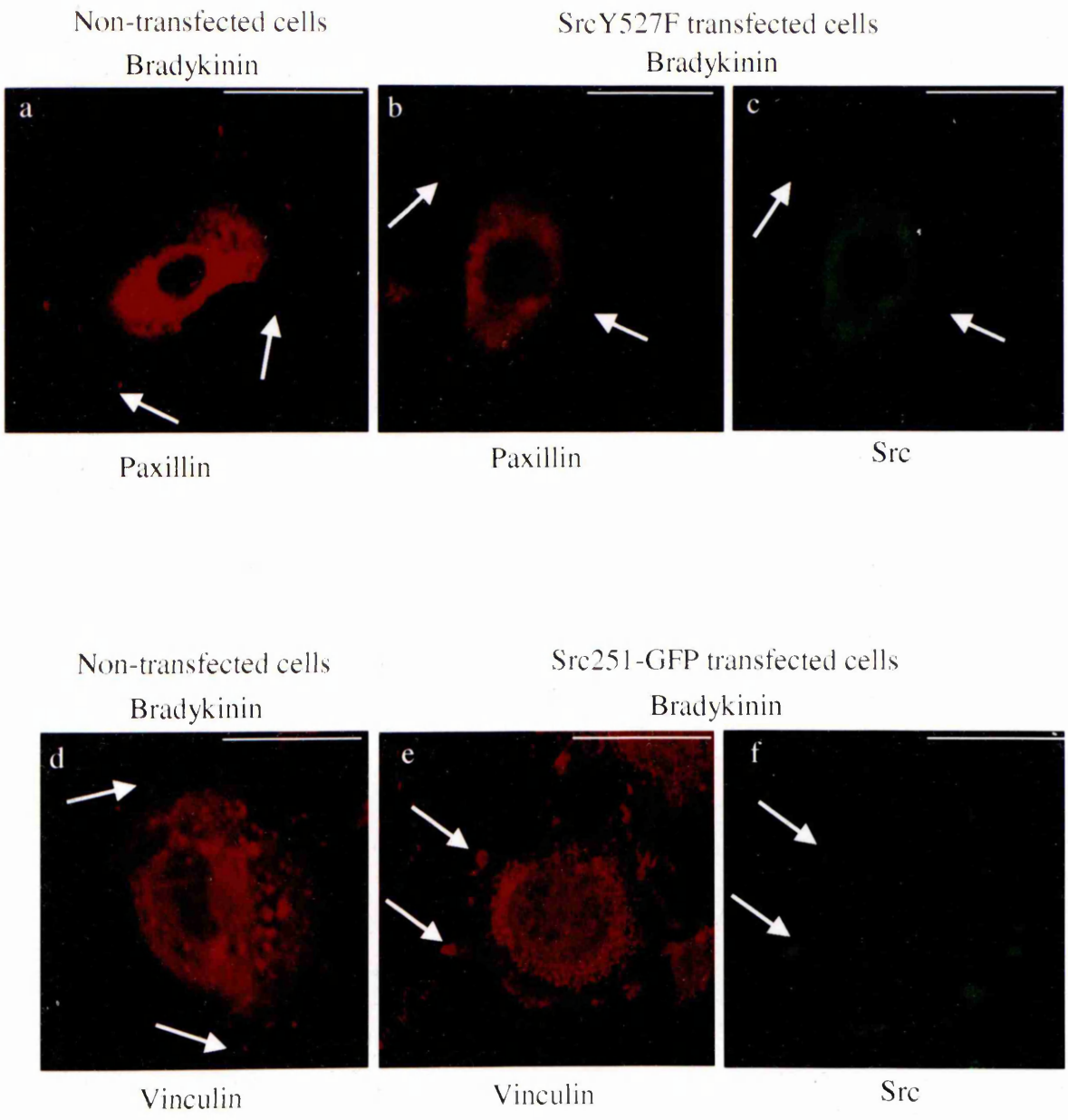
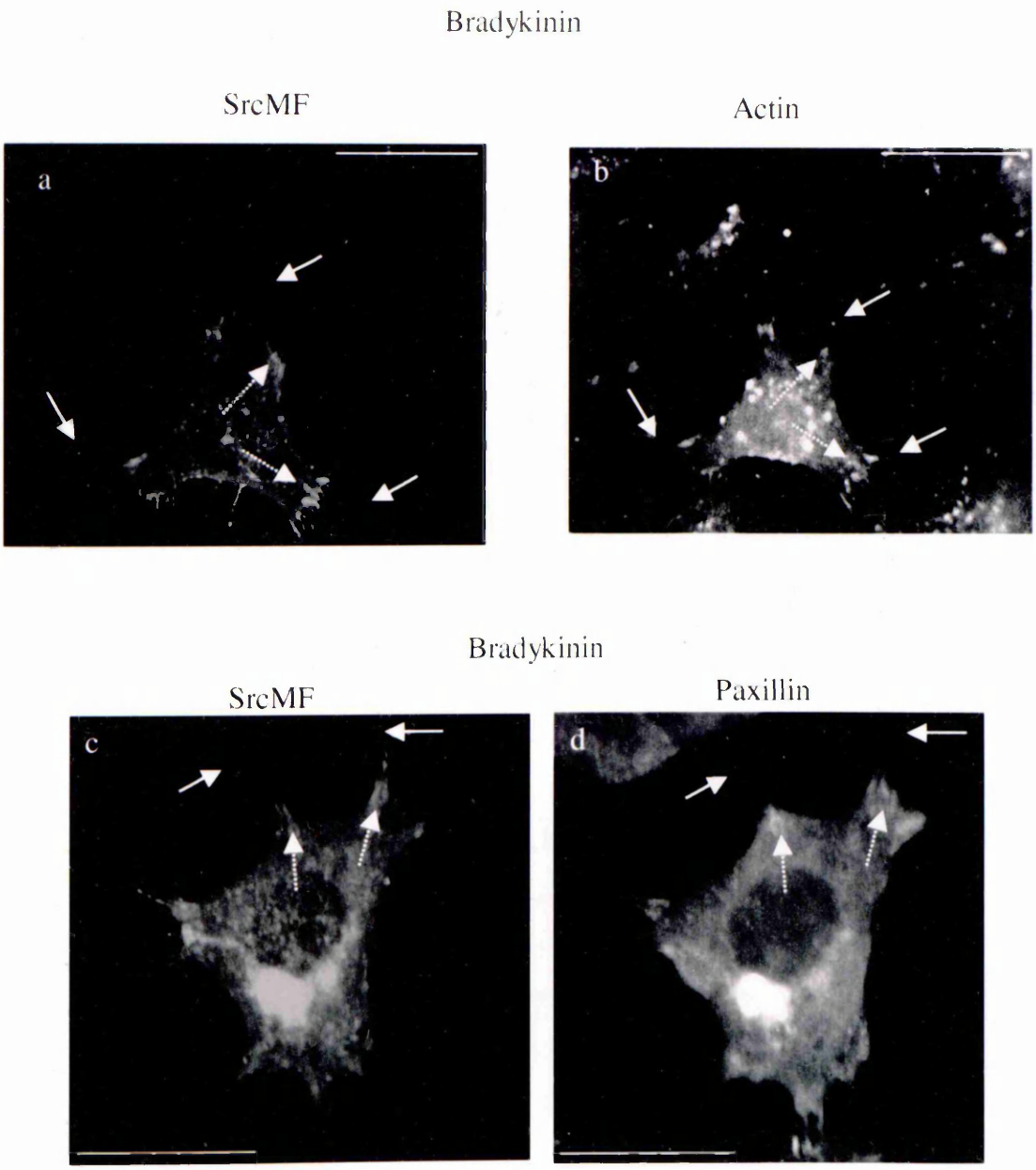

Non-transfected cells
Bradykinin
SrcY527F transfected cells
Bradykinin
a
b
c
Paxillin
Paxillin
Src
Non-transfected cells
Bradykinin
Src251-GFP transfected cells
Bradykinin
d
e
f
Vinculin
Vinculin
Src

Figure 43

SrcMF expression in focal adhesions prior to bradykinin stimulation inhibits actin and focal adhesion remodelling during filopodia formation. Cells were transfected with SrcMF and placed in serum-free medium overnight. Cells were then treated with the protein synthesis inhibitor emetine (55 μg ml^{-1}) for 30 min prior to stimulation with bradykinin (100 nM) for 5 min. This protocol allowed us to reduce the heterogeneity in the cells following growth factor stimulation while maintaining the Src proteins in the focal adhesions prior to stimulation. SrcMF was visualised using an anti-Src antibody (a,c) and actin was visualised with rhodamine-phalloidin (b). Paxillin was visualised with rhodamine-conjugated anti-paxillin (d). Scale bars 25 μm.

Figure 43



5.2.6 Expression of Src251-GFP or SrcMF in focal adhesions prevents Y-27632 induced focal adhesion disassembly.

Further evidence that Src251-GFP was acting to prevent the turnover of peripheral adhesion structures during lamellipodia or filopodia formation was provided by treating cells with Y-27632 following expression of Src251-GFP. Src251-GFP is found in focal adhesions in cells grown in serum and treatment of these cells with Y-27632 resulted in the loss of actin stress fibres (Figure 44b). However, Src251-GFP remained at the cell periphery under these conditions (Figure 44a,c, broken arrows). Treatment with Y-27632 also resulted in the breakdown and loss of focal adhesion structures, with few vinculin-containing adhesion structures evident (Figure 44e, solid arrows). In contrast, in cells expressing Src251-GFP, large focal adhesion-like structures remained (Figure 44d,e, broken arrows) although no stress fibres were present under these conditions. As a result of the loss of stress fibres and the majority of their focal adhesions, the surrounding cells appeared to have retracted and are therefore much smaller than the Src251-GFP expressing cell. Similarly cells expressing SrcMF prevented focal adhesion breakdown induced by Y-27632. The Rho-kinase inhibitor caused the loss of actin stress fibres (Figure 45b) in cells expressing SrcMF which remained in focal adhesions in the presence of Y-27632. However, Y-27632 did not induce loss of focal adhesions in cells expressing SrcMF (Figure 45d,e). Compare cells expressing SrcMF (broken arrows) to non-expressing cells (solid arrows), which have a marked reduction in paxillin staining compared to cells expressing SrcMF. Thus, expression of the kinase-deficient Src251-GFP or SrcMF proteins stabilised focal adhesion structures that normally disassemble when the RhoA effector pathway is inhibited. This finding is consistent with the requirement for c-Src's catalytic activity during focal adhesion breakdown and turnover (Fincham & Frame, 1998), where kinase defective Src expressing cells have been shown to have enlarged focal adhesions. This may provide a potential insight into the inefficient Rac1- and Cdc42-mediated peripheral adhesion remodelling observed in cells expressing Src251-GFP or SrcMF.

Figure 44

Stabilisation of focal adhesions in cells expressing Src251-GFP. Cells were transfected with Src251-GFP and upon expression, treated with Y-27632 (10 μM) for 1 h. Broken arrows indicate cells expressing Src251-GFP, while solid arrows indicate vinculin staining in non-expressing cells. Actin was visualised with rhodamine-phalloidin (b,c). Vinculin was visualised using an anti-vinculin antibody (e) and Src251-GFP by direct fluorescence (a,c,d). Scale bars 25 μm.

Figure 44

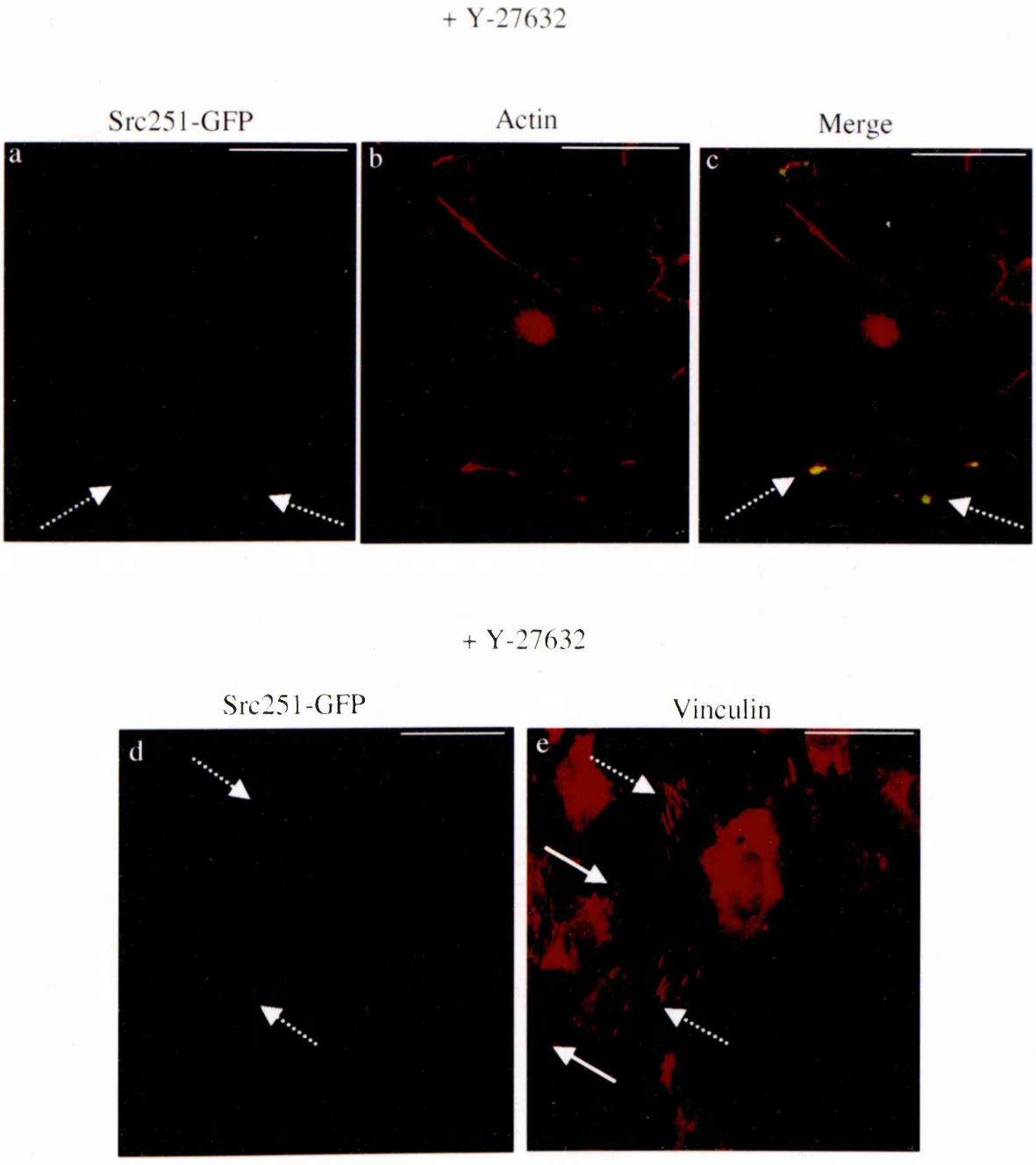
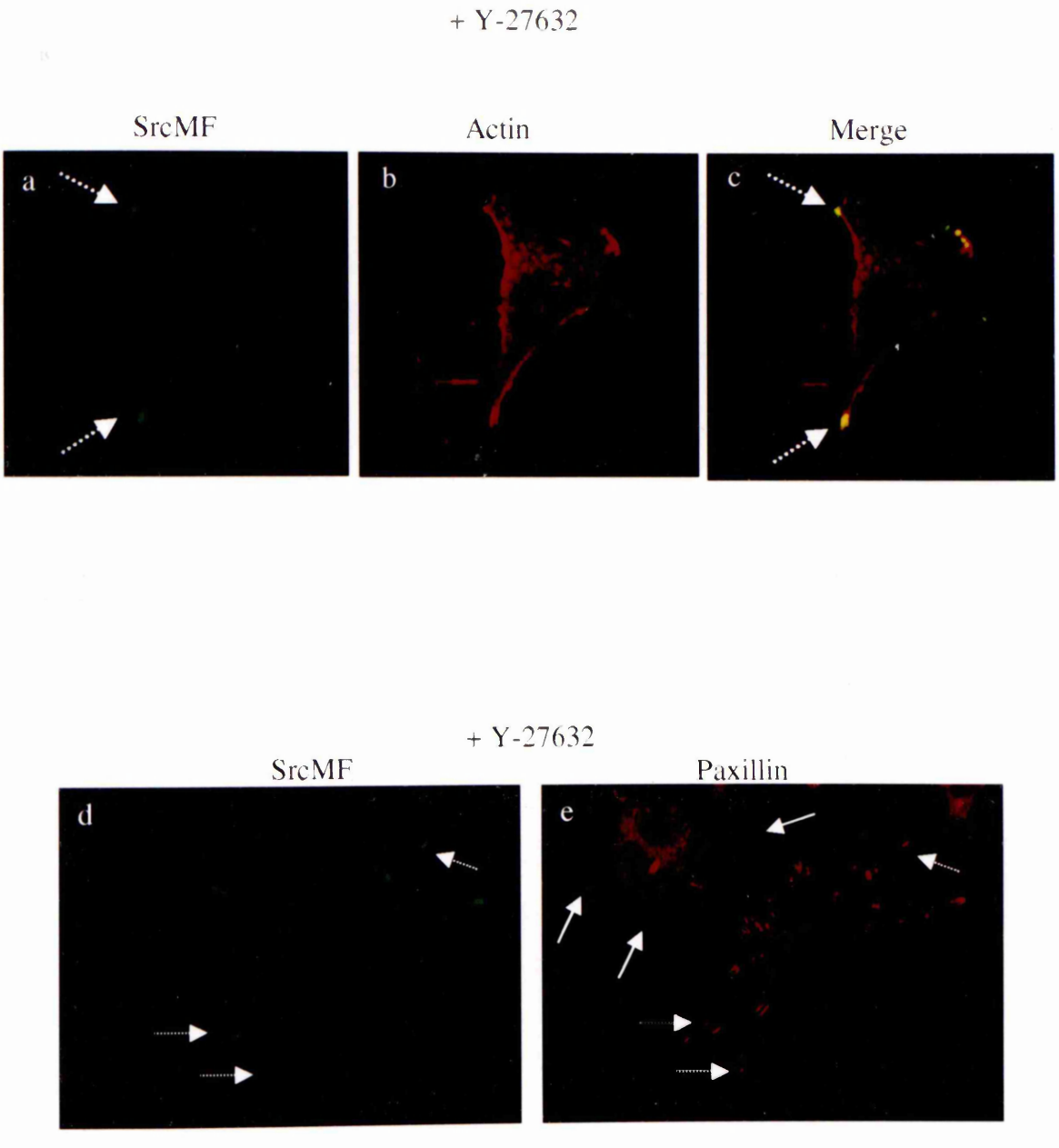

+ Y-27632
Src251-GFP
Actin
Merge
a
b
c
+ Y-27632
Src251-GFP
Vinculin
d
e

Figure 45

Stabilisation of focal adhesions in cells expressing SrcMF. Cells were transfected with SrcMF and following its expression, treated with Y-27632 (10 μM) for 1 h. Broken arrows indicate cells expressing SrcMF, while solid arrows indicate vinculin staining in non-expressing cells. Actin was visualised with rhodamine-phalloidin (b,c). Paxillin was visualised with rhodamine-conjugated anti-paxillin (e) and SrcMF was visualised using a anti-Src antibody (a,c,d). Scale bars 25 μm.

Figure 45



5.2.7 c-Src kinase activity is required for cell motility and polarity

As the formation of lamellipodia and filopodia have been associated with the regulation of cell motility and chemotaxis (Allen et al., 1997; Nobes & Hall, 1999) we examined whether the impaired focal adhesion remodelling in the cells lacking kinase activity, was associated with an inhibition of cell movement or the ability of cells to polarise in response to a chemotactic agent. Using time-lapse video microscopy we found that the random migration of cells expressing Src251-GFP and SrcMF was inhibited by around 70-75%, compared to non-transfected cells, while expression of GFP alone had no effect on cell motility (Figure 46). Cells were co-transfected with the respective Src mutants and GFP for visualisation. Expression of activated Src (SrcY527F) had no significant effect on the migration of the cells suggesting that the level of endogenous Src within the cells is not limiting for migration.

To examine the capacity of cells to respond to a chemotactic gradient, we measured the ability of cells to polarise towards PDGF using a Dunn chamber (see Materials and Methods Section 2.9.11) (Allen et al., 1997). Less than 10% of cells expressing Src251-GFP or SrcMF were able to polarise along the PDGF gradient, while around 80% of the non-transfected cells exhibited polarity (Figure 47). Expression of GFP or SrcY527F had no effect on the ability of cells to polarise in response to PDGF (Figure 47). Thus, both cell migration and cell polarisation in response to a chemotactic stimuli, biological responses that require the co-ordinated activities of Rho GTPases, are impaired in cells expressing either the Src251-GFP or SrcMF kinase-deficient mutants of c-Src. Our findings indicate that RhoA, Rac1 and Cdc42 not only control the precise sub-cellular localisation of c-Src, but co-operate with the catalytic activity of c-Src to mediate the adhesion remodelling that is required for polarisation and cell migration.

Figure 46

Src251-GFP and SrcMF inhibit random cell migration. Random cell migration of cells expressing Src251-GFP, GFP, SrcY527F (+ GFP) or SrcMF (+ GFP) in 10% serum. Values are mean $\pm$ S.E.M. taken from at least 5 independent experiments and represent data from between 30 and 60 cells in each case.

Figure 46

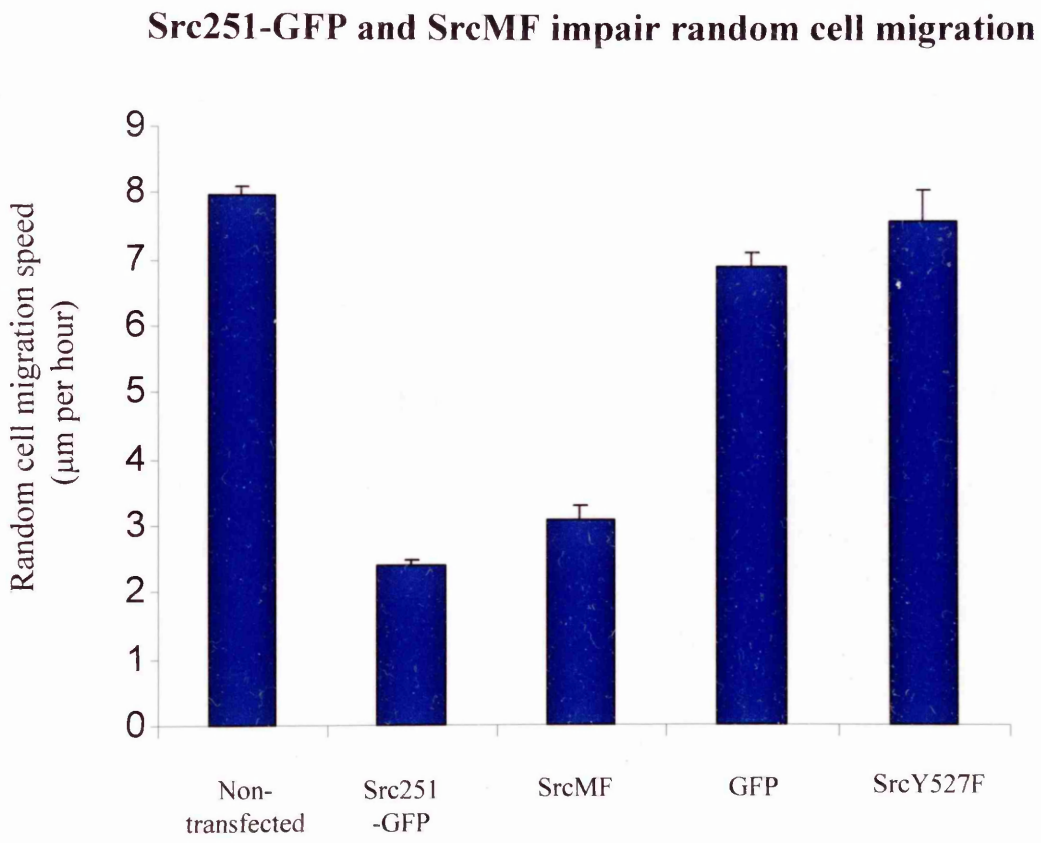
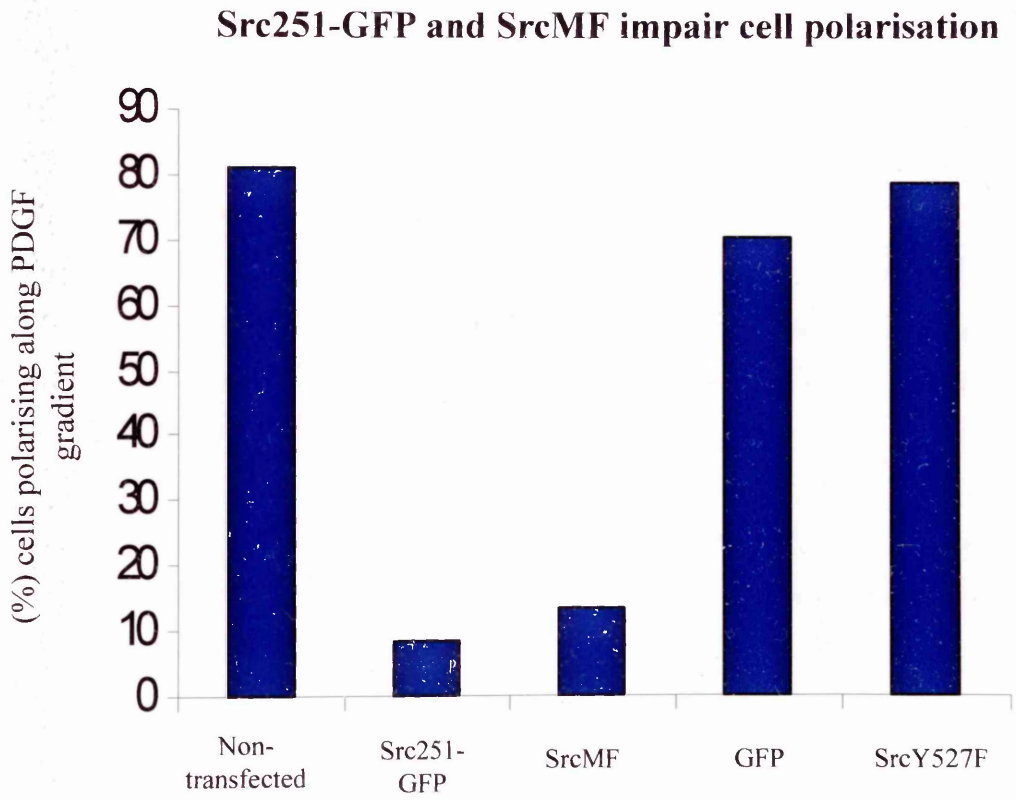

Src251-GFP and SrcMF impair random cell migration
Random cell migration speed (μm per hour)
0
1
2
3
4
5
6
7
8
9
Non-transfected
Src251-GFP
SrcMF
GFP
SrcY527F

Figure 47

Src251-GFP and SrcMF inhibit cell polarisation. Polarity of cells expressing Src251-GFP, GFP, SrcY527F (+GFP) or SrcMF (+GFP) in response to a PGDF gradient (10ng ml^{-1}). Values are expressed as the percentage of cells in a population able to polarise towards PDGF using the criteria described in the Methods Section 2.10.11, generated from at least 5 independent experiments.

Figure 47


Src251-GFP and SrcMF impair cell polarisation
(%) cells polarising along PDGF gradient
90
80
70
60
50
40
30
20
10
0
Non-transfected
Src251-GFP
SrcMF
GFP
SrcY527F

5.3 Discussion.

The involvement of RhoA, Rac1 and Cdc42 in cell motility is undoubtedly linked to their ability to rearrange the actin cytoskeleton. However, a secondary consequence of this actin remodelling is apparently to target other cellular proteins, such as c-Src, to these peripheral sites. We show here that once located at the cell periphery, in focal adhesions, c-Src acts in concert with the Rho GTPases to regulate directed cell motility. Our data supports a critical role for the catalytic activity of c-Src in the Rac1- and Cdc42-induced peripheral adhesion remodelling that results in the breakdown of larger focal adhesions and the generation of smaller focal complexes to allow lamellipodia or filopodia formation respectively (see Figure 48).

In this study we introduced constitutively active and inactive-Src mutants into the cells and used them as tools to establish the importance of Src kinase activity during adhesion remodelling. In addition, we also used a pharmacological agent to inhibit Src kinase activity during focal adhesions remodelling.

The Src kinase inhibitor specificity however is not confined to Src kinase, as it has also been reported to have weak inhibitory effects on other kinases such as ZAP-70 and Jak2 (Hanke et al., 1996; Yoshizumi et al., 2000). In the context of these experiments however, PP2 was used at concentrations below the threshold reported to block these kinases, therefore ruling out their involvement in the impaired focal adhesions remodelling and Src localisation observed. More significant however, is the reported inhibitory effect PP2 has on the PDGF and EGF receptors (Hanke et al., 1996; Yoshizumi et al., 2000). As PDGF was used here to cause Rac1-induced lamellipodia it is possible that the fragmented lamellipodia and Src localisation observed in the presence of PP2 occurs as a result of partial inhibition of the PDGF receptor. This however is not the only explanation, as the kinase deficient Src mutant SrcMF, like Src251-GFP, caused the same impairment in Src localisation and lamellipodia formation as seen in cells treated with the Src kinase inhibitor. Moreover, PP2 has been reported to block phosphorylation of tyrosine-577 on FAK, which is required for FAK kinase activity (Hanke et al., 1996) (Salazar & Rozengurt, 1999). FAK is thought to play and important role in focal adhesion turnover (Parsons et al., 2000) and therefore inhibition of FAK activity may also explain the impaired focal adhesion remodelling observed in cells treated with PP2. It should be noted that this impairment in tyrosine-577 phosphorylation maybe an indirect result of Src kinase inactivation as Src has been reported to phosphorylate FAK on this site (Calalb et al.,

1995). The potential involvement of Src-induced phosphorylation on various residues of FAK, including tyrosine-577, in focal adhesion remodelling will be discussed later in Chapter 7. Finally, it has been previously demonstrated that PP2 blocks Src kinase activity as well as the activity of other ubiquitously expressed Src family kinase such as c-Fyn and Yes (Hanke et al., 1996; Yoshizumi et al., 2000). This suggests that we cannot rule out the inhibition of other family members in this impaired focal adhesion to focal complexes remodelling observed in PP2 treated cells. In these experiments, expression of truncated or kinase defective Src has a similar effect as PP2 treatment, verifying the use of this inhibitor in assessing the role of Src kinase activity in this process. In keeping with this however, Src251-GFP and SrcMF expression may also be inhibiting other Src family members.

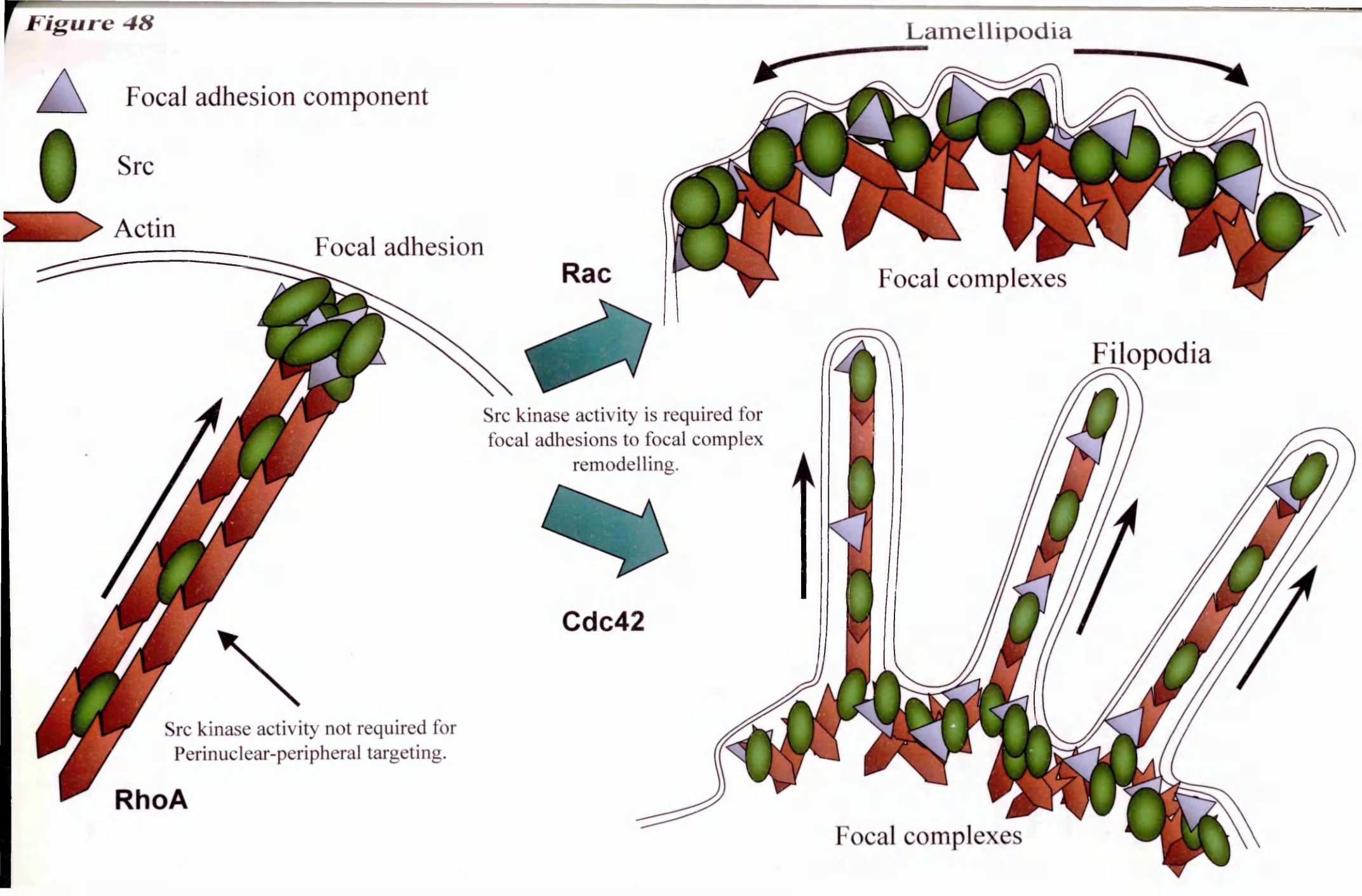
The kinase defective Src mutant, SrcMF has regularly been used to assess the role Src kinase activity plays in various cellular processes (Snyder et al., 1985). A potential pitfall however in using this dominant negative mutant is that the impairment of focal adhesion to focal complex remodelling observed during lamellipodia formation in cells expressing this mutant may be caused by the over-expression of available SH2/SH3 domains and could therefore be acting as a non-regulated adaptor molecule sequestering and maintaining various proteins in focal adhesions. It is therefore possible that Src plays an adaptor function in focal adhesion formation via the SH2/SH3 domains but ultimately its kinase activity is required for the turnover of these sites. This would explain why the absence of Src kinase activity in Src251-GFP and SrcMF expressing cells have enlarged focal adhesions and inefficiently remodel focal adhesions during lamellipodia or filopodia formation.

In this study we also used the constitutively active Src mutant (SrcY527F), to assess the role Src kinase activity has on focal adhesions remodelling. Previous reports using v-Src have demonstrated that unrestrained kinase activity of v-Src leads to enhanced cytoskeletal disruption and adhesion turnover (Fincham et al., 1999), where there is a breakdown and loss of stress fibres resulting in cell rounding and detachment associated with a morphologically transformed phenotype. Although SrcY527F, has unrestrained kinase activity and is a transforming mutant of c-Src, in the time scale of these experiments the cells do not undergo morphological transformation and therefore transformation-associated actin rearrangements or focal adhesion disruption does not interfere with the formation of lamellipodia or filopodia. Therefore SrcY527F was used in these experiments to monitor the involvement of Src kinase activity in focal adhesion to focal complex remodelling.

Figure 48

Schematic representation of Src's targeting from the perinuclear region of the cell to lamellipodia or filopodia. Src kinase activity is not required for Src's translocation from the perinuclear region of the cell to the cell periphery at focal adhesion but its kinase activity is required for the efficient remodelling of focal adhesion to focal complexes during lamellipodia or filopodia formation respectively.

Figure 48


Focal adhesion component
Src
Actin
Focal adhesion
Rac
Lamellipodia
Focal complexes
Src kinase activity is required for focal adhesions to focal complex remodelling.
Cdc42
Filopodia
Focal complexes
Src kinase activity not required for Perinuclear-peripheral targeting.
RhoA

The use of the protein synthesis inhibitor emetine in these experiments excluded the possibility of any newly synthesised Src being involved in the focal adhesions remodelling during lamellipodia or filopodia formation. This therefore implies that Rac1 or Cdc42 activation led to the re-location of c-Src that pre-existed in focal adhesions into complexes along lamellipodia or filopodia respectively. However, in these experiments we cannot rule out the possibility that some c-Src pre-existing in the perinuclear region after emetine treatment could move out into the newly formed lamellipodia or filopodia. In the case of bradykinin-stimulated filopodia however, the time scale of these experiments would not be sufficient to allow Src to translocate from the perinuclear region of the cell to the cell periphery, which has been reported to take between 15-30 min (V. Fincham, unpublished observation). In this study filopodia formation was observed within 5 min of stimulation with bradykinin. Data presented in this chapter on Src targeting to lamellipodia or filopodia, suggests that it is the presence of kinase-defective c-Src at focal adhesions prior to PDGF or bradykinin stimulation that interferes with the efficient formation of these structures rather than Src from the perinuclear region of the cell.

A key component of the co-ordinated movement of cells is their ability to polarise towards different migratory signals and in this study we have addressed the role of c-Src in this process. The formation of Rac1 dependent lamellipodia at the leading edge of cells is required for cell motility (Allen et al., 1998; Nobes & Hall, 1999; Ridley et al., 1995). In addition, while Cdc42 is not essential for cell movement per se, it is required for directionality and the maintenance of cell polarity (Allen et al., 1998; Nobes & Hall, 1999; Stowers et al., 1995). Swiss 3T3 cells have frequently been reported to have a low migration rate and in our system the Swiss 3T3 fibroblast migration rate was relatively low compared to other cell types such as macrophages (Allen et al., 1997). Moreover, the migration rate was significantly reduced upon expression of kinase defective Src mutants in these cells. It should be noted that a reduction in cell migration does not automatically confer a loss in the cells chemotactic capacity. Cell chemotaxis can be defined as the ability of cells to polarise and move towards a chemical gradient in a persistent manner. Using the Dunn chamber assay we can visualise cells under the influence of a constant chemical gradient, which can be generated rapidly and can persist for 5-20 h depending on the chemoattractant used. This assay allows the option of determining the persistence and directionality of cells during chemotaxis separately from migration rates. Other methods of measuring chemotaxis such as the Boyden chamber assay do not take this into account and can therefore potentially lead to false negative or positive results. For example, a reduction in cell migration (as observed in this study) could be misinterpreted as a failure to undergo chemotaxis, as cells that do not migrate fast enough may not reach the other

side of the filter during the course of the experiment thereby resulting in a false negative. On the other, hand cells with a significantly enhanced random migration rate may give rise to false positive results. Inhibition of Cdc42 in macrophages for example has been reported to significantly increase cell migration rates due to the loss of directional control normally enforced by Cdc42 (see Introduction Section 1.5.2 (Allen et al., 1997)). Using the Boyden chamber assay, it can be envisaged that this impairment of Cdc42, which is known to be necessary for macrophage chemotaxis (Allen et al., 1998), could lead to false positives regarding cell chemotaxis due to the enhanced random migration leading to an increased number of cells reaching the other side of the filter. As Swiss 3T3 cells expressing Src251-GFP or SrcMF have a marked reduction in cell migration, the Dunn chamber assay allowed us to examine cell polarity separately form cell migration and eliminate these potential problems. Other methods of examining directed cell migration or chemotaxis include visualising golgi orientation during a wound healing assay (Nobes & Hall, 1999). Cell migration in a wound healing assay however is not chemotactic cell migration, as cells are randomly moving into an open space as opposed to reacting to a chemical gradient. We have shown here, using the Dunn chamber assay, that c-Src kinase activity is required for cells to polarise and form a leading edge.

We propose that the ability of the RhoGTPases to direct the localisation of Src to these peripheral sites is required for the remodelling of the more static focal adhesions into smaller more dynamic focal complexes found at the leading edge of motile cells. c-Src activity is not required downstream of migratory signals to initiate cell shape changes associated with lamellipodia and filopodia formation, but is required for the remodelling of focal adhesions to smaller focal complexes associated with these structures. This is consistent with studies in which cells lacking all three ubiquitously expressed Src family members (Src, Fyn and Yes), are able to assemble focal adhesions (Klinghoffer et al., 1999), indicating that Src is not required for their formation. However, cells in which kinase defective mutants of Src are expressed have enlarged focal adhesions (Fincham & Frame, 1998; Kaplan et al., 1994), consistent with our observation with Src251-GFP and SrcMF expressing cells, suggesting a role for Src kinase activity in the turnover of these structures that is needed for cells to move (Fincham & Frame, 1998).

As discussed in Chapter 1 (see Section 1.6.1) a number of studies have also demonstrated a key role for Src family kinases in cell migration other than its role in focal adhesions turnover. For example, Src has been shown to be required for growth factor-induced cell migration which has also been attributed to Src's role in growth factor induced signalling pathways (Liu et al., 1999; Rahimi et al., 1998; Rodier et al., 1995). It is therefore possible

that in cells expressing kinase defective Src mutants that the impaired cell migration could be caused by an impairment in signalling pathways involving Src kinase activity.

As described earlier v-Src leads to the disruption of cellular adhesions and loss of actin organisation, resulting in cell rounding and detachment (Fincham et al., 1999; Fincham et al., 1995). This disruption of cellular adhesions and loss of actin organisation induced by v-Src may be an extreme version of a normal, spatial and temporally controlled process that allows adhesion remodelling to occur during cell migration. In cells expressing constitutively active SrcY527F, paxillin locates to uniform focal complexes along the lamellipodia, which were difficult to visualise as discrete structures, suggesting that costitutive Src's activity may be further disrupting the focal complexes. Interestingly, expression of activated SrcY527F did not enhance the migration of cells, indicating that the level of endogenous Src within the cell is not limiting for cell migration. This has also been seen upon re-introduction of activated Src into Src -/- cells where activated Src restored migration to a similar level as re-introduction of the wild type protein (Hall et al., 1996). Similarly, in this study SrcY527F did not enhance the ability of cells to form filopodia and polarise. Together, this indicates that adhesion dynamics must be strictly regulated during cell migration and polarisation, where there is sufficient adhesion turnover to allow focal adhesions remodelling and retraction, while maintaining enough adhesion for cell traction during cell polarisation and locomotion.

The presence of functional SH2 and SH3 domains in Src suggests that, in addition to Src's kinase activity, Src may be acting as an adaptor protein allowing its recruitment into focal adhesion structures and/or the recruitment of cellular partners to these sites. In line with this, kinase-deficient Src mutants can rescue both defects in cell spreading and cytoskeletal organisation in Src -/- fibroblasts and osteoclasts respectively (Kaplan et al., 1995; Schwartzberg et al., 1997). Moreover, cells expressing kinase defective Src have been shown to enhance the adhesive strength of cells by re-enforcing the linkage between the actin cytoskeleton and the vitronectin receptor (Felsenfeld et al., 1999). These studies imply that at least some effects of Src on cell adhesions and the cytoskeleton are kinase-independent and may rely on the adaptor function of Src. Spatial and temporal regulation of this adaptor function by RhoGTPases could potentially serve to transiently strengthen adhesions during cell traction possibly by recruiting various scaffolding proteins to these sites and increasing interactions between the ECM and the actin cytoskeleton.

One interesting aspect of Src's functional interactions with cytoskeletal regulators is the apparently paradoxical requirement for RhoA to mediates Src's peripheral targeting (see

Chapter 3 (Fincham et al., 1996)), and the Src-induced antagonism of RhoA via activation of p190RhoGAP (Fincham et al., 1999). p190RhoGAP is phosphorylated in a Src-kinase dependent manner (Ellis et al., 1990), leading to its association with p120RasGAP (Chang et al., 1995; McGlade et al., 1993). This association, in turn, results in increased RhoA-GTPase activity of p190RhoGAP, and the disruption and remodelling of the actin cytoskeleton (Chang et al., 1995). The antagonism of RhoA as a key event in Src's de-regulation of the actin cytoskeleton was highlighted by the RhoA-induced reversion of the Src-mediated actin changes in rat fibroblasts (Mayer et al., 1999). In keeping with this, the complex between p190RhoGAP and p120RasGAP is required for stress fibre turnover and efficient cell migration (Kulkarni et al., 2000). Thus it seems that Src is recruited to peripheral adhesions in a RhoA and actin dependent manner where the kinase activity of Src leads to cytoskeletal disruption and adhesion turnover necessary for cell migration (Fincham & Frame, 1998). In line with this, Src selectively modulates integrin-dependent traction forces that are important for cell migration. In particular, linkages between integrin vitronectin receptors and the actin cytoskeleton are strengthened in Src-deficient fibroblasts (Felsenfeld et al., 1999), indicating that Src activity is required to weaken, or disrupt, links at the integrin-cytoskeleton interface thus modulating cell motility. Interestingly, integrin signalling induces early Src-dependent tyrosine phosphorylation and activation of p190RhoGAP that is thought to relieve RhoA-mediated contractile forces thus allowing protrusions to form at the leading edge of motile cells (see section 1.6.2) (Arthur et al., 2000).

In our experiments, the presence of Src251-GFP or SrcMF in focal adhesions stabilised these adhesion sites that normally disassemble when the RhoA effector pathway is inhibited. The failure to relieve RhoA-induced contractile forces may partially explain the inefficient formation of protrusive structures such as lamellipodia or filopodia in these cells. Moreover failure to inhibit this contraction at focal adhesions may also explain the impaired focal adhesion to focal complex remodelling observed in these cells. In keeping with this, further studies have demonstrated that inhibition by p190RhoGAP enhances cell spreading and migration by promoting cell protrusion and polarity (Arthur & Burridge, 2001). This may provide an insight as to why Src kinase activity is required in cell migration and more specifically why cells expressing Src251-GFP or SrcMF cannot efficiently co-ordinate cell polarisation or migration in the absence of Src kinase activity (Klinghoffer et al., 1999). Therefore although RhoA is required for Src targeting to focal adhesions and the generation of contractile forces during cell motility, Src-induced antagonism of RhoA-induced contractility is probably also required at specific times and places within migrating cells. c-Src therefore seems to have both positive and negative

effects on cellular adhesions and may act as a switch between their assembly and disassembly placing it in a central position from which to co-ordinate the multi-faceted process of cell motility.

Src induces tyrosine phosphorylation of a number of adhesion substrates, including both structural and regulatory proteins such as, ERM proteins, cortactin, FAK, paxillin (Burridge et al., 1992; Huang et al., 1997; Ilic et al., 1995; Krieg & Hunter, 1992; Martin et al., 1995b; Wu et al., 1991). The exact function of each individual phosphorylation event is not fully understood, however, alterations, in the tyrosine phosphorylation of a number of these proteins could potentially contribute to focal adhesion disruption and the regulation of cell motility (see Introduction Section 1.5.2). It should be noted that tyrosine phosphorylation of various proteins have also been observed in cells expressing kinase defective Src mutants (Kaplan et al., 1994) (E Avizienyte unpublished). This tyrosine phosphorylation could potentially be attributed to the adaptor function of Src's SH2/SH3 domains serving to localise various proteins in focal adhesions within the vicinity of potential binding partners/kinases thereby inducing their phosphorylation. The enhanced tyrosine phosphorylation observed in cells expressing constitutively active Src or v-Src may be involved in focal adhesion turnover and therefore some potential candidates linked to Src-induced focal adhesions turnover and remodelling are examined in more detail in the next chapter.

5.4 Summary

We have demonstrated that co-ordinated regulation of RhoGTPases not only serves to control cell contraction or protrusion during cell migration but also has a secondary role in determining the localisation of Src kinase to functionally distinct structures involved in directed cell migration. Moreover, we have shown that Src does not play a passive role in this process and demonstrate that Src kinase activity participates in the efficient remodelling of focal adhesions to allow the formation of lamellipodia and filopodia which are both necessary for polarised cell migration. In the next chapter we begin to address why Src kinase activity is required during this process and examine the involvement of some potential binding partners/substrates of Src in this process.

Chapter 6

6 Identification of potential binding partners/substrates of Src specifically linked to focal adhesion remodelling.

6.1 Purpose

The different abilities of active and kinase defective Src to re-model Src-containing focal adhesions into lamellipodia- or filopodia- associated complexes is most likely due to the fact that kinase-null or defective forms of Src inhibit the normal function of endogenous Src family kinases. This is consistent with previous findings that kinase-defective v-Src can inhibit focal adhesion turnover (Fincham & Frame, 1998), and suggests that the mechanism of focal adhesion turnover during v-Src transformation and during peripheral re-modelling in response to Rac and Cdc42 may be similar, in that they both require kinase activity. In this chapter we aim to identify binding partners/substrates that are specifically linked to the impaired focal adhesion remodelling observed in Src251-GFP and SrcMF expressing cells, thereby, providing an insight as to why the kinase domain or activity of Src is required for efficient lamellipodia or filopodia formation and ultimately for biological processes such as cell migration and chemotaxis. Since there are many proteins reported to bind to Src, we gave priority to candidates which are of interest to our research and whose likely cellular functions are consistent with our current model of the role Src kinase activity plays in adhesion turnover and remodelling (see Chapter 5). Each potential candidate has been selected on the basis of their reported binding to Src and links to the actin cytoskeleton or focal adhesions.

6.1.1 Scar family proteins as potential candidates involved in focal adhesion remodelling.

Scar proteins are related to the WASP family proteins and like WASP they control actin dynamics by both binding to actin and inducing actin nucleation and polymerisation via the Arp2/3 complex (Machesky & Insall, 1998). The mammalian Scar family include at least four member named Scar-1 - Scar-4 (Bear et al., 1998). Recently, preliminary data obtained by our group indicates that Scar-1 associates with Src in immune complexes, which is mediated by the SH3 domain of Src (V. Brunton, unpublished). Consistent with this link between Src and Scar-1, there has been a great deal of evidence linking Src family kinases to the related protein N-WASP and the actin cytoskeleton. Vaccina virus for

example, utilises the viral protein ρ 36R, which via Src kinase, recruits N-WASP to the cell surface and propels it through the host cell (Frischknecht et al., 1999). Furthermore, the Src family kinase Fyn and the Src related kinase Fgr can both bind to WASP via proline rich sequences in WASP (Banin et al., 1996; Finan et al., 1996; Ramesh et al., 1999), although the consequences for actin nucleation are not known. As Scar-1 has been reported to play a key role in actin remodelling (Machesky & Insall, 1998), especially associated with the formation of PDGF/Rac1-induced lamellipodia, we wanted to establish whether Scar was involved in Src dependent adhesion remodelling events during lamellipodia formation.

In this section we therefore set out to determine whether Scar localises to focal adhesions and focal complexes, following stimulation of RhoA and Rac1. Cells stimulated with LPA, to activate RhoA, resulted in the formation of actin stress fibres (Figure 49b) and using an anti-Scar antibody, Scar was located at sites at the ends of these actin cables (Figure 49a,c, solid arrows). Similarly, following treatment with PDGF, to induce Rac1 activity, Scar was targeted to lamellipodia (Figure 49d, solid arrow) continuous with actin staining (Figure 49e,f, solid arrows). Cells were then stained with vinculin as a monitor of adhesion complexes and vinculin co-localised with Scar in focal adhesions following stimulation with LPA (Figure 50a,c, solid arrows). Moreover, PDGF stimulation resulted in the diffuse localisation of vinculin along the base of lamellipodia (Figure 50e, solid arrows) where it co-localised with Scar (Figure 50d,f). This data demonstrates that Scar localised to similar sub-cellular compartments, as previously described for Src in response to the activation of RhoA or Rac1 (see chapter 4). It is possible that the antibody used in this study could be detecting other isoforms of Scar (Bear et al., 1998; Miki et al., 1998b). As conformation of Scar localisation at these peripheral sites we therefore transfected cells with Myc-tagged Scar-1 and subsequently stimulated cells with LPA or PDGF. Cells stimulated with LPA, resulted in the formation of stress fibres (Figure 51b, broken arrow) and using an anti-myc antibody, myc-tagged Scar-1 was found at the ends of these stress fibres (Figure 51a,c, solid arrow). Furthermore, PDGF stimulation induced the diffuse localisation of myc-tagged Scar-1 along the base of lamellipodia (Figure 51d,e,f, solid arrows). Together, this data indicates that Scar is found at the same peripheral sites as Src in response to RhoA or Rac1 activation. It is therefore possible that Scar-1 along with Src may potentially play a role in the remodelling of focal adhesions to lamellipodia. As Scar has been shown to associate with Src (V Brunton, unpublished) we therefore set out to determine whether Scar co-localised with Src after the stimulation of peripheral targeting of Src from focal adhesions to lamellipodia. Src251-GFP was found to co-localise with Scar in focal adhesions following LPA treatment of cells (Figure 52a-c, solid arrows).

Similarly, SrcY527F co-localised with Scar in focal adhesions after LPA stimulation (Figure 52d-e, solid arrows). Upon stimulation of focal adhesion remodelling to produce lamellipodia both, Src251-GFP and Scar where found at large discrete structures along the base of forming lamellipodia induced by PDGF (Figure 53a-c, solid arrows). In contrast in SrcY527F expressing cells Scar localised with SrcY527F along the base of uniform lamellipodia (Figure 53d-f, solid arrows). This data indicates that in cells with both, activated or kinase-defective forms of Src, Scar co-localised with Src even in cells that were unable to remodel their focal adhesions efficiently to form uniform lamellipodia. This suggests that the binding or co-localisation of Src and Scar does not seem to be determined by the kinase activity of Src and implies that more subtle differences in Scar function may be involved in the impaired lamellipodia formation observed in kinase defective Src expressing cells. One explanation for this is that Scar's activity may be compromised in cells expressing the kinase defective Src mutants.

Figure 49.

Scar localises to focal adhesion and lamellipodia induced by LPA and PDGF. Cells were grown in serum-free conditions overnight prior to stimulation with LPA (200 ng ml^{-1}) for 30 min (a-c) or PDGF (25 ng ml^{-1}) for 30 min (d-f). Solid arrows indicate Scar localisation. Actin was visualised with rhodamine-phalloidin (b,c,e,f). Scar was visualised using an anti-Scar antibody. Scale bars 25 μm.

Figure 49

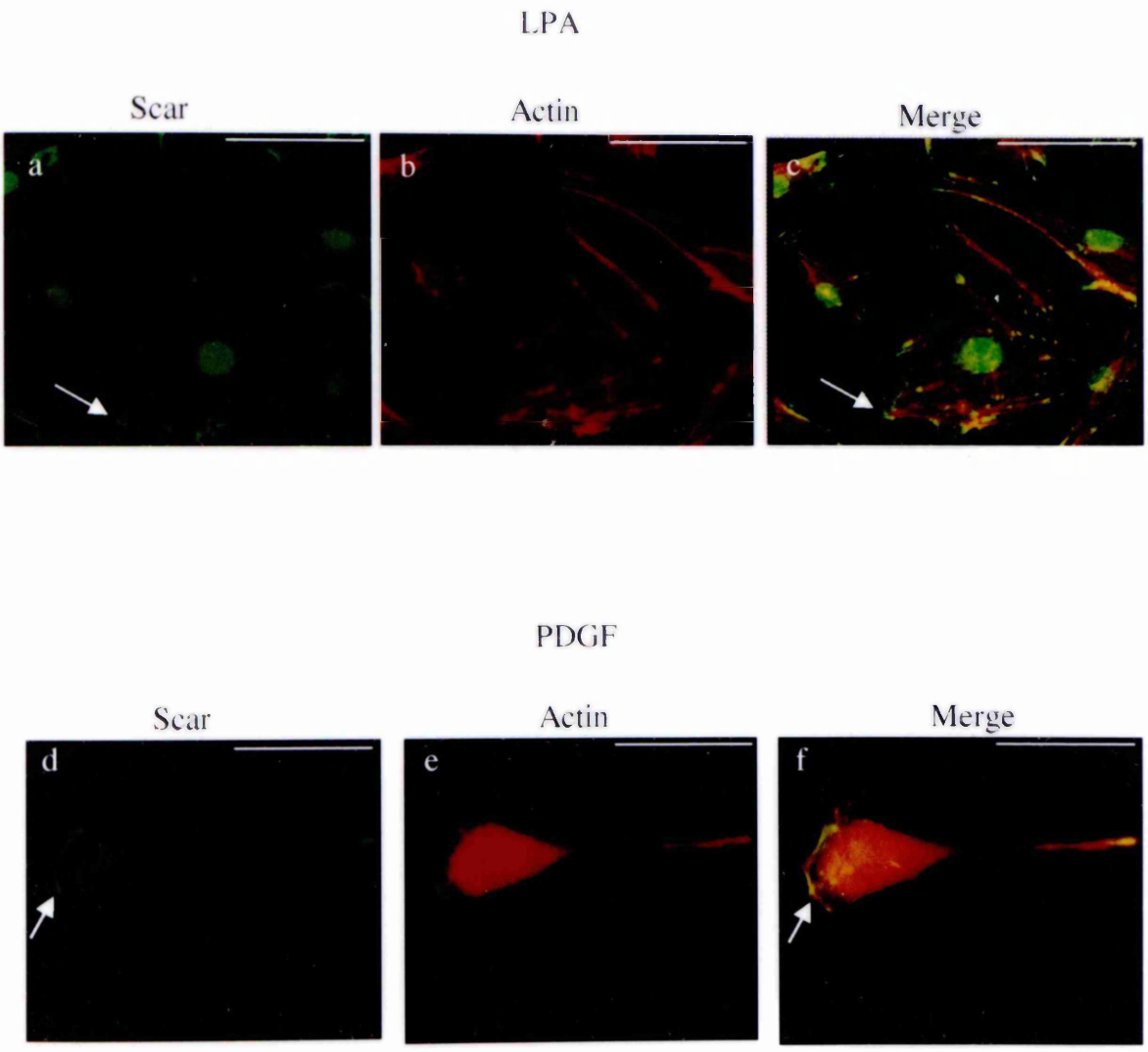


Figure 50

Scar co-localises with vinculin at focal adhesions and lamellipodia induced by LPA and PDGF. Cells were grown in serum-free conditions overnight prior to stimulation with LPA (200 ng ml^{-1}) for 30 min (a-c) or PDGF (25 ng ml^{-1}) for 30 min (d-f). Solid arrows indicate Scar localisation. Vinculin was visualised using an anti-vinculin antibody (b,c,e,f) and Scar was visualised using an anti-Scar antibody (a,c,d,f). Scale bars 25 μm.

Figure 50

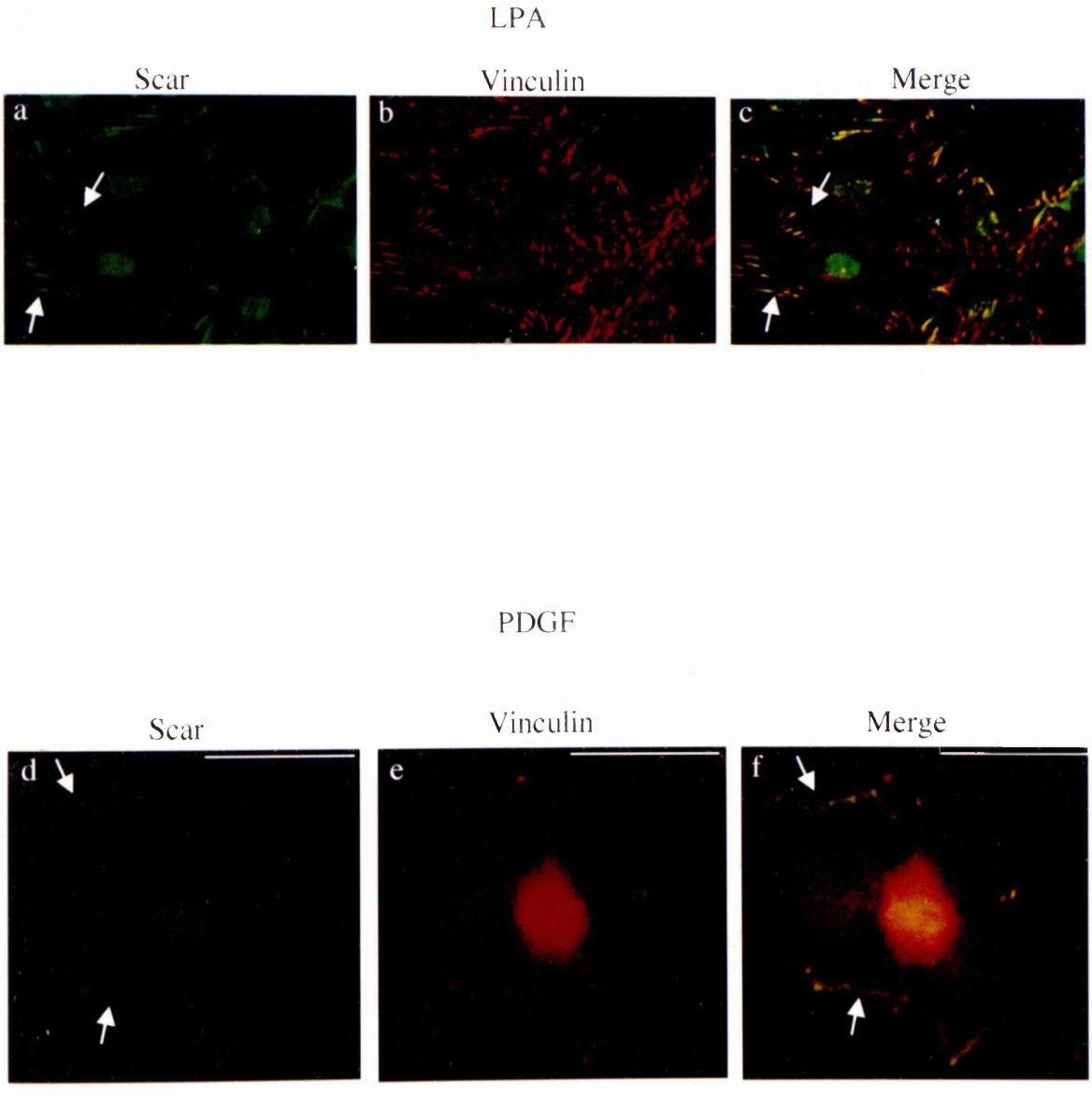


Figure 51

Myc-tagged Scar-1 localises to focal adhesions and lamellipodia induced by LPA and PDGF. Cells were grown in serum-free conditions overnight prior to stimulation with LPA (200 ng ml^{-1}) for 30 min (a-c), or PDGF (25 ng ml^{-1}) for 30 min (d-f). Actin was visualised with rhodamine-phalloidin (b,c,e,f). Myc-tagged Scar-1 was visualised using an anti-myc antibody (a,c,d,f). Scale bars 25 μm.

Figure 51

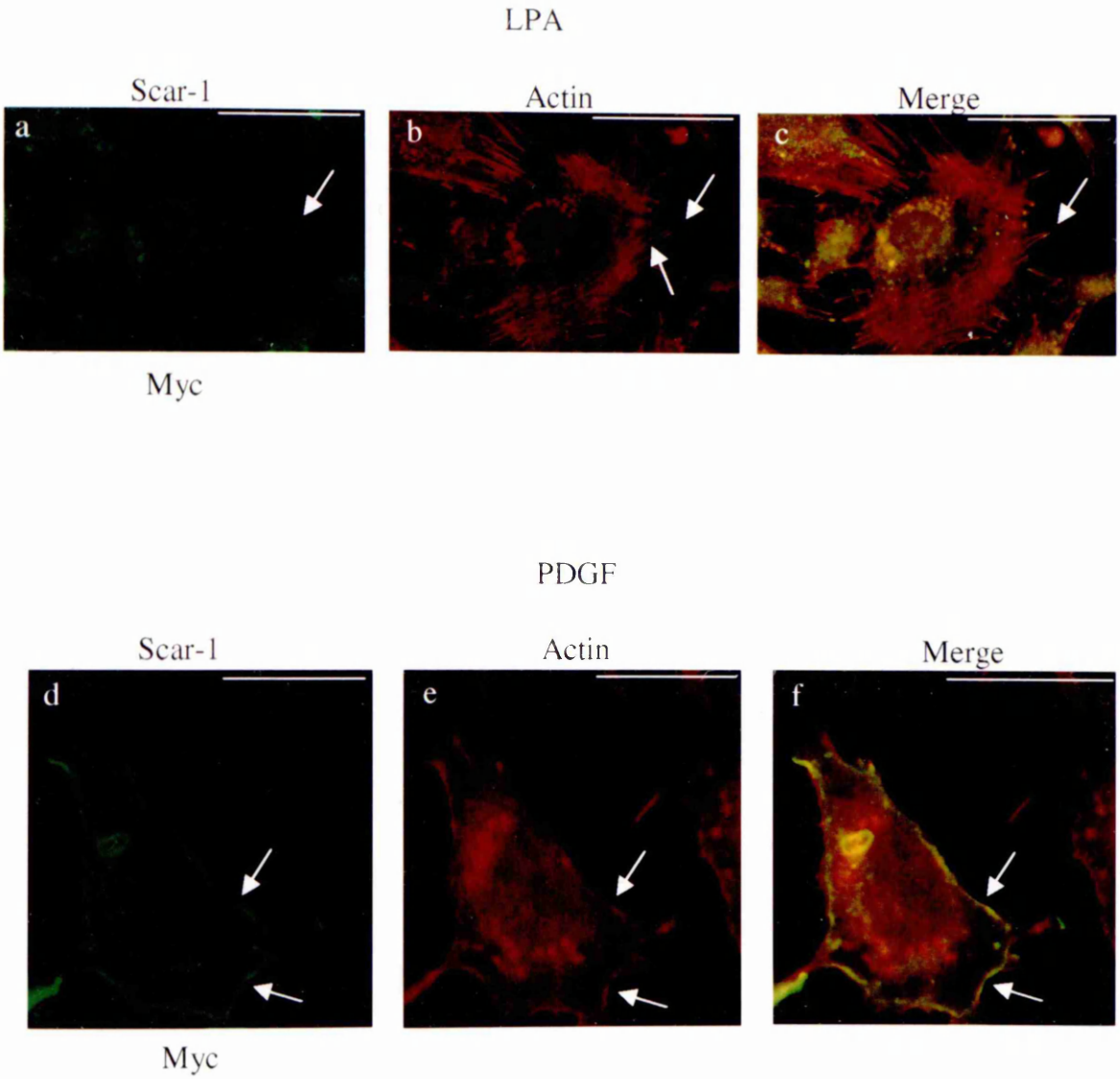


Figure 52

Scar co-localises with Src251-GFP and SrcY527F at focal adhesions induced by RhoA activation. Cells transfected with Src251-GFP (a-c) or SrcY527F (d-f) were placed in serum-free medium overnight and then treated with the protein synthesis inhibitor emetine (55 μg ml^{-1}) for 30 min prior to stimulation with LPA (200 ng ml^{-1}) for 30 min (a-f). This protocol allowed us to reduce the heterogeneity in the cells prior to LPA stimulation while maintaining the Src proteins in the focal adhesions prior to stimulation. Scar was visualised using an anti-Scar antibody (b,c,e,f), Src251-GFP by direct fluorescence (a,c) and SrcY527F using anti-Src antibody (d,f). Scale bars 25 μm.

Figure 52

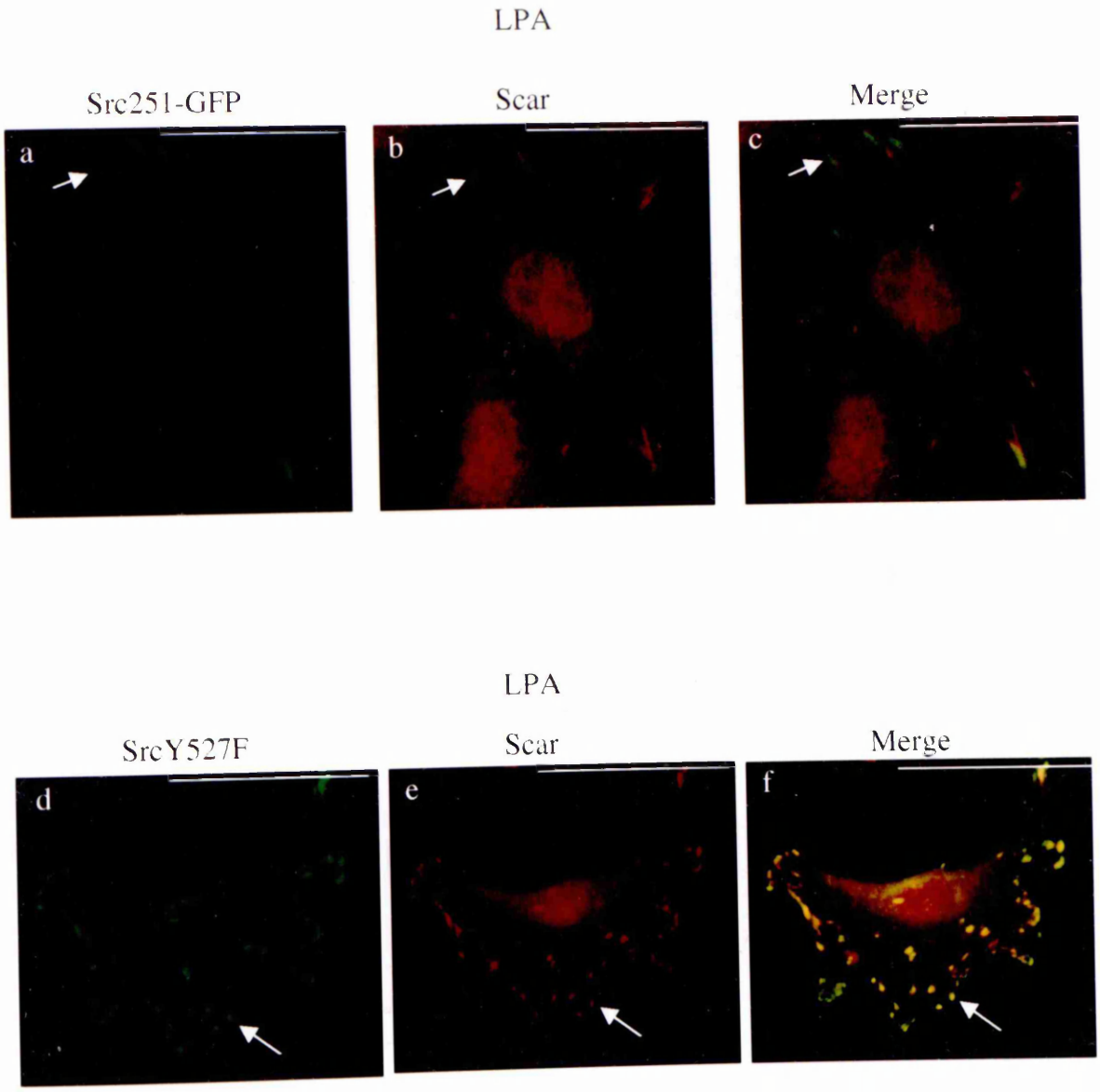
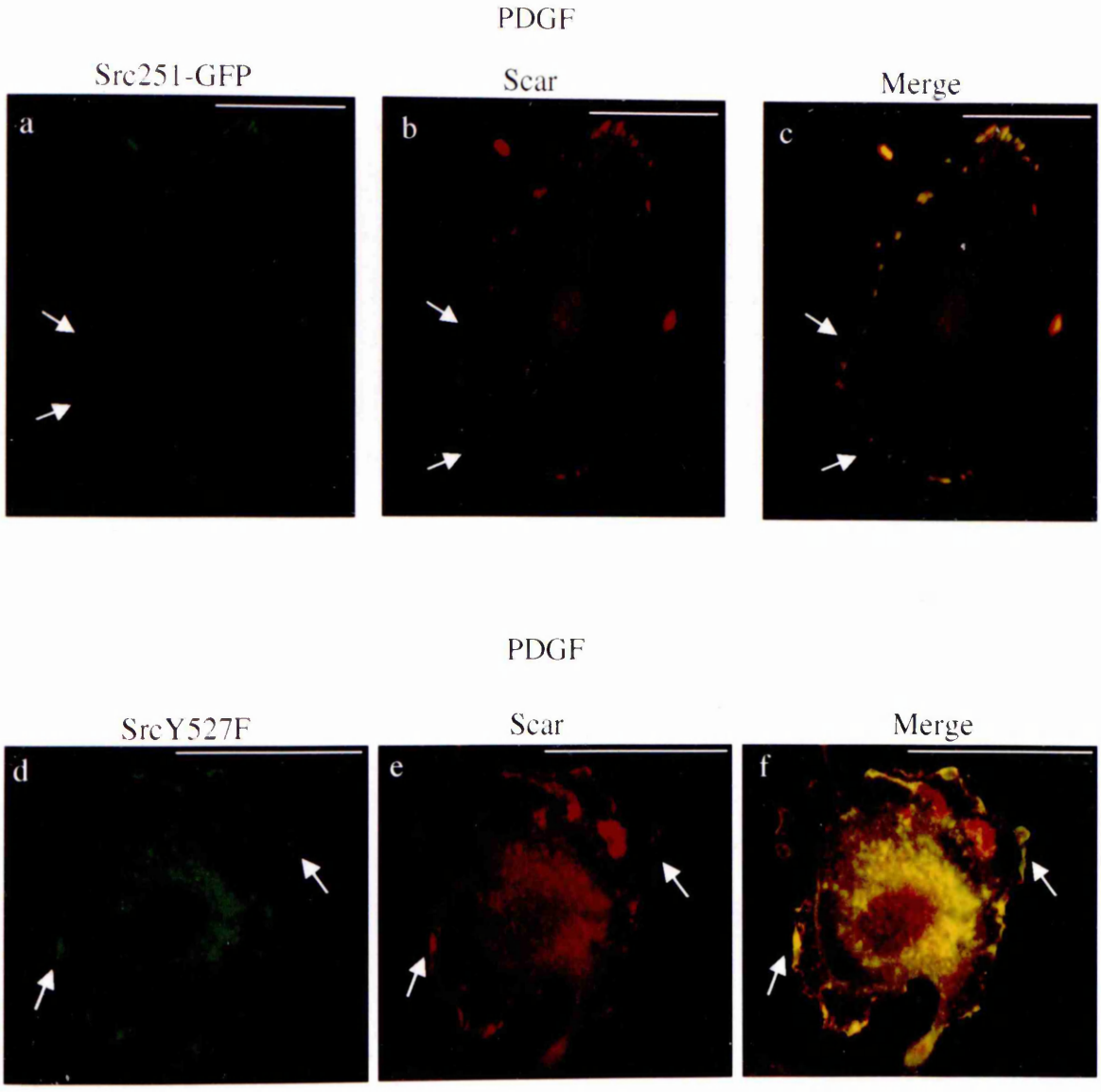


Figure 53

Scar co-localises with Src251-GFP and SrcY527F in lamellipodia induced by Rac-1 activation. Cells transfected with Src251-GFP (a-c) or SrcY527F (d-f) were placed in serum-free medium overnight and then treated with the protein synthesis inhibitor emetine (55 μg ml^{-1}) for 30 min prior to stimulation with PDGF (25 ng ml^{-1}) for 30 min (a-f). This protocol allowed us to reduce the heterogeneity in the cells prior to PDGF stimulation while maintaining the Src proteins in the focal adhesions prior to stimulation. Scar was visualised using an anti-Scar antibody (b,c,e,f), Src251-GFP by direct fluorescence (a,c) and SrcY527F using anti-Src antibody (d,f). Scale bars 25 μm.

Figure 53



6.1.2 The effect of Src kinase activity on Scar-1 function.

As discussed earlier Scar is involved in actin cytoskeletal remodelling by binding to actin and the Arp2/3 complex (Machesky & Insall, 1998). We therefore examined Scar's ability to regulate these processes where there was inefficient actin and focal adhesion remodelling during lamellipodia formation. In particular, as Scar is known to bind directly to the Arp2/3 complex and stimulate its ability to promote the nucleation of new actin filaments (Machesky & Insall, 1998), we determined whether a functional kinase domain of c-Src was required for Arp2/3 binding. We examined the binding of Myc-tagged Scar-1 and Arp2/3 in cells expressing kinase-defective mutants of Src that where unable to remodel focal adhesions in response to PDGF and displayed impaired motility (see Chapter 5) and compared this with cells expressing constitutively active Src. Swiss 3T3 cells expressing Myc-tagged Scar-1 or co-expressing Scar-1 with Src251-GFP, SrcMF or SrcY527F were stimulated with PDGF. (Machesky et al., 1997; Welch et al., 1997). Using one component of the Arp2/3 complex, Arp3, as a surrogate marker of Arp2/3 binding, we found that the presence of either constitutively active Src or kinase defective Src mutants within the cell had no effect on the ability of Scar-1 to bind to Arp3 (Figure 54). As Scar is known to bind directly to actin monomers (Machesky & Insall, 1998) and via the Arp2/3 complex induce actin nucleation, we assessed whether a functional kinase domain of Src could influence the capacityof Scar to bind to actin, which could potentially alter lamellipodia formation (Machesky & Insall, 1998; Miki et al., 1998b). Swiss 3T3 cells expressing Scar-1 or co-expressed with Src251-GFP, SrcMF or SrcY527F were stimulated with PDGF. Myc-tagged Scar-1 was immunoprecipitated from cell lysates and incubated with monomeric actin for 30 min. Using an antibody to actin, we found that the actin binding capacity of Scar-1 was not altered in cells co-expressing the constitutively active or kinase defective Src mutants (Figure 55).

As Scar is known to act as an intermediate between growth factor receptors and the actin cytoskeleton, via adapter molecules such as Grb2 and Nck (Frischknecht et al., 1999; Miki et al., 1999), we investigated whether Src kinase activity effected the adapter function of Scar by examining its ability to bind to Grb2. Cells expressing kinase-defective mutants of Src that where unable to remodel focal adhesions in response to PDGF were compared to cells expressing constitutively active Src. Cells expressing Scar-1 or co-expressing Scar-1 with Src251-GFP, SrcMF or SrcY527F were stimulated with PDGF. Using an antibody to Grb2, we discovered that the presence of a Src kinase domain had no effect on the adaptor

function of Scar-1 (Figure 56). Thus we have no evidence to date that the Src kinase domain can influence the function of Scar-1 within the cell.

Figure 54

Src kinase activity does not effect Arp2/3 binding to Scar-1. Cells were placed in serum-free conditions overnight and then stimulated with PDGF (25 ng ml^{-1}) for 30 min. Myc-tagged Scar-1 was immunoprecipitated from extracts of cells co-transfected with SrcY527F, SrcMF, Src251-GFP or Scar-1 alone using conjugated 9E10 anti-serum. The proteins were resolved by SDS-PAGE and the levels of Arp2/3 binding to Scar-1 were detected following blotting with an anti-Arp3 antibody, upper panel. Levels of Scar-1 immunoprecipitated from cell extracts was determined using an anti-Scar-1 antibody, lower panel.

Figure 54

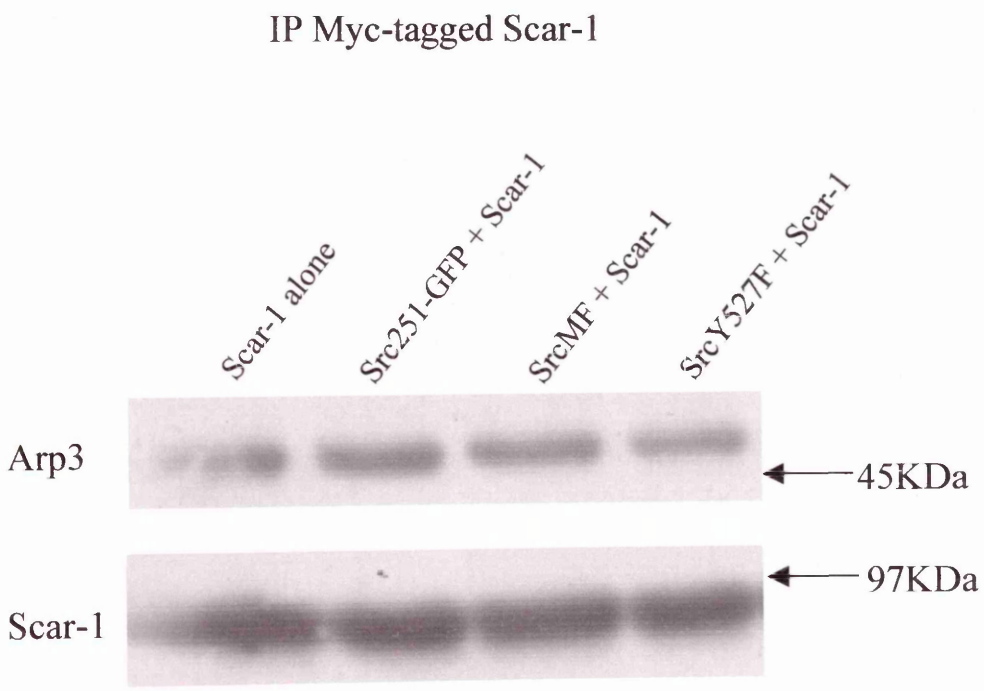

IP Myc-tagged Scar-1
Scar-1 alone
Src251-GFP + Scar-1
SrcMF + Scar-1
SrcY527F + Scar-1
Arp3
45KDa
Scar-1
97KDa

Figure 55

Src kinase activity does not effect actin monomer binding to Scar-1. Cells were placed in serum-free conditions overnight and then stimulated with PDGF (25 ng ml^{-1}) for 30 min. Myc-tagged Scar-1 was immunoprecipitated from extracts of cells co-transfected with SrcY527F, SrcMF, Src251-GFP or Scar-1 alone using conjugated 9E10 anti-serum and incubated with monomeric actin for 30 min at 4°C. The proteins were resolved by SDS-PAGE and the levels of actin binding to Scar-1 detected following blotting with an anti-actin antibody, upper panel. Levels of Scar-1 immunoprecipitated from cell extracts was determined using an anti-Scar-1 antibody, lower panel.

Figure 55

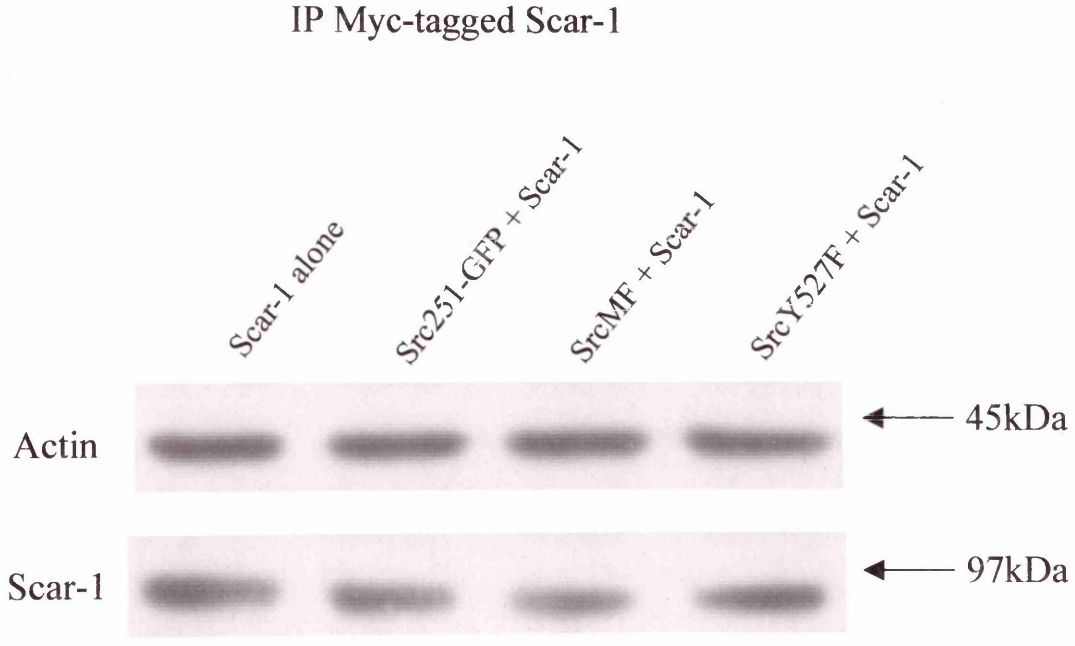
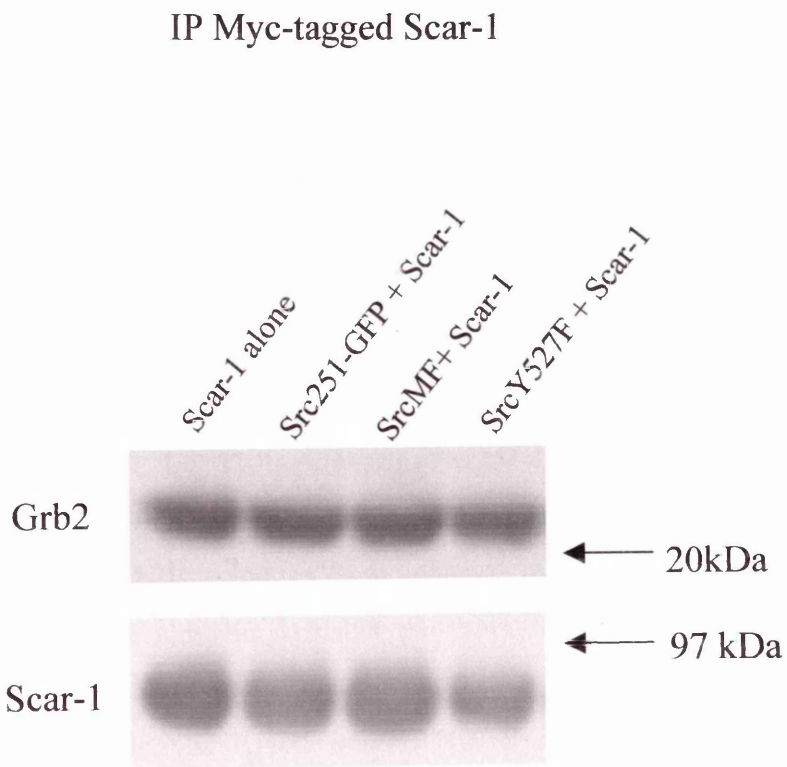

IP Myc-tagged Scar-1
Scar-1 alone
Src251-GFP + Scar-1
SrcMF + Scar-1
SrcY527F + Scar-1
Actin
45kDa
Scar-1
97kDa

Figure 56

Src kinase activity does not effect Grb2 binding to Scar-1. Cells were placed in serum-free conditions overnight and then stimulated with PDGF (25 ng ml^{-1}) for 30 min. Myc-tagged Scar-1 was immunoprecipitated from extracts of cells co-transfected with SrcY527F, SrcMF, Src251-GFP or Scar-1 alone using conjugated 9E10 anti-serum. The proteins were resolved by SDS-PAGE and the levels of Grb2 binding to Scar-1 detected following blotting with an anti-Grb2 antibody, upper panel. Levels of Scar-1 immunoprecipitated from cell extracts was determined using an anti-Scar-1 antibody, lower panel.

Figure 56


IP Myc-tagged Scar-1
Scar-1 alone
Src251-GFP + Scar-1
SrcMF+ Scar-1
SrcY527F + Scar-1
Grb2
20kDa
Scar-1
97 kDa

6.1.3 FAK as a candidate in Src induced focal adhesions remodelling.

Src phosphorylates and associates with a number of proteins involved in the regulation of focal adhesion dynamics. FAK for example, is a known Src substrate at adhesion sites and there is considerable evidence that FAK plays a central role in the regulation of focal adhesion disassembly and cell motility (see Section 1.6.2). We therefore, initially set out to determine whether phosphorylation of FAK was altered during PDGF-induced focal adhesion remodelling and lamellipodia formation. Swiss 3T3 cells were stimulated with PDGF, and FAK was immunoprecipitated from cell lysates with an anti-FAK antibody. Using an phosphotyrosine antibody we found that there was an increase in the tyrosine phosphorylation of FAK in PDGF stimulated cells, compared to non-stimulated cells (Figure 57), indicating that the PDGF-induced focal adhesion remodelling seen in Swiss 3T3 cells is accompanied by FAK phosphorylation events. We therefore, tested whether phosphorylation of FAK was regulated by Src kinase activity, by examining FAK phosphorylation in cells expressing Src kinase defective mutants that were unable to remodel focal adhesions in response to PDGF and compared this to cells expressing constitutively active Src. Cells expressing Src251-GFP, SrcMF or SrcY527F, in which the Src mutants localised to focal adhesions in the presence of serum, were subsequently stimulated with PDGF to induce focal adhesion remodelling and lamellipodia formation. Under these conditions the tyrosine phosphorylation of FAK in response to PDGF stimulation, was compromised in cells expressing Src251-GFP and SrcMF, compared to cells expressing SrcY527F and was reduced to similar levels as non-transfected cells (Figure 58). This indicates that the presence of kinase defective Src in focal adhesions is inhibiting the normal phosphorylation of FAK during focal adhesion remodelling induced by PDGF.

FAK has been reported to be phosphorylated on a number of different sites that are potentially involved in mediating various properties of FAK (Calalb et al., 1995; Owen et al., 1999). Src for example, has been shown to phosphorylate FAK on many sites including tyrosine-925 (Schlaepfer & Hunter, 1996). Recent data obtained by our group however demonstrates that in a colorectal cancer cell line expressing Src251-GFP, FAK is phosphorylated on all tyrosine phosphorylation sites except for tyrosine-925 (E Avizienyte, unpublished). It is therefore possible that this site is a potential phosphorylation site linking the activities of Src and FAK to the impaired focal adhesions turnover and remodelling observed in this study. We therefore tested whether FAK phosphorylated on tyrosine-925 was located at peripheral focal adhesions or focal complexes described

earlier, following stimulation of RhoA or Rac1. In cells stimulated with LPA, FAK phosphorylation on tyrosine-925 (visualised using a phospho-Y925 FAK antibody) could be seen to localise to focal adhesions (Figure 59a, solid arrows), while FAK phosphorylated on tyrosine-925 was evenly distributed at the base of lamellipodia in cells stimulated with PDGF (Figure 59b, solid arrows). Total (non-phosphorylated and phosphorylated) FAK localisation was also examined under these conditions and was found in focal adhesions or focal complexes along the base of lamellipodia in response to LPA or PDGF respectively (Figure 59c,d). This data, indicates that phosphorylation of tyrosine-925 of FAK occurs at these peripheral adhesion sites and we therefore used tyrosine-925 phosphorylation as a marker of Src-induced, phosphorylation events during the remodelling process. We wished to establish whether, in the context of this remodelling, the phosphorylation of FAK on tyrosine-925 was differentially regulated in cells expressing constitutively active or kinase defective Src. Cells expressing Src251-GFP or SrcY527F, in which the Src mutants localised to focal adhesions in the presence of serum, were subsequently stimulated with PDGF to induce focal adhesion remodelling and lamellipodia formation. Under these conditions FAK phosphorylation on tyrosine-925 could be seen to localise to discrete patches around the edge of the lamellipodia in Src251-GFP expressing cells (Figure 60a), while in SrcY527F expressing cells FAK phosphorylation on tyrosine-925 was found to be continuous along the lamellipodia (Figure 60b). Thus a different spatial regulation in FAK phosphorylation on tyrosine-925 can be seen between cells expressing kinase active or defective Src however differences in FAK phosphorylation levels were difficult to establish. Subsequent experiments employing a retroviral transfection system allowed us to examine differences between kinase defective and active Src expressing cells (see Disccusion Section 6.2.2).

Figure 57

PDGF stimulation increases FAK phosphorylation. Cells were placed in serum-free conditions overnight and then stimulated with PDGF (25 ng ml^{-1}) for 30 min. Control cells were not stimulated with PDGF. FAK was immunoprecipitated from whole cell extracts using an anti-FAK antibody. The proteins resolved by SDS-PAGE and the levels of tyrosine phosphorylation were detected following blotting with an anti-phosphotyrosine antibody, upper panel. Levels of FAK immunoprecipitated from cell extracts was determined using an anti-FAK antibody, lower panel.

Figure 57

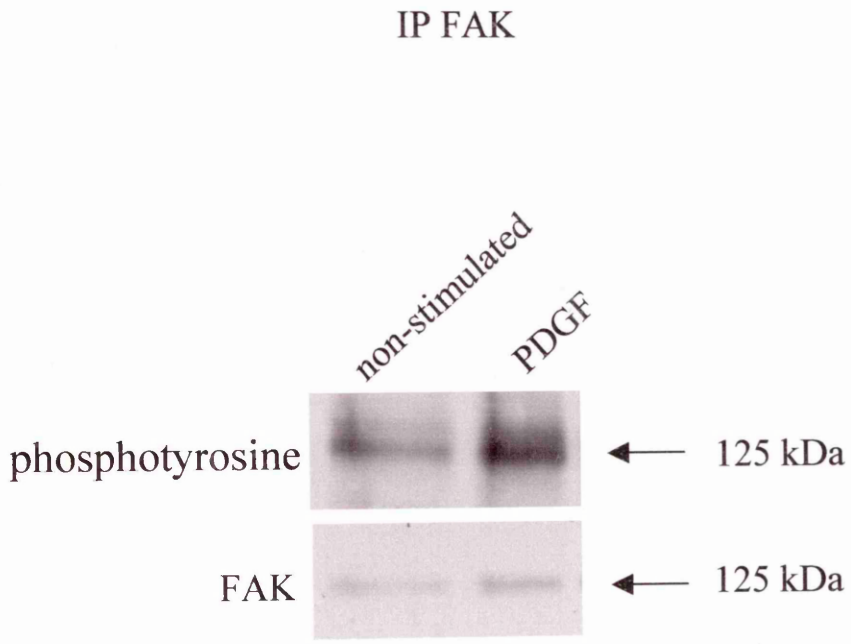

IP FAK
non-stimulated
PDGF
phosphotyrosine
125 kDa
FAK
125 kDa

Figure 58

Phosphorylation of FAK is inhibited in cells expressing kinase defective Src. Cells were placed in serum-free conditions overnight and then stimulated with PDGF (25 ng ml^{-1}) for 30 min (lane 2-4). FAK was immunoprecipitated from extracts of cells transfected with SrcY527F, SrcMF, or Src251-GFP or non-transfected cells using an anti-FAK antibody. The proteins were resolved by SDS-PAGE and the levels of tyrosine phosphorylation detected following blotting with an anti-phosphotyrosine antibody, upper panel. Levels of FAK immunoprecipitated from cell extracts was determined using an anti-FAK antibody, lower panel.

Figure 58

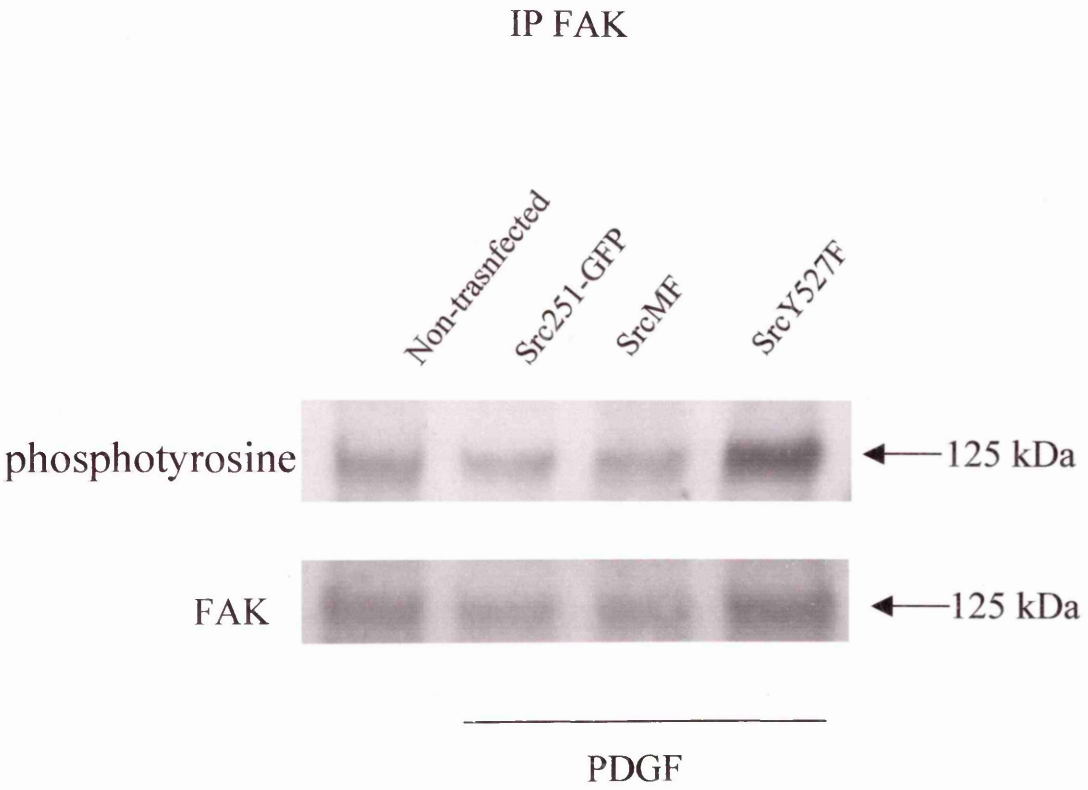

IP FAK
Non-trasnfected
Src251-GFP
SrcMF
SrcY527F
phosphotyrosine
125 kDa
FAK
125 kDa
PDGF

Figure 59

FAK phosphorylated on tyrosine-925 is found in focal adhesions and lamellipodia. Cells were grown in serum-free conditions overnight prior to stimulation with LPA (200 ng ml^{-1}) for 30 min (a,c) or PDGF (25 ng ml^{-1}) for 30 min (b,d). FAK phosphorylated on tyrosine-925 was visualised using a phospho-FAK-Y925 specific antibody (a,b), while FAK was visualised using an anti-FAK antibody (c,d). Arrows indicate FAK localisation. Scale bars 25 μm.

Figure 59

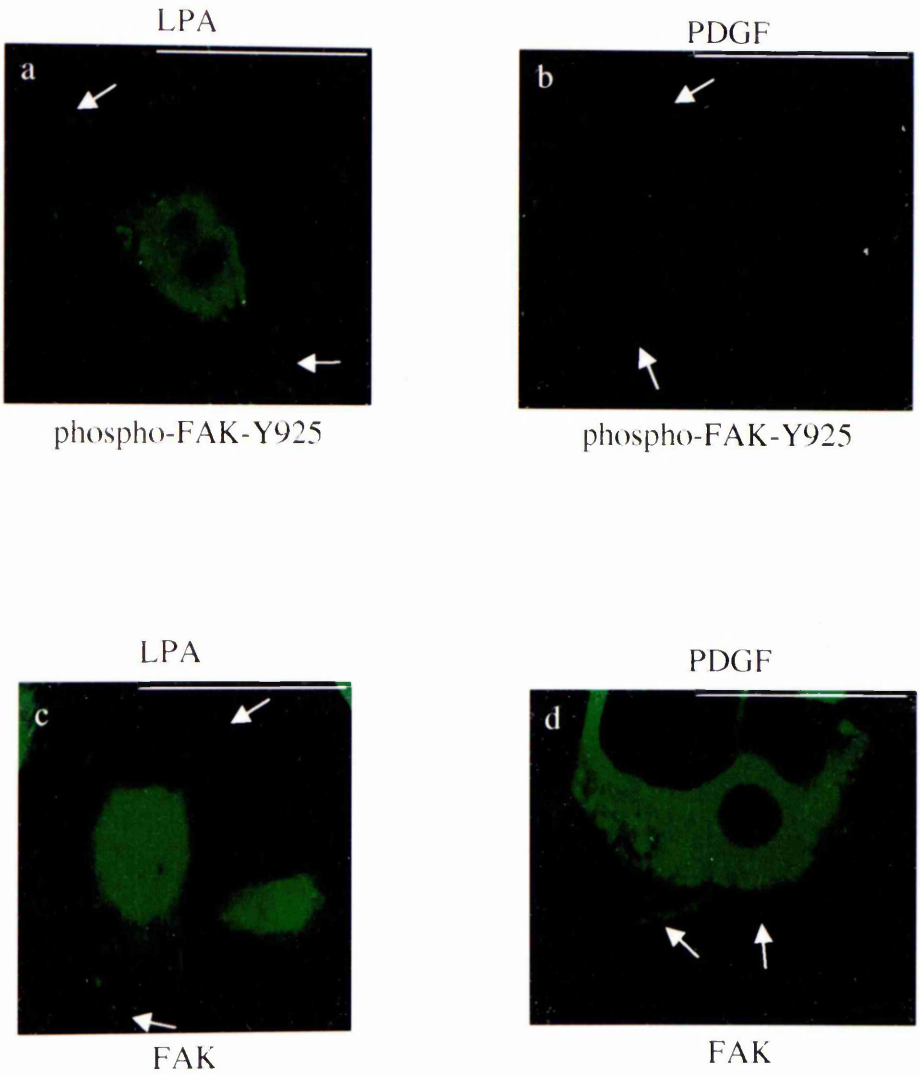
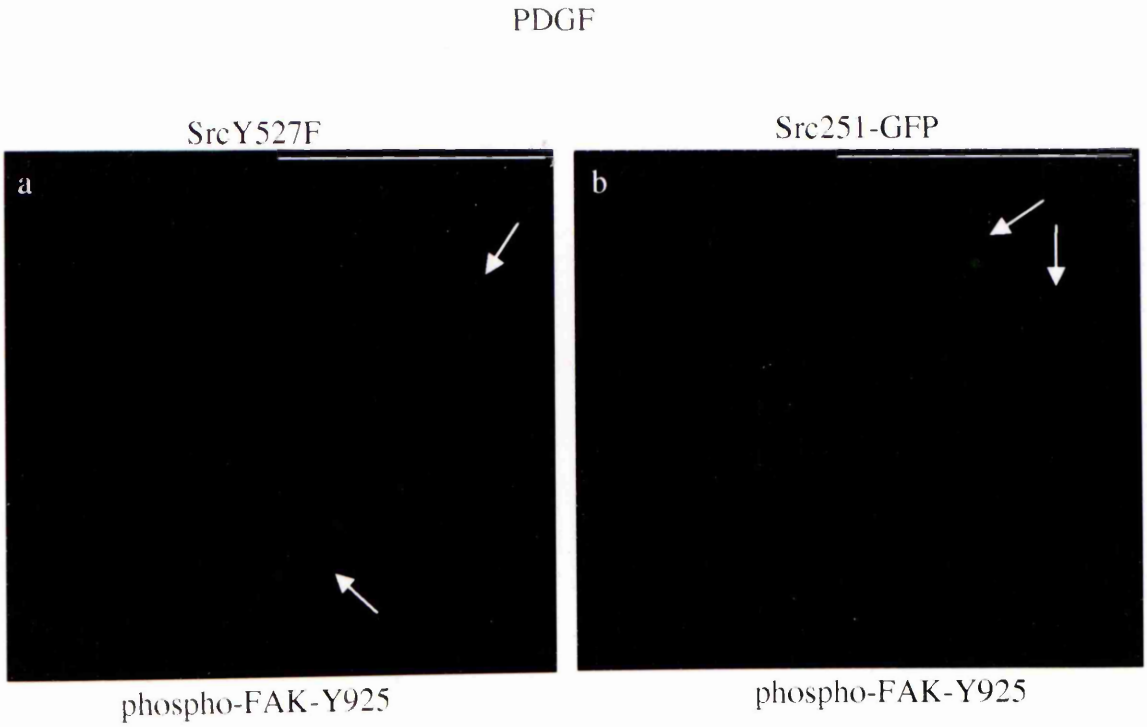

LPA
a
phospho-FAK-Y925
PDGF
b
phospho-FAK-Y925
LPA
c
FAK
PDGF
d
FAK

Figure 60

FAK phosphorylation on tyrosine-925 localises to discrete patches around the edge of the lamellipodia in Src251-GFP expressing cells and is found to be continuous along the lamellipodia in SrcY527F expressing cells. Cells transfected with SrcY527F (a) or Src251-GFP (b) were grown in serum-free conditions overnight prior to stimulation with PDGF (25 ng ml^{-1}) for 30 min. FAK phosphorylated on tyrosine-925 was visualised using a phospho-FAK-Y925 specific antibody (a,b). Scale bars 25 μm.

Figure 60


PDGF
SrcY527F
Src251-GFP
a
b
phospho-FAK-Y925
phospho-FAK-Y925

6.2 Discussion

In this chapter we addressed whether the impaired remodelling observed in cells expressing kinase defective Src could be linked to two potentially important Src binding partners/substrates, Scar and FAK.

6.2.1 Scar as a candidate in Src induced focal adhesions remodelling.

We initially addressed whether the impaired remodelling in cells expressing kinase-defective Src could be related to Src-induced changes in the localisation of Scar. Scar is involved in Rac1 mediated actin polymerisation, which is a prerequisite for lamellipodia formation and could potentially influence the efficiency of lamellipodia formation (Machesky & Insall, 1998; Machesky et al., 1997; Miki et al., 1998b). Previous reports for example, have demonstrated that Scar plays a critical role in Rac1 induced lamellipodia formation (Machesky & Insall, 1998; Miki et al., 1998b). Scar was found at the same peripheral sites as Src in response to RhoA and Rac1 stimuli, indicating that Scar's spatial regulation within the cell is consistent with a potential role in remodelling events during lamellipodia formation. After inducing focal adhesion remodelling to form lamellipodia, we found that Scar was still localised with Src even in the kinase-defective cells that were unable to efficiently remodel their focal adhesions to induce uniform lamellipodia. This suggested that Scar was at the correct sites within the cell to initiate the actin remodelling, required for lamellipodia formation, however efficient remodelling of the actin cytoskeleton did not occur. One explanation for this is that Scar activity may be compromised in cells expressing the kinase defective Src mutants. We therefore examined whether the function of Scar within the cell was altered in cells expressing constitutively active or kinase defective mutants of Src. The ability of Scar to bind to the Arp2/3 complex and thereby control actin polymerisation during lamellipodia formation however was not altered in cells expressing kinase defective Src mutants. Consistent with this, Src kinase activity had no effect on the ability of Scar to bind to actin monomers in vitro. We can therefore conclude from this that the inhibition of focal adhesion remodelling observed in kinase defective Src expressing cells may not be mediated by alterations in Scar-1-induced actin binding events.

Other factors involving Scar-1 however could also influence the remodelling events during lamellipodia formation. Grb2 for example, has recently been shown to bind to Scar (Miki et al., 1999) and has been shown to be required for lamellipodia formation, as inhibitory

antibodies to Grb2 block PDGF-induced lamellipodia formation (Matuoka et al., 1993). Moreover, Grb2 has also been shown to bind and sequester proteins to the plasma membrane (Li et al., 1993; Miki et al., 1999; Rozakis-Adcock et al., 1993; She et al., 1997). The binding therefore of Grb2 to Scar could potentially be involved in the peripheral targeting of Scar and the Arp2/3 complex to the cell membrane during lamellipodia formation. Alterations in the binding of Grb2 to Scar in cells expressing active or kinase defective Src could therefore effect the efficient formation of lamellipodia (Miki et al., 1999). In this study however we demonstrate that expression of kinase active or deficient Src mutants in cells has no effect on Grb2 binding to Scar thereby ruling out this possibility in our model. Tyrosine phosphorylation of the related protein WASP has been reported in various cell systems to alter its function and thereby the actin dynamics within the cell during a number of processes and this phosphorylation-induced alteration in WASP function could therefore act as a precedent for Scar regulation during lamellipodia formation (Oda et al., 1998; She et al., 1997; Wu et al., 1998). Under the conditions of our study however we have not detected any alteration in the tyrosine phosphorylation status of Scar within the cells expressing kinase active or defective Src mutants. Scar was thought to be a potential candidate involved in linking Src to the actin cytoskeleton during lamellipodia formation. To date however we have no evidence that Scar function is altered in cells expressing constitutively active or kinase defective Src mutants, suggesting that alterations in Scar localisation or activity are not responsible for the impaired remodelling observed in cells expressing kinase defective Src. We cannot however completely rule out the possibility that Scar may be involved in this process by mechanisms that we have not addressed.

6.2.2 FAK as a candidate in Src induced focal adhesions remodelling.

Src binds to and phosphorylates a number of other cytoskeletal associated proteins and here we have addressed the involvement of FAK in focal adhesion remodelling. There is convincing evidence that the linked activity of Src and FAK, controls adhesion turnover and cell migration (see Section 1.6.2). Direct evidence for a link between Src and FAK in adhesion turnover comes from studies where over-expression of exogenous FAK in CHO cells stimulates cell migration, which is dependent on its ability to associate with Src family members (Cary et al., 1996). Furthermore, expression of FAK mutants unable to bind Src, hinders focal adhesion turnover and cell migration (Cary et al., 1996). Finally, evidence that dominant-inhibitory Src proteins and the absence of FAK both give rise to enlarged cellular adhesions and impaired migration (see Section 1.6.1), strongly suggests that the critical role of their activity is to induce adhesion turnover and remodelling. In this

regard, mutants of Src that suppress cell motility, including kinase defective Src (see Chapter 3) have previously been shown to impair FAK proteolysis and adhesion turnover (Fincham & Frame, 1998). In this study we have shown that tyrosine phosphorylation of FAK is suppressed in cells expressing kinase defective Src. Recently, regulatory events that lead to Src-mediated adhesion disassembly via FAK have come from studies on v-Src-induced focal adhesion disruption, in which v-Src induced tyrosine phosphorylation of FAK precedes its calpain-mediated proteolytic cleavage. This results in FAK cleavage, adhesion disassembly and cell detachment (Carragher et al., 2001; Carragher et al., 1999; Fincham et al., 1995). In keeping with this, calpain is present at focal adhesions and is required for migratory responses downstream of EGFR (Beckerle et al., 1987; Glading et al., 2000). Moreover, recent work by our group has also demonstrated that inhibition of calpain blocks cell migration (Carragher et al., 2001). It is therefore possible that FAK phosphorylation and the calpain-mediated proteolysis of FAK is a tightly controlled process that triggers adhesion turnover and remodelling when cell/matrix adhesions are being dynamically regulated during Src dependent cell migration. In this regard, the reduced phosphorylation of FAK in kinase defective cells may explain why cells expressing kinase defective Src have reduced focal adhesion turnover and remodelling and as a result cell migration and polarisation is impaired. In line with this, previous work by our group has shown that un-phosphorylated FAK, in the presence of kinase defective v-Src, forms a more stable complex with Src and remains un-degraded in stabilised focal adhesions (Fincham & Frame, 1998).

FAK has been documented to be phosphorylated on a number of different sites (Calalb et al., 1995; Owen et al., 1999). We chose to study the phosphorylation of FAK on tyrosine-925 as it has previously been shown to be the only site on FAK that is not phosphorylated in Src251-GFP expressing colorectal cancer cells (E Avizienyte, unpublished) and is therefore a potential link between the impaired focal adhesion turnover and remodelling observed in cells expressing kinase defective Src and the activities of Src and FAK in this process. In the context of focal adhesion remodelling to lamellipodia, we examined whether the phosphorylation of FAK on tyrosine-925 is differentially regulated in cells expressing SrcY527F or SrcMF. We found a different spatial regulation in FAK phosphorylation on tyrosine-925 between cells expressing kinase active or defective Src however differences in FAK phosphorylation levels by immunofluorescence were difficult to visualise. Subsequently, experiments employing a retroviral transfection system to increase the percentage of cell expressing the respective Src constructs allowed us to see more subtle differences between cells expressing kinase defective and active Src mutants. Cells expressing the empty vector or SrcMF in which SrcMF was localised to focal

adhesions, were stimulated with PDGF. Using an antibody against phosphorylated tyrosine-925 in FAK, we found that PDGF stimulated the phosphorylation of tyrosine-925, while in cells expressing SrcMF there was very little phosphorylation on this site (Figure 7, (Timpson et al., 2001). Therefore, using FAK tyrosine 925 phosphorylation as a marker of Src-dependent phosphorylation events at peripheral adhesions, we have shown that kinase defective Src expression suppresses both peripheral phosphorylation events and adhesion re-modelling. The potential link between FAK-925 phosphorylation and focal adhesion remodelling and turnover will be discussed in more detail in chapter 7 (see section 7.1.4, future studies). Reduced FAK phosphorylation on tyrosine-925, like the general reduction in phosphorylation of FAK in kinase defective cells, in response to PDGF stimulation, may effect its susceptibility to calpain cleavage and explain the impaired remodelling and directed cell migration observed in these cells. The potential involvement of FAK-specific phospho-acceptor sites and their involvement in FAK degradation, focal adhesion disruption and remodelling could be examined in the future. For this, we currently have constructs encoding epitope tagged mutant FAK proteins with each of FAK's tyrosine phosphorylation sites mutated to phenylalanine, both individually and in combinations, thereby inhibiting possible tyrosine phosphorylation on these sites. Over-expression of these proteins would allow us to determine for example, whether Src-induced phosphorylation of FAK on tyrosine-925 observed here, regulates focal adhesion turnover and remodelling, as well as allowing us to assess the role other phosphorylation sites play in this process. It is possible that phosphorylation on one or more of these sites could be involved in focal adhesion turnover during cell migration. On the other hand phosphorylation has been shown to stabilise FAK, for example, integrin-induced phosphorylation of FAK on tyrosine-397 is associated with focal adhesions assembly, which interestingly has recently been shown to protect FAK and focal adhesions from degradation induced by v-Src (N Carragher, unpublished). The potential for site specific phosphorylation events in controlling focal adhesions disassembly or assembly, makes FAK an exciting candidate to examine further as a key player in adhesion dynamics induced by Src during cell migration.

Phosphorylation of FAK on tyrosine-925 also recruits the SH2 domain of the adaptor protein Grb2 (Schlaepfer & Hunter, 1996) which in turn can result in activation of the Ras-extracellular signal regulated kinase (ERK) pathway. Several lines of evidence suggest a role for this pathway in the control of cell motility and polarity. Firstly, as described earlier inhibitory antibodies to Grb2 block PDGF-induced lamellipodia (Matuoka et al., 1993). Secondly, activated ERK has been found in newly forming focal adhesions where it is thought to play a role in their assembly (Fincham et al., 2000). Thirdly, inhibition of

ERK activation blocks cell migration in different cell types (Klemke et al., 1997; Nguyen et al., 1999) which has been attributed to the ability of ERK to phosphorylate and activate MLCK (Nguyen et al., 1999). Moreover, we have recently shown that inhibition of MEK blocks Src-induced focal adhesion turnover (V. Fincham unpublished). MLCK is important for the formation of peripheral adhesions and the generation of tension during cell motility (Schoenwaelder & Burridge, 1999). Therefore, the reduced phosphorylation on tyrosine-925 could potentially explain the impaired lamellipodia and cell migration observed in cells expressing kinase defective Src by a mechanism which involves an impaired FAK-Grb2-ERK signalling pathway.

6.3 Summary

In this chapter we have initiated studies to address the involvement of two potentially interesting binding partners/substrates of Src in focal adhesion to focal complex remodelling during lamellipodia formation. Src however, also phosphorylates and binds to a number of other cytoskeletal and focal adhesion associated proteins and these events along with Src's potential link to yet unidentified proteins may also be involved in adhesion turnover, remodelling and directed cell motility (Arthur & Burridge, 2001; Arthur et al., 2000; Thomas et al., 1995). The impaired FAK phosphorylation reported in this study are therefore likely to represent only part of a number of Src mediated phosphorylation events regulating focal adhesion remodelling and directed cell motility.

In these experiments we have focused on focal adhesion remodelling events during lamellipodia formation. It should be noted that time permitting we would also like to address the involvement of potential candidates in the focal adhesion remodelling during bradykinin (Cdc42)-induced filopodia formation. It is possible that different candidates may play a role in focal adhesion remodelling depending on the structure being formed and therefore some candidates may be specifically involved in lamellipodia formation while others could be involved in filopodia formation or both.

Chapter 7

7 Conclusion

7.1 Summary and future work.

7.1.1 Summary

The ability of a cell to move depends on a cycle of adhesion, loss of attachment and re-adhesion, which is governed by, and co-ordinated with, changes in the actin cytoskeleton arrangement (Schoenwaelder & Burridge, 1999; Small et al., 1999). The actin structures, lamellipodia and filopodia assemble and disassemble at the leading edge of cells under the control of Rac1 and Cdc42, providing a means of polarised cell movement. Small focal complexes form at lamellipodia and filopodia, which are rapidly turned over as the cell moves forward, while the RhoA-dependent assembly of stress fibres provide contractility and propulsion required for forward cell movement. Since it is the balance between contractility and adhesion that governs the rate of cell motility, relative activities of RhoA, Rac1 and Cdc42 are critical in determining whether and how a cell moves. Moreover, alterations in adhesive interactions between the cell and the substratum by protein-protein interactions, via proteins such as Src, during this process also influence cell migration. In the current study we have examined both the role of the RhoGTPases in directing the intracellular localisation of the tyrosine kinase c-Src, that is known to be required for adhesion turnover and cell motility (Fincham & Frame, 1998), and secondly examined the role of Src kinase activity in controlling cell movement.

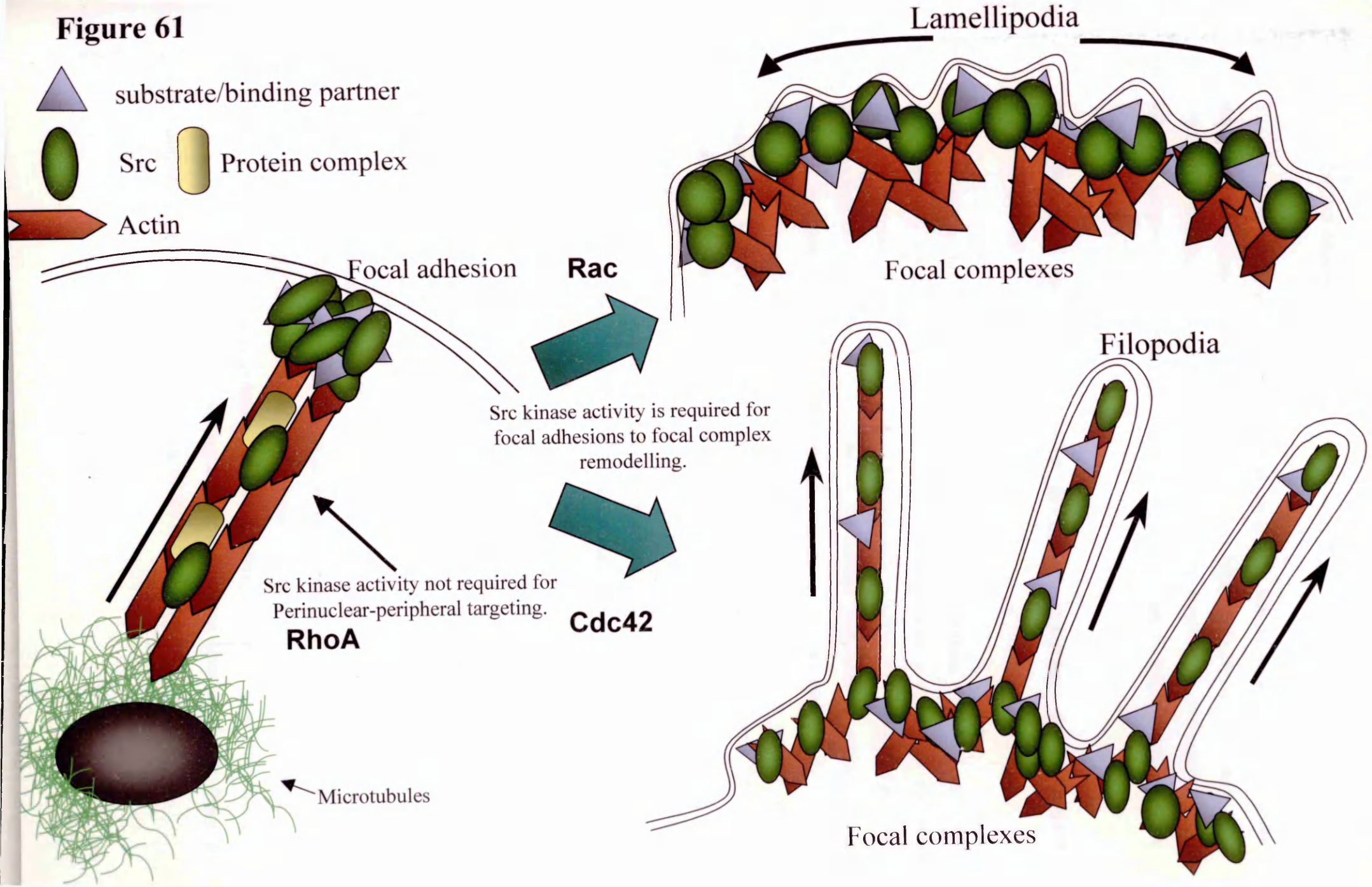
The current data presented here and in previous studies with v-Src (Fincham et al., 2000; Fincham & Frame, 1998; Fincham et al., 1996) has helped us define a number of stages in Src's peripheral targeting. Firstly, Src is transferred from the microtubule-dependent perinuclear region of the cell into multi-protein complexes, yet to be fully identified, which associate with the actin cytoskeleton (Fincham & Frame, 1998; Fincham et al., 1996). Subsequently Src translocates to the cell periphery to focal adhesions in a RhoA and actin-dependent manner (see Figure 61) (See Chapter 4, Section 4.1.1 (Fincham et al., 1996). Moreover, we have illustrated here, that the co-ordination of RhoGTPases is required to specifically relocate Src from RhoA-induced focal adhesions to lamellipodia or filopodia following Rac1 or Cdc42 activation, respectively (see Chapter 4, section 4.1.1). Furthermore, the kinase activity of Src has previously been shown to be dispensable for Src's peripheral targeting to focal adhesions (Fincham & Frame, 1998; Kaplan et al., 1994).

We show in this study that Src kinase activity is required for focal adhesion turnover and remodelling to induce the disassembly of large focal adhesions to smaller focal complexes needed for efficient lamellipodia or filopodia formation and directed cell migration (Chapter 5). Finally, we have identified FAK as a potential binding partner/substrate involved in Src-induced focal adhesion remodelling during lamellipodia formation (Chapter 6).

Figure 61

Schematic representation of Src's intracellular targeting from the perinuclear region of the cell to lamellipodia or filopodia. Illustrating some questions yet to be addressed including which potential chaperon proteins are involved in the peripheral targeting of Src and which potential candidates are involved in Src's focal adhesion remodelling during lamellipodia or filopodia formation.

Figure 61


substrate/binding partner
Src
Protein complex
Actin
Lamellipodia
Focal adhesion
Rac
Focal complexes
Src kinase activity is required for focal adhesions to focal complex remodelling.
Filopodia
Src kinase activity not required for Perinuclear-peripheral targeting.
RhoA
Cdc42
Microtubules
Focal complexes

7.1.2 Future studies.

The two main areas for future investigations are (a) the role of various proteins in the initial peripheral targeting of Src from a perinuclear location to the cell periphery (see Figure 61) and (b) examination of proteins potentially involved in Src-induced focal adhesion turnover and remodelling during lamellipodia and filopodia formation (See Figure 61).

7.1.3 Potential candidates involved in Src's perinuclear to peripheral targeting

As discussed in the introduction (see Section 1.1.1), Src contains both SH2 and SH3 protein–protein interacting domains, which can associate with a variety of different chaperon proteins. It has been postulated that sequential binding of a sub-set of proteins may mediate the translocation of Src between sub-cellular compartments within the cell and it is therefore likely that different proteins will be specifically involved in different stages of Src's intracellular targeting. To date, we have ruled out a number of key components in Src's peripheral targeting including AFAP, HSP90 and FAK (see Introduction Section 1.2.2) (Fincham et al., 2000). We have however, identified PI3-kinase as an SH3 binding protein required for Src's peripheral targeting to focal adhesions, most likely mediated by its ability to maintain actin stress fibre integrity (Fincham et al., 2000). In addition to this, there are many proteins that bind to Src, whose likely cellular functions are consistent with a role in Src's peripheral targeting. The Scar family of proteins that control actin polymerisation for example, were assessed in Chapter 6 (see Section 6.1.1) as potential candidates involved in focal adhesions remodelling and actin reorganisation during lamellipodia formation. Interestingly, Machesky and Hall have previously demonstrated that long-term expression of inhibitory Scar-1 mutants result in a loss of actin stress fibres, indicating that there is a need for actin nucleation and new polymerisation, induced by Scar-1, for long-term maintenance of stress fibres (Machesky & Hall, 1997). This requirement for actin polymerisation to maintain stress fibres has been demonstrated by the incorporation of new polymerised actin into stress fibres (Machesky & Hall, 1997). It is therefore possible that Scar-1s ability to control actin polymerisation may be involved in Src's peripheral targeting by maintaining actin stress fibre integrity during RhoA and actin dependent targeting of Src to focal adhesions.

To address the role of actin polymerisation in Src targeting to the cell periphery, we could inhibit actin polymerisation and examine Src translocation and new actin assembly in real time using time lapse microscopy. Cells could be microinjected with rhodamine-labelled actin (Chan et al., 1998; Small et al., 2002), to allow us to monitor actin dynamics in real time. This will allow us to visualise both, Src251-GFP translocation to focal adhesions and the incorporation and remodelling of new actin in real time. From this we could determine whether Src translocation occurs along with new actin polymerisation or whether Src is targeting to the cell periphery along pre-formed actin stress fibres.

With regards to RhoA-induced peripheral targeting of Src to focal adhesions we have also demonstrated that inhibition of the Rho-effector ROCK (see Section 1.4.1), blocks this peripheral targeting process (see Chapter 4). ROCK is thought to co-operate with various other RhoA effector to induce stress fibres (see Section 1.4.1). Recently the diaphanous related formin proteins, mDia 1/2 have attracted attention as important effectors co-operating with ROCK to induce actin stress fibres (see Section 1.4.1). Interestingly, a direct association between c-Src and mDia1/2, most likely mediated by the SH3 domain of Src and the proline-rich sequences of the Dia protein FH domains has been reported (Tominaga et al., 2000). Moreover, the expression of a dominant inhibitory Src protein, or deletion of the FH1 domain of mDia2 has been reported to abolish the co-operative effects of the Dia proteins with ROCK in mediating RhoA-induced stress fibre formation (Tominaga et al., 2000). These properties of mDia1/2 make them exiting candidates that potentially play a role in Src's peripheral targeting. It has therefore been proposed that RhoA may localise Src to focal adhesions via mDia1/2 (Tominaga et al., 2000). A number of DRF proteins and mutants are currently available to address the involvement of diaphanous proteins in Src's peripheral targeting (provided by Art Albert's group at the Van Andel Institute, Michigan). Furthermore, mouse cells that are functionally -/- for mDia1 or mDia2 are being generated by Art Alberts group, which will help establish the involvement of Dia in Src's peripheral transport to focal adhesions.

There are almost certainly more Src binding partners and methods of Src transport to the cell periphery to be identified. For example, recent work by our group has suggested that, like its binding partner paxillin, there may be a vesicle-mediated delivery component to the intracellular targeting of Src. Mutants of v-Src which do not target to the cell periphery can be observed located in the perinulcear region of the cell in structures that resemble large vesicles (V Fincham, unpublished observation), suggesting that these non-targeting mutants are unable to be released from this mode of transport to the actin cytoskeletal transport mechanisms described in this study. The ARF-GTP-binding proteins could be

potential candidates involved in this process, as they are well known regulators of intracellular membrane traffic (Serafini et al., 1991) and have previously been identified as Src binding partners (Brown et al., 1998). Moreover, ARF proteins have been shown to be involved in the vesicular transport of various proteins to the cell periphery, such as paxillin (Norman et al., 1998), as well as being implicated in regulation of the actin cytoskeleton (Randazzo et al., 2000). Furthermore, ARF proteins have been documented to localise in the perinuclear region of the cell and to focal adhesions, or to peripheral lamellipodia during cell migration (Brown et al., 1998; Randazzo et al., 2000). These properties of ARF proteins make them exciting candidates that may potentially play a role in Src's intracellular targeting, both as a link between the potential vesicle-mediated targeting of Src and the known actin-dependent targeting of Src described in this study. As discussed earlier, it is likely that the sequential binding of a sub-set of proteins may mediate the transport of Src between sub-cellular compartments. Our ability to regulate the intracellular localisation of Src by modulating the actin cytoskeleton (described in this study) or by utilising the versatile properties of the temperature sensitive (ts) v-Src system, which incorporates both targeting and non-targeting mutants of Src (Fincham et al., 2000; Westphal et al., 1997), provides us with excellent tools to address the involvement of various proteins at different stages of Src's intracellular targeting and may reveal more about the interactions between Src, the actin cytoskeleton and other potentially modes of transport, including vesicle based targeting within the cell. A greater understanding of the nature and mechanisms involved in the defined stages of Src's intracellular transport may lead to the identificatio of new key players in this process in the future.

7.1.4 Potential candidates involved in Src's focal adhesion remodelling during lamellipodia or filopodia formation.

In this study we have demonstrated a further stage in Src's intracellular targeting, by illustrating the remodelling of Src containing focal adhesions to lamellipodia or filopodia in response to the respective RhoGTPase (see Figure 61) (see chapter 4 section 4.1.4 and 4.1.5). Similar to the strategy discussed above, we have employed a candidate approach here to address the involvement of potential proteins involved in this specific remodelling and our preliminary results suggest that FAK might be involved in the regulation and partial disassembly of large focal adhesions to smaller focal complexes necessary for lamellipodia formation (see chapter 5 section 5.1.4 and 5.1.5 and chapter 6 section 6.1.3).

FAK is unlikely to be the sole substrate of Src involved in focal adhesion turnover. Cortactin, paxillin and ERM proteins are amongst some of the other potential candidates involved in Src induced focal adhesion remodelling. The effects on FAK phosphorylation reported in this study are therefore likely to represent only part of a number of Src mediated phosphorylation and binding events regulating adhesion remodelling in response to biological stimuli that induce directed cell migration. The list of potential candidates involved in Src's perinuclear to peripheral targeting or in Src-induced phosphorylation events during adhesion remodelling, is not exhaustive, and on a long term basis we plan to screen for other potential candidates involved in these processes. Standard procedures will be used, including co-immunoprecipitation, metabolic labelling and 2D gel electrophoresis to identify binding partners/substrates that are specifically linked to the impaired focal adhesion remodelling observed in cells expressing kinase defective Src. Cells expressing activated or kinase deficient Src, treated with the respective stimuli (PDGF or bradykinin), will be used to examine the differential Src protein binding partners/substrates involved in Src's intracellular targeting at different stages within the targeting process. This will allow us to specifically link known and potentially unknown binding proteins to the impaired focal adhesions remodelling observed in this study during lamellipodia of filopodia formation respectively. Unidentified proteins that are differentially expressed will then be subjected to further examination and identification by mass spectroscopy.

A great deal of structural and functional studies with members of the Src family kinases has led to a detailed understanding of Src activation and inactivation at the molecular level (see Introduction Section 1.1.3). Conformational activation, usually induced by de-phosphorylation of Src tyrosine-527, or displacement of Src's intramolecular interactions, simultaneously stimulates catalytic activity and frees the protein binding domains for protein-protein interactions, which could potentially control Src's targeting within the cell. The co-ordinated regulation of Src targeting by RhoGTPases and the simultaneous kinase activation ensures that inadvertant relocalisation of active Src is avoided. This allows Src to respond for example, to migratory stimuli such as PDGF, and specifically incorporate into adhesions as they form. Src activity at these sites however, ultimately induces the disassembly and remodelling of adhesions (see Introduction Section 1.6.1) which is necessary for example, to produce protrusive structures such as lamellipodia and filopodia. Since the formation of these structures is intimately involved in controlling cell migration and chemotaxis, the major challenge now is to establish how motile cells spatially and temporally integrate the activity of RhoA, Rac1 and Cdc42, which in turn spatially regulates c-Src during cell migration. In future work, monitoring Src by direct fluorescence may allow us to begin to address how the integrated effects of RhoGTPases

relates to the spatial and temporal regulation of Src at different stages during the process of directed cell migration. Recently, we have intitiated studies to address this by visualising Src's intracellular targeting in real time using a C-terminal linker sequence to tag full length Src with GFP (a kind gift from G. Superti Furga). Furthermore, understanding which proteins are key regulators in this process and determining how Src-kinase dependent events at focal adhesions regulate remodelling in response to biological stimuli that induce directed cell migration will be of great interest in the future. It should be noted that RhoA, Rac1 and Cdc42 may also regulate the localisation of other proteins within the cell that, like Src, play an important role in adhesion dynamics during cell migration.

Appendices

1836 Research Paper

Coordination of cell polarization and migration by the Rho family GTPases requires Src tyrosine kinase activity

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Background: The ability of a cell to polarize and move is governed by remodeling of the cellular adhesion/cytoskeletal network that is in turn controlled by the Rho family of small GTPases. However, it is not known what signals lie downstream of Rac1 and Cdc42 during peripheral actin and adhesion remodeling that is required for directional migration.

Results: We show here that individual members of the Rho family, RhoA, Rac1, and Cdc42, direct the specific intracellular targeting of c-Src tyrosine kinase to focal adhesions, lamellipodia, or filopodia, respectively, and that the adaptor function of c-Src (the combined SH3/SH2 domains coupled to green fluorescent protein) is sufficient for targeting. Furthermore, Src's catalytic activity is absolutely required at these peripheral cell-matrix attachment sites for remodeling that converts RhoA-dependent focal adhesions into smaller focal complexes along Rac1-induced lamellipodia (or Cdc42-induced filopodia). Consequently, cells in which kinase-deficient c-Src occupies peripheral adhesion sites exhibit impaired polarization toward migratory stimuli and reduced motility. Furthermore, phosphorylation of FAK, an Src adhesion substrate, is suppressed under these conditions.

Conclusions: Our findings demonstrate that individual Rho GTPases specify Src's exact peripheral localization and that Rac1- and Cdc42-induced adhesion remodeling and directed cell migration require Src activity at peripheral adhesion sites.

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Background

Adhesive interactions between cells and the extracellular environment, together with regulation of the intracellular cytoskeleton, control a number of key biological functions, including cell motility and invasion. Focal adhesions consist of clustered integrins and proteins that link the extracellular matrix (ECM), through the integrins, to the actin cytoskeleton and to proteins involved in adhesion-dependent signal transduction [1]. One such protein is the non-receptor tyrosine kinase c-Src that can also associate with endosomal membranes in the perinuclear region of the cell [2, 3]. Mutation of tyrosine-527 to phenylalanine in c-Src, which results in catalytic activation, as well as release of constraints on the SH3 and SH2 domains, leads to c-Src being constitutively associated with focal adhesions [4]. Similarly, temperature-sensitive variants of the viral oncoprotein v-Src localize to focal adhesions when activated by switch to permissive temperature [5]. The kinase activity of v-Src or c-Src is not required for focal adhesion localization, but mutational analysis has revealed a critical role for the SH3 domain of both proteins in their intracellular targeting [4, 6, 7]. Although the localization of Src to focal adhesions does not require Src's catalytic activity, kinase activity is required for v-Src-induced focal adhesion turnover during transformation and motility [6, 8]. Thus, in order for v-Src to induce its biological effects, it must first be translocated from its site of synthesis to its site of action at peripheral cell-matrix adhesions.

The localization of v-Src to focal adhesions requires the integrity of the actin cytoskeleton and is under the control of RhoA GTPase [5]. In fibroblasts, activation of Rho GTPases, RhoA, Rac1, and Cdc42 by different transmembrane receptors leads to distinct rearrangements of the actin cytoskeleton. Activation of RhoA stimulates actomyosin-based contractility, which leads to the assembly of actin stress fibers and focal adhesions found at the end of stress fibers [9, 10]. Rac1 activation leads to localized actin polymerization at the cell periphery, resulting in the formation of lamellipodia, while activation of Cdc42 results in the formation of fine actin-rich protrusions, known as filopodia [11–13]. Rac1 and Cdc42 stimulate the assembly of focal complexes that are associated with lamellipodia and filopodia, respectively. They contain a number of the same proteins found in Rho-induced focal adhesions, including vinculin, paxillin, and focal adhesion kinase (FAK) [13].

Here, we addressed how RhoA-, Rac1-, or Cdc42-induced changes in the actin cytoskeleton influence the intracellu-

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lar localization of c-Src and Src's biological effects at these peripheral sites. As the first 251 amino acids of c-Src, comprising the site of myristylation, unique region, and SH3 and SH2 domains, are sufficient to localize c-Src to focal adhesions, we used green fluorescent protein (GFP) fused to the first 251 amino acids of c-Src (Src251-GFP) to monitor subcellular localization following stimulation of the Rho GTPases. Upon stimulation of RhoA, Rac1, or Cdc42, actin reorganization is accompanied by recruitment of c-Src to peripheral adhesion sites at stress fiber termini, lamellipodia, or filopodia, respectively. Furthermore, modulation of the actin cytoskeleton showed that RhoA activity and actin stress fibers are needed for Src's translocation from the cell interior to the membrane, with the precise peripheral localization being determined by local actin organization that is in turn controlled by RhoA, Rac1, and Cdc42 activities. In addition, Src's catalytic activity is required for the Rac1- and Cdc42-induced adhesion remodeling that is required for cell polarization and migration. We also show that impaired adhesion remodeling in the presence of Src proteins that lack kinase activity is accompanied by reduced peripheral phosphorylation events, as shown by suppression of FAK phosphorylation at tyrosine 925.

Results

The regulation of the actin cytoskeleton by the Rho family of GTPases is well documented and readily manipulated in Swiss 3T3 cells, making them an ideal model system in which to study the role of RhoA, Rac1, and Cdc42 in the intracellular targeting of c-Src. We therefore determined whether Src251-GFP (Figure 1) was a suitable tool for studying the targeting of c-Src in these cells. We introduced Rho, Rac, and Cdc42 expression plasmids and Src251-GFP by transient transfection or microinjection (further details on the conditions used are given in the Supplementary methods section available with this article online).

Translocation of Src251-GFP to focal adhesions is dependent on the actin cytoskeleton

When Src251-GFP was microinjected into serum-starved cells, it was located in the perinuclear region (Figure 1b, arrow). However, upon addition of serum, which resulted in reformation of stress fibers, Src251-GFP translocated to sites at the end of the actin cables (Figure 1c, arrows), where it colocalized with the focal adhesion protein vinculin (data not shown). Similar results were seen following treatment with lysophosphatidic acid (LPA) (Figure 1d), which initiates the reformation of actin stress fibers in serum-starved Swiss 3T3 cells in a RhoA-dependent manner [9]. Treatment with the actin-disrupting drug cytochalasin D prevented the reformation of the actin stress fibers in response to serum and the translocation of Src251-GFP from the perinuclear region to the peripheral focal adhesions (Figure 1e), further confirming that the translocation of Src251-GFP to focal adhesions was actin dependent. Thus, the regulation of Src251-GFP in Swiss 3T3 fibroblasts is indistinguishable from the actin-dependent translocation of v-Src to focal adhesions that we have described previously [5]. These results imply that Src251-GFP is a useful tool to monitor the control of c-Src's intracellular targeting by direct fluorescence.

Localization of Src251-GFP to specific peripheral sites is under the control of Rho family GTPases

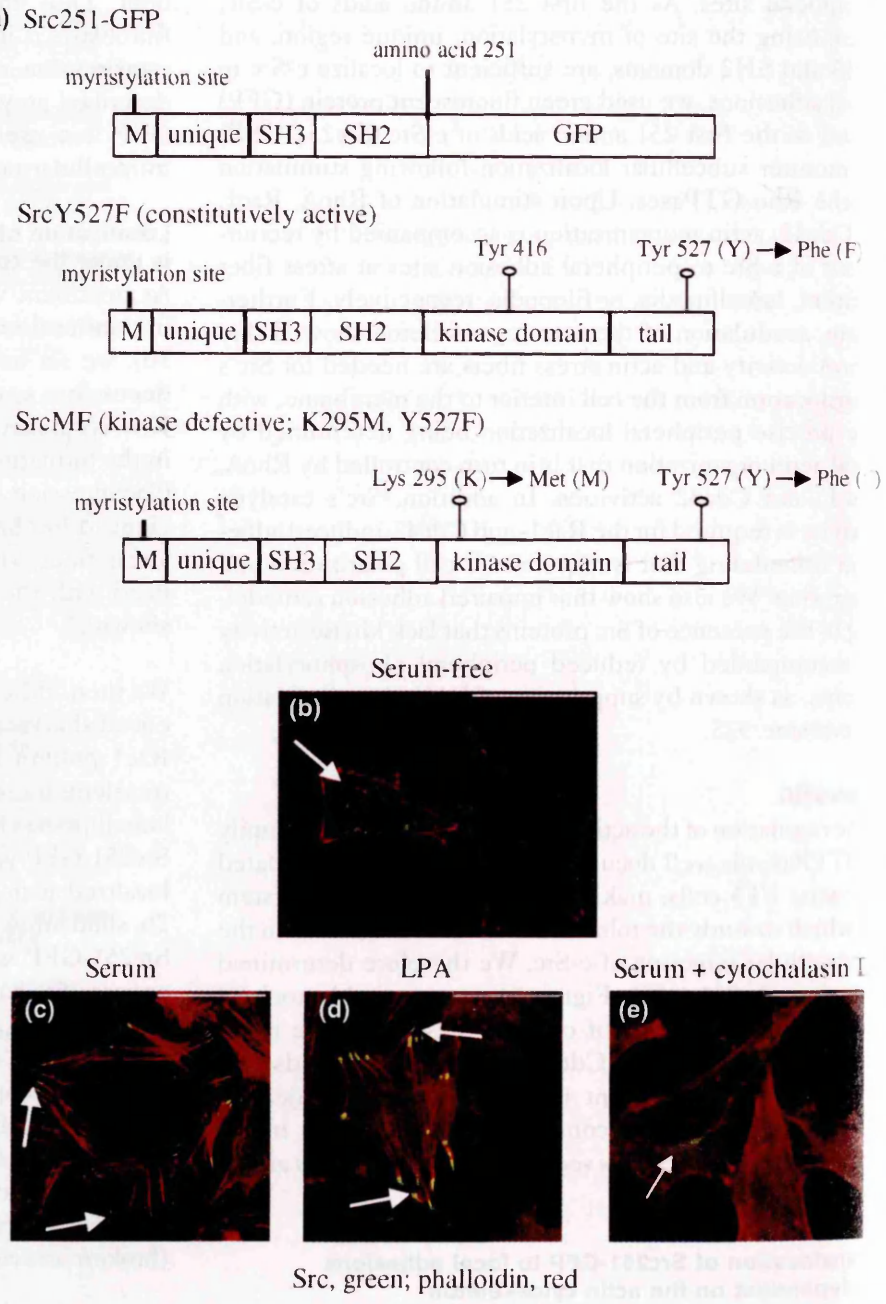
As treatment with LPA, which activates RhoA, resulted in translocation of Src251-GFP to focal adhesions (Figure 1d), we set out to determine whether this was a RhoA-dependent event. Expression of a constitutively active RhoA protein (V14RhoA) in the absence of serum resulted in the formation of stress fibers (Figure 2a, broken arrow). Coexpression of V14RhoA with Src251-GFP was sufficient to localize Src to the cell periphery at the ends of stress fibers (Figure 2a, broken arrow), where it colocalized with the focal adhesion protein vinculin (data not shown).

We then addressed how activation of Rac1 or Cdc42 influenced the localization of c-Src within the cell. Activated Rac1 protein (L61Rac1) was expressed in the cells by transient transfection and resulted in the formation of lamellipodia (Figure 2b, broken arrows). Coexpression of Src251-GFP with L61Rac1 showed that Src251-GFP was localized at an actin ring around the cell periphery (Figure 2b, solid arrow) beyond which an actin ruffle had formed. Src251-GFP was also found at the tip of the ruffle that appeared to have folded back on itself (Figure 2b, broken arrows). Coexpression of a constitutively active Cdc42 protein (V12Cdc42) with Src251-GFP resulted in the formation of filopodia, and some of the expressed Src251-GFP moved from the perinuclear region to membrane regions at the base of filopodia (Figure 2c, broken arrow), and at enhanced resolution (Figure 2c, inset) it can be seen in distinct complexes spaced along the actin filopodia (broken arrows, inset).

The role of the individual Rho GTPases in the subcellular targeting of c-Src was further probed in experiments using specific inhibitors of RhoA, Rac1, and Cdc42. Expression of a dominant-negative RhoA protein (N19RhoA) prevented the reformation of stress fibers in response to serum (compare Figure 3a with 3b). The broken arrow shows a cell expressing N19RhoA, which is consequently devoid of actin stress fibers and in which Src251-GFP remained perinuclear (Figure 3b). This was in contrast to its location at peripheral focal adhesions in cells stimulated with serum (Figure 3a). Y-27632 is a chemical inhibitor of Rho kinase (ROCK) [14], which is a downstream effector of RhoA implicated in stress fiber formation [15, 16]. Treatment of cells with Y-27632 results in the loss of stress fibers, and, when cells were treated with Y-27632

Figure 1

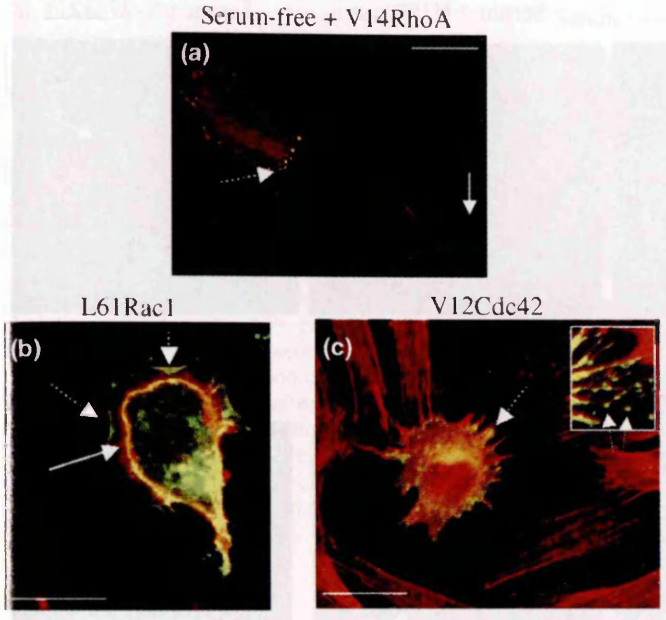
Translocation of Src251-GFP to focal adhesions is dependent on the actin cytoskeleton. **(a)** Schematic representation of the structural domains of Src251-GFP, SrcY527F, and SrcMF constructs used. **(b)** Cells were serum starved for 48 hr prior to microinjection of Src251-GFP. **(c,d)** Serum-starved cells were microinjected with Src251-GFP and, following expression of the protein, were stimulated with serum (c) or LPA (d). **(e)** Serum-starved cells were microinjected with Src251-GFP and pretreated with cytochalasin D for 2 hr before stimulation with serum. Actin was visualized with rhodamine-phalloidin, and Src251-GFP was localized by direct fluorescence. Scale bars = 25 μm.



prior to expression of Src251-GFP, the cells were devoid of stress fibers even though serum was present and translocation of Src251-GFP to peripheral focal adhesions in response to serum was blocked (Figure 3c). Y-27632 has also been reported to inhibit other kinases, and we cannot rule out the possibility that Y-27632 is inhibiting kinases other than ROCK. However, in the context of these experiments, treatment with Y-27632 results in the loss of stress fibers and taken together with the ability of N19RhoA to block translocation of Src251-GFP to focal adhesions we conclude that the actin-dependent localization of Src251-GFP to these peripheral sites is controlled by RhoA.

Platelet-derived growth factor (PDGF) stimulates the formation of lamellipodia at the cell periphery [17] via a Rac1-dependent mechanism [11]. To address the relationship between RhoA and Rac1 signaling in the localization of c-Src within the cell, we stimulated cells with PDGF while inhibiting the formation of stress fibers by treating the cells with Y-27632. As above, Y-27632 was added to the cells prior to expression of Src251-GFP, resulting in loss of stress fibers. After stimulation with PDGF, Src251-GFP remained perinuclear (Figure 3d, broken arrow) and did not translocate to the cell periphery, although the shape of the cell did change in response to

Figure 2



Src, green; phalloidin, red

Src localization is under the control of Rho family GTPases. **(a)** Cells were serum starved for 48 hr to remove stress fibers prior to microinjection of V14RhoA and Src251-GFP (broken arrow). Solid arrow indicates noninjected cell. **(b)** Cells were cotransfected with L61Rac and Src251-GFP. **(c)** Cells were cotransfected with V12Cdc42 and Src251-GFP. The broken arrow indicates Src251-GFP at the base of filopodia, and the broken arrows (inset) show an enhanced resolution of Src251-GFP localizing along filopodia. Actin was visualized with rhodamine-phalloidin and Src251-GFP by direct fluorescence. Scale bars = 25 μm.

PDGF at the cell periphery (Figure 3d, solid arrows). However, if Y-27632 was washed out prior to stimulation with PDGF, Src251-GFP localized to lamellipodia (Figure 3e, solid arrows). Expression of dominant-negative N17Rac1 blocked the formation of lamellipodia in response to PDGF (Figure 3f), confirming that PDGF was working through activation of Rac1. No lamellipodia were formed in cells expressing N17Rac1 (broken arrow) as compared to the nontransfected cells (solid arrows). Although cells expressing N17Rac1 did not form lamellipodia, these cells still contained actin stress fibers, and Src251-GFP was consequently located at the ends of these stress fibers in peripheral focal adhesions (Figure 3f, broken arrow). Thus, Rac1 drives the targeting of c-Src to lamellipodia but is not involved in the initial movement of c-Src from the cell interior to the cell periphery, which is mediated by RhoA-dependent formation of stress fibers.

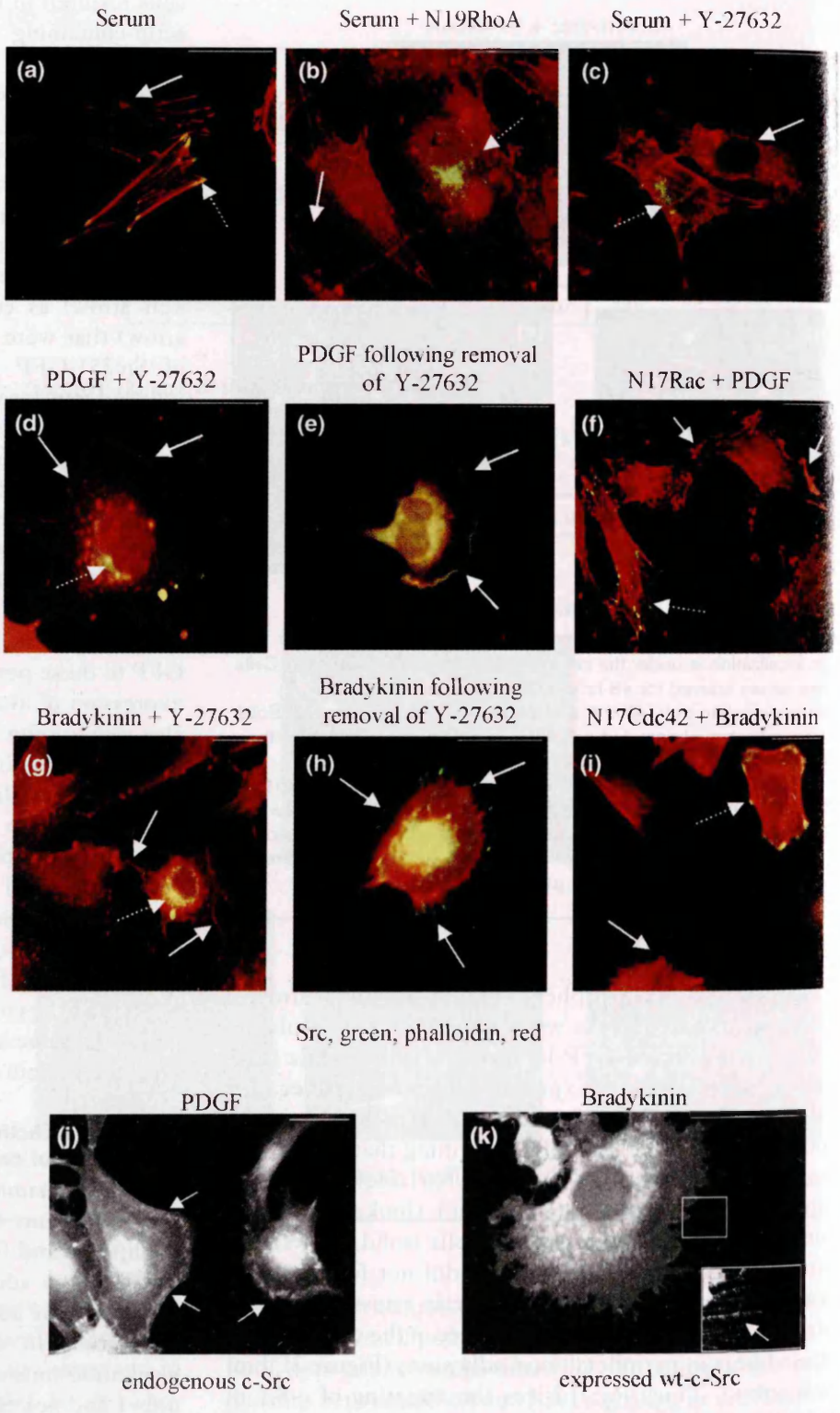
Similar experiments were carried out to define the relationship between RhoA signaling and Cdc42. Bradykinin stimulates the formation of actin microspikes, known as filopodia, at the cell periphery, by a Cdc42-mediated pathway [12, 13]. Addition of bradykinin to Y-27632-treated cells resulted in the formation of filopodia seen as small actin-containing microspikes protruding from the cell body (Figure 3g, solid arrows), although Src251-GFP remained perinuclear in the presence of Y-27632 (Figure 3g, broken arrow). However, removal of Y-27632 prior to addition of bradykinin resulted in the translocation of Src251-GFP to the peripheral microspikes in response to bradykinin (Figure 3h, solid arrows). The expression of a dominant-negative Cdc42 protein (N17Cdc42) blocked bradykinin-induced filopodia formation (Figure 3i, broken arrow) as compared to nonexpressing cells (solid arrow) that were able to induce filopodia. Coexpression of Src251-GFP with N17Cdc42, blocked bradykinin-induced translocation of Src to filopodia; however, Src251-GFP was not retained in the perinuclear region but was present in peripheral focal adhesions under these conditions (Figure 3i, broken arrow). Thus, the RhoA pathway is also required to target c-Src from the perinuclear region to the cell periphery, prior to its localization to filopodia induced by Cdc42.

To eliminate the possibility that the localization of Src251-GFP to these peripheral sites was a consequence of overexpression of available SH3 and SH2 domains, we were also able to show that endogenous Src was able to localize to lamellipodia in response to PDGF (Figure 3j). It was difficult to visualize endogenous Src in filopodia following stimulation with bradykinin, which is most likely due to problems in detection of the low levels of endogenous proteins in small structures. However, this could be overcome by expressing full-length wild-type Src protein where treatment with bradykinin resulted in Src being readily seen in filopodia (Figure 3k, inset). These data imply that the control of Src251-GFP targeting within the cell is the same as for endogenous Src or expressed wild-type Src protein.

c-Src kinase activity is required for the efficient remodeling of cellular adhesions

It is the dynamic remodeling and interchange between focal adhesions and focal complexes associated with lamellipodia and filopodia that regulates cell motility, and we therefore addressed whether the activity of c-Src at these cellular adhesions altered their remodeling. Cells were grown in serum, where Src251-GFP is localized at focal adhesions as a consequence of RhoA activity (Figures 1 and 4c). Subsequent treatment of these cells with PDGF resulted in PDGF-induced shape changes and formation of lamellipodia. Although expression of Src251-GFP did not significantly alter the number of cells able to form lamellipodia (around 80% of cells expressing Src251-GFP underwent shape changes associated with the formation of lamellipodia), we found that the Src was localized only to discrete patches around the forming ruffle (broken arrow in Figure 4d) where it colocalized with actin (data

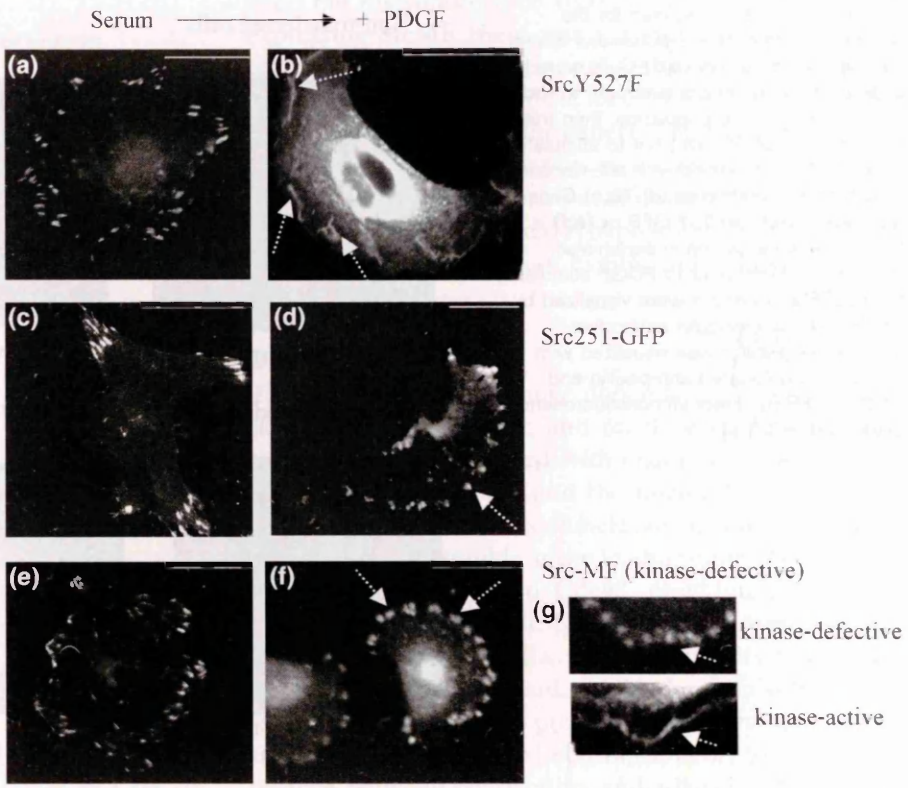
Figure 3



Coordinated regulation of the actin cytoskeleton by Rho family GTPases is required for Src targeting. **(a–c)** Cells were serum starved for 48 hr to remove stress fibers prior to microinjection of Src251-GFP (a,c) or comicroinjection of Src251-GFP with N19RhoA (b). Following expression of the proteins, cells were stimulated with serum (a,b) or preincubated with Y-27632 for 1 hr prior to stimulation with serum (c). Broken arrows indicate microinjected cells, while solid arrows are nonexpressing cells. **(d,e,g,h)** Cells were transfected with Src251-GFP and prior to expression of the protein were treated overnight with Y-27632 to abolish stress fibers. The cells were then stimulated with PDGF (d) or bradykinin (g) or were stimulated with PDGF or bradykinin following wash out of the Y-27632 (e,h). **(f,i)** Cells were cotransfected with Src251-GFP and N17Rac or N17Cdc42 and then placed in serum-free medium overnight to reduce the heterogeneity in the population prior to PDGF (f) or bradykinin (i) stimulation. Solid arrows show nontransfected cells, while broken arrows show cells expressing the constructs. Actin was visualized with rhodamine-phalloidin and Src251-GFP by direct fluorescence. **(j)** Cells were placed in serum-free conditions overnight, stimulated with PDGF and endogenous Src visualized using the N2-17 Src antibody. **(k)** Cells expressing wild-type Src were placed in serum-free conditions overnight and stimulated with bradykinin. Inset shows enhanced resolution of wt-Src localizing along filopodia. Exogenously expressed Src was visualized using the chicken-specific Src EC10 antibody. Scale bars = 25 μm.

not shown). This is in contrast to the uniform lamellipodia formed when Src251-GFP is held in the perinuclear region of the cell prior to stimulation with PDGF (Figure 3e), where Src251-GFP is found in small structures continuous along the lamellipodia. This suggests that it is the presence of Src251-GFP at focal adhesions prior to addition of PDGF that interferes with the actin rearrangements required for efficient formation of uniform lamellipodia. Since Src251-GFP lacks a c-Src kinase domain (Figure 1), we examined whether the presence of an activated c-Src kinase at focal adhesions facilitated the Rac1-dependent formation of uniform, continuous lamellipodia. For

Figure 4



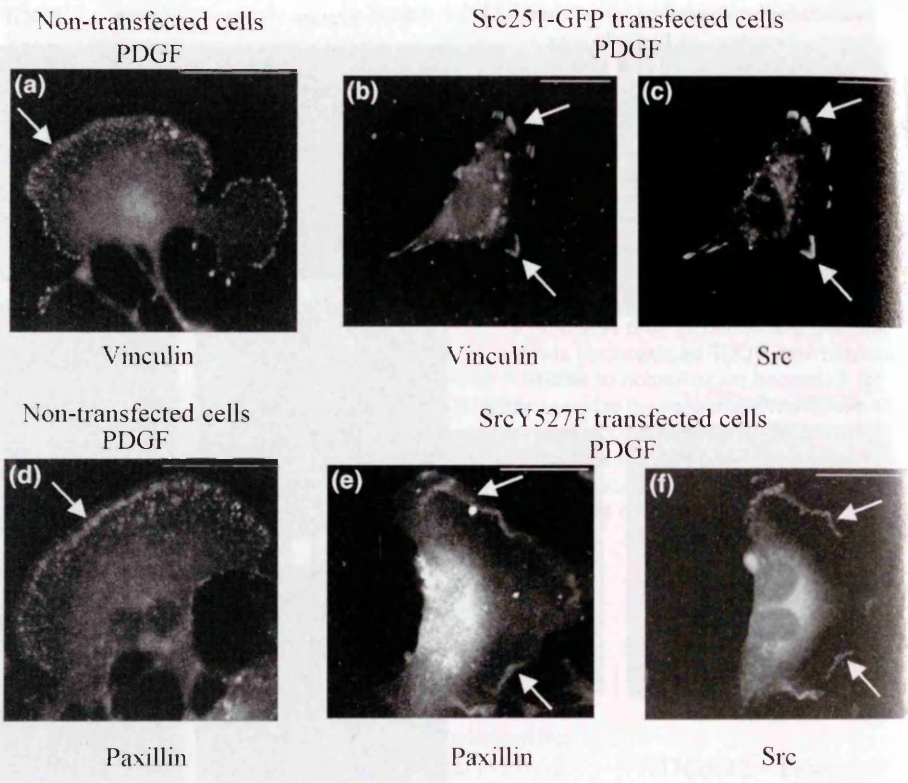
Src kinase activity is required for efficient lamellipodia formation. **(a,b)** Cells were transfected with SrcY527F and maintained in 10% serum to localize SrcY527F in peripheral focal adhesions (a). Cells were then placed in serum-free medium overnight to reduce heterogeneity in the population and treated with emetine for 30 min prior to PDGF stimulation (b). **(c,d)** Cells were transfected with Src251-GFP and maintained in 10% serum (c) or stimulated with PDGF as described above (d). **(e,f)** Cells were transfected with SrcMF and maintained in 10% serum (e) or stimulated with PDGF as described above (f). **(g)** Enhanced magnification of kinase-defective (SrcMF) (upper panel) or kinase-active Src (SrcY527F) (lower panel) localization in PDGF-induced lamellipodia. Src251-GFP was visualized by direct fluorescence, SrcY527F and SrcMF using an anti-Src antibody. Scale bars = 25 μm.

this, we expressed a c-Src protein, which has an activating tyrosine to phenylalanine mutation at position 527 in the c-Src coding sequences (SrcY527F) (reviewed in [18]) (Figure 1). SrcY527F localized to focal adhesions in the presence of serum (Figure 4a). Following treatment with PDGF, peripheral SrcY527F was located at lamellipodia that appeared continuous and relatively uniform (Figure 4b, broken arrows), in contrast to the discontinuous structures formed in cells expressing Src251-GFP (Figure 4d, broken arrow). These data imply that the efficient relocation of c-Src from RhoA-induced focal adhesions into Rac1-induced structures along lamellipodia requires Src's catalytic activity, although expression of kinase-deficient Src251-GFP did not block the initial PDGF-induced shape changes that accompany lamellipodia formation (Figure 4d). Furthermore, the protein synthesis inhibitor emetine was added prior to PDGF treatment in these experiments, implying that Rac1 activation led to relocalization of c-Src that preexisted in focal adhesions into complexes that are continuous along lamellipodia. However, we cannot rule out that some c-Src preexisted in the perinuclear region after emetine treatment and moved out into the newly forming lamellipodia upon activation of Rac1.

To further test whether the kinase activity of Src was required for efficient remodeling, we expressed a kinase-defective Src mutant, SrcMF, in the cells and examined the ability of these cells to form lamellipodia in response to PDGF. SrcMF has a point mutation in the kinase domain (K295M) rendering it kinase inactive (Figure 1). SrcMF localizes to focal adhesions in cells grown in serum (Figure 4e), and subsequent stimulation of these cells with PDGF resulted in the relocalization of SrcMF to discrete patches around the edge of the forming lamellipodia (Figure 4f) rather than to continuous lamellipodia as seen in cells expressing the activated Src protein (Figure 4b). Magnification of the edge of the lamellipodia in SrcMF and activated Src-expressing cells (Figure 4g) shows more clearly the influence of Src kinase activity on the formation of the lamellipodia. Src colocalizes with actin both in the discrete patches in the SrcMF-expressing cells and the continuous lamellipodia formed in cells expressing activated Src (data not shown). Treatment of cells with the Src kinase inhibitor PP2 also resulted in the formation of fragmented lamellipodia in response to PDGF (results shown in Supplementary materials). Taken together, these data imply a role for Src kinase activity in the efficient Rac1-induced actin remodeling associated with lamellipodia formation.

To demonstrate that the discontinuous lamellipodia found in Src251-GFP-expressing cells was associated with impaired focal adhesion remodeling into focal complexes, we compared Rac1-induced peripheral adhesion remodeling in cells expressing Src251-GFP with cells expressing

Figure 5



Src kinase activity is required for the remodeling of peripheral adhesions during lamellipodia formation. **(a,d)** Cells were grown in serum-free conditions overnight to reduce heterogeneity in the population, then treated with emetine for 30 min prior to stimulation with PDGF, and stained with anti-vinculin (a) or anti-paxillin antibodies (d). **(b,c)** Cells transfected with Src251-GFP or **(e,f)** SrcY527F were placed in serum-free medium overnight prior to PDGF stimulation. SrcY527F and vinculin were visualized by anti-Src and anti-vinculin antibodies, respectively. Paxillin was visualized with rhodamine-conjugated anti-paxillin and Src251-GFP by direct immunofluoresence. Scale bars = 25 μm.

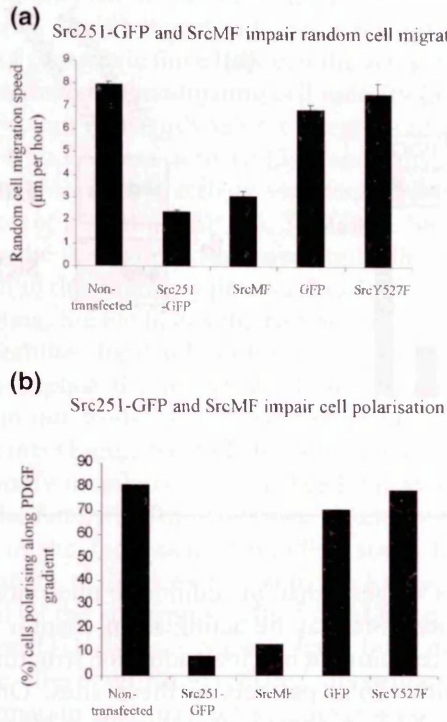
the activated c-Src mutant SrcY527F, monitoring adhesion complexes by staining with anti-vinculin or anti-paxillin. (Anti-paxillin staining was used for colocalization studies with anti-SrcY527F because anti-vinculin and anti-Src sera used were from the same species, restricting colocalization studies.) Anti-vinculin and anti-paxillin staining were indistinguishable, and, in growing cells treated with PDGF, we observed small vinculin- and paxillin-containing complexes around the base of lamellipodia (Figure 5a,d, respectively). In PDGF-treated cells expressing Src251-GFP, vinculin colocalized with Src251-GFP at the cell periphery but was located in larger discrete structures along the length of the lamellipodia (Figure 5b,c). In contrast, in activated SrcY527F-expressing cells, c-Src colocalized with paxillin along the base of uniform lamellipodia (Figure 5e,f). Paxillin localizes to focal complexes at the leading edge of lamellipodia as described by Nobes and Hall [13]; however, in cells expressing activated Src, these were difficult to visualize as discrete structures, suggesting that Src's overactivity may be further breaking down the focal complexes.

Similar to these observations with PDGF, we found that bradykinin-induced focal adhesion remodeling to produce focal complexes at filopodia was impaired in Src251-GFP-expressing cells (results shown in Supplementary materials). Thus, our data support a critical role for the catalytic activity of c-Src in the Rac1- and Cdc42-induced peripheral adhesion remodeling that results in the breakdown of larger focal adhesions and the generation of smaller focal complex adhesion structures at lamellipodia and filopodia, respectively. Further details given in Supplementary materials.

c-Src kinase activity is required for cell motility and polarization

As the formation of lamellipodia and filopodia have been associated with the regulation of cell motility and chemotaxis [19, 20], we examined whether the impaired focal adhesion remodeling in the cells lacking kinase activity inhibited cell movement or the ability of cells to polarize in response to a chemotactic agent. We found that the random migration of cells expressing Src251-GFP or SrcMF was inhibited by around 70%, while expression of GFP alone had no effect on cell motility (Figure 6a). Expression of activated Src (SrcY527F) had no effect on the migration of the cells, suggesting that the level of endogenous Src within the cells is not limiting for migration. This has also been reported for reintroduction of activated Src into $Src^{-/-}$ cells where activated Src restored migration to a similar level as reintroduction of the wild-type protein [21]. To examine the capacity of cells to respond to a chemotactic gradient, we measured the ability of cells to polarize toward PDGF using a Dunn chamber [19]. Approximately 10% of cells expressing Src251-GFP or SrcMF were able to polarize along the PDGF

Figure 6



Src kinase activity is required for random cell migration and the ability of cells to remodel in response to a chemoattractant gradient. **(a)** Random cell migration of cells in 10% serum expressing Src251-GFP, GFP, SrcY527F (+ GFP), or SrcMF (+ GFP). Values are mean ± SEM taken from at least five independent experiments and represent data from between 30 and 60 cells in each case. **(b)** Polarity of cells expressing Src251-GFP, GFP, SrcY527F (+ GFP), or SrcMF (+ GFP) in response to a 10 ng/ml^{-1} PGDF gradient. Values are expressed as the percentage of cells in a population able to polarize, using the criteria described in the Supplementary materials section, generated from at least five independent experiments.

gradient, while around 80% of the nontransfected cells exhibited polarity (Figure 6b). Expression of GFP or SrcY527F had no effect on the polarity of the cells in response to PDGF (Figure 6b). Thus, both cell migration and cell polarization in response to a chemotactic stimuli, biological responses that require the coordinated activities of Rho GTPases, are impaired in cells expressing either the Src251-GFP or SrcMF kinase-deficient mutants of c-Src.

Src activity is required for phosphorylation of FAK

FAK is a known Src substrate at adhesion sites, and there is considerable evidence that FAK plays a central role in the regulation of cell motility (reviewed in [22]). We therefore addressed whether phosphorylation of FAK was suppressed in cells expressing Src kinase-defective mutants that were unable to remodel focal adhesions in response to PDGF and displayed impaired motility. Using an antibody against phosphorylated tyrosine 925 in FAK, which is a site specifically phosphorylated by Src ([23] and our unpublished data), we found that PDGF stimulated the phosphorylation of tyrosine 925, while in cells expressing SrcMF there was very little phosphorylation on this site (Figure 7a). Expression of SrcMF (Figure 7b, lower panel) did not alter the level of FAK protein within the cells (Figure 7b, upper panel). Therefore, using FAK tyrosine 925 phosphorylation as a marker of Src-dependent phosphorylation events at adhesions, we have shown that kinase-defective SrcMF expression suppresses both peripheral phosphorylation events and adhesion remodeling.

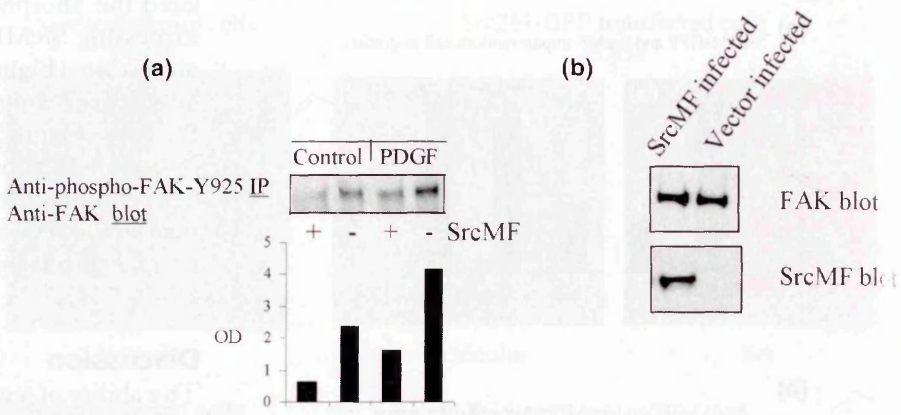
Discussion

The ability of a cell to move depends on a cycle of adhesion, loss of attachment, and readhesion, which is governed by and coordinated with changes in the actin cytoskeleton arrangement and the microtubule system [1, 24]. The actin structures, lamellipodia, and filopodia assemble and disassemble at the leading edge of cells under the control of Rac1 and Cdc42, providing a means of polarized cell movement. Small focal complexes form at lamellipodia and filopodia, which are rapidly turned over as the cell moves forward, while the RhoA-dependent assembly of stress fibers provide contractility and propulsion required for forward cell movement. Since it is the balance between contractility and adhesion that governs the rate of cell motility, relative activities of RhoA, Rac1, and Cdc42 are critical in determining whether and how a cell moves. In this study, we examined the role of the Rho GTPases in directing the intracellular localization of the tyrosine kinase c-Src, which is known to be required for cell motility, and the role of its kinase activity in controling cell movement.

The intracellular localization of c-Src is modulated by RhoA, Rac1, and Cdc42 activity

The localization of v-Src and c-Src to the cell periphery in fibroblasts requires an intact actin cytoskeletal network [5]. Here, we show that the peripheral localization of Src251-GFP, a fusion protein that contains Src's SH3 and SH2 domains but lacks a kinase domain, is also dependent on RhoA-mediated assembly of actin stress fibers. Although Src251-GFP was found in lamellipodia and filopodia following activation of Rac1 or Cdc42, this was also dependent on the activity of RhoA, which mediates transit from the perinuclear region to the cell periphery. This implies that Src requires actin stress fibers to incorporate into focal adhesions or into smaller focal complexes at lamellipodia or filopodia, depending on the balance of specific Rho GTPase signals within the cell. As yet, little is known of the mechanism involved in the transport of Src from the perinuclear region to the cell periphery. We and others have demonstrated that it is not dependent on the catalytic activity of c-Src but does require the Src SH3 domain [4, 6, 7]. In vitro experiments have demonstrated that the Src SH3 domain binds to a number of

Figure 7



Phosphorylation of FAK is suppressed in cells expressing kinase-defective Src. **(a)** Cells were placed in serum-free conditions overnight and then stimulated with PDGF. Control cells were not stimulated with PDGF. FAK phosphorylated on tyrosine 925 was immunoprecipitated (IP) from extracts of cells infected with SrcMF or the empty vector using a phospho-FAK-Y925-specific antibody. The proteins were resolved by SDS-PAGE, and the levels of phosphorylated FAK were detected following blotting with an anti-FAK antibody (blot). **(b)** Expression of FAK and SrcMF in the SrcMF and vector-infected cells was determined in whole-cell extracts using an anti-FAK antibody (top panel), and exogenously expressed SrcMF was visualized using the chicken-specific Src EC10 antibody (bottom panel).

cellular partners and that the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) is one binding partner of the v-Src SH3 domain that is important for its peripheral targeting [7]. Although the catalytic activity of Src is not required for its targeting, a number of stimuli that induce its peripheral targeting also activate Src kinase activity (e.g., PDGF and bradykinin). These stimuli can therefore release constraints on both the kinase domain and the SH3 domain of c-Src, ensuring that the activated protein is not aberrantly localized within the cell.

Regulation of cell motility requires coordinated activity of Rho GTPases and Src

Involvement of RhoA, Rac1, and Cdc42 in cell motility is undoubtedly linked to their ability to rearrange the actin cytoskeleton. However, a secondary consequence of this actin remodeling is apparently to target other cellular proteins, such as c-Src, to these peripheral sites. We show here that once located at the cell periphery c-Src acts in concert with the Rho GTPases to regulate cell polarity and motility. Several studies have suggested that Src family kinases play a role during cell motility. For example, c-Src-deficient fibroblasts have a decreased random migration [21], and cells deficient in all three ubiquitously expressed family members, Src, Fyn, and Yes, are unable to migrate into a wound [25]. Furthermore, c-Src kinase activity is required for growth factor-induced migration and scattering in a number of cell types [26–28]. The ability of Src to influence cell motility in fibroblasts has been linked to the regulation of focal adhesion turnover. Cells lacking Src family members are able to assemble focal adhesions [25], indicating that Src is not required for their formation. However, cells in which kinase-defective mutants of Src are expressed have enlarged focal adhesions [4, 6], suggestive of a role for Src kinase activity in the turnover of these structures that is needed for cells to move [6]. The presence of functional SH2 and SH3 domains in these Src proteins suggests that, in addition to phosphorylatin key substrates, Src may be acting as an adaptor protei allowing its recruitment into focal adhesion structures and or to recruit cellular partners to these sites. One suc candidate is FAK, as there is considerable evidence link ing Src activity to FAK-regulated cell motility [8, 29]. is known that in fibroblasts lacking the FAK protein ther is an increased number of focal adhesions and an impaire ability to move, suggesting that FAK is required for th turnover of focal adhesions during cell motility [30], whil the ability of Src to induce adhesion turnover has bee linked to the phosphorylation and possibly subsequen proteolysis of FAK [6, 8]. In this study, we have show that phosphorylation of FAK on tyrosine 925, which is site known to be phosphorylated by Src [23], is suppresse in cells which are unable to remodel their peripheral adhe sions after PDGF treatment, correlating with impaire cell motility and polarity. Thus, SrcMF expression sup presses both Src-dependent phosphorylation events at th cell periphery and stimulus-induced adhesion remod eling.

A key component of the coordinated movement of cell is their ability to polarize toward different migratory sig nals, and we addressed the role of c-Src in this process. The formation of Rac1-dependent lamellipodia at the leading edge is required for cell motility [19, 20, 31]. In addition, while Cdc42 is not essential for cell movement per se, it is required for directionality and the maintenance of cell polarity [19, 20, 32]. We have shown that c-Src kinase activity is required for cells to polarize and form a leading edge, and we propose that the ability of the Rho GTPases to direct the localization of Src to these peripheral sites is required for the remodeling of the more static focal adhesions into smaller more dynamic focal complexes found at the leading edge of motile cells.

The involvement of Src kinases in the regulation of integrin-ECM interactions has been reported in a number of studies [25, 33, 34]. Specifically, Src can regulate the generation of contractile force between the actin cytoskeleton and integrins, thus modulating cell motility [33]. In addition, c-Src can transiently inhibit integrin-induced activation of Rho GTPase activity [34], and oncogenic v-Src can antagonize RhoA activity via mechanisms involving activation of p190RhoGAP [35, 36]. Thus, Src may act to regulate the turnover of Rho-mediated adhesions, which is critical to the migratory process. In addition to adhesion remodeling, Src facilitates the turnover of actin filaments which stabilize focal adhesion sites [7, 36], and this may partially explain the reduction of random migration observed in our study in cells expressing kinase-defective Src mutants (Figure 6a) and also cells lacking Src and the other family members Fyn and Yes [25]. More recently, the mDia family of Rho effectors, which cooperate with ROCK in the formation of bundled stress fibers, have been shown to bind to Src, and Src kinase activity is required for the cooperative effect of mDia on stress fiber formation [37]. Thus, c-Src can have both positive and negative effects on Rho-mediated stress fiber and adhesion formation and may act at a pivotal point between their assembly and disassembly, placing it in a central position from which to coordinate the multifaceted process of cell motility.

The major challenges are now to determine (1) how motile cells integrate different signals that regulate the activity of RhoA, Rac1, and Cdc42, which in turn regulate the localization of c-Src and possibly other Src family kinases within the cell, and (2) how Src-dependent tyrosine phosphorylation regulates adhesion remodeling in response to biological stimuli that induce directed cell migration.

Materials and methods

Cells and plasmids

CA10-Src251-GFP and CA10-Src were kind gifts from P. Schwartzberg (NIH, Bethesda, Maryland), and CA10-SrcY527F and CA10-SrcMF (K295M,Y527F) were a kind gift from K. Kaplan (MIT, Cambridge, Massachusetts). pEGFP (Clontech, Basingstoke, UK) was used for expression of GFP. N19- and V14RhoA, V12- and N17Cdc42, and L61- and N17Rac, all in the pRK5myc plasmid, were kindly provided by Alan Hall (UCL, London, UK). Swiss 3T3 cells were routinely grown in DMEM supplemented with 10% fetal calf serum. Plasmids were introduced into the cells by nuclear microinjection or transient transfection as indicated in the text, with further details given in the Supplementary materials.

Immunofluorescence

Cells were plated onto glass chamber slides 24 hr prior to transfection or onto glass coverslips 24 hr prior to microinjection. For experiments in which cells were serum starved to remove stress fibers prior to expression of the proteins, the cells were grown for 48 hr in serum-free conditions prior to microinjection of the appropriate plasmids and analysis carried out 3–5 hr later after expression of the proteins. For all other experiments, the proteins were expressed by transient transfection. Growth factor treatment of the cells were as follows: 10% serum for 30 min, PDGF, 25 ng/ml^{-1} for 30 min (TCS Biologicals, Botolph Claydon, UK), bradykinin, 100 nM for 5 min or LPA 200 ng/ml^{-1} for 30 min (both Sigma, Poole, UK). Y-27632 (10 μM, Welfide Corporation, Japan), cytochalasin D (0.1 μg/ml^{-1}), or emetine (55 μg/ml^{-1}, both Sigma, Poole, UK) were added as indicated. Cells were fixed in 3% paraformaldehyde, washed PBS/100 nM glycine, then permeabilized with PBS/0.1% saponin/20 mM glycine. After blocking with PBS/0.1% saponin/10% fetal calf serum, cells were incubated with anti-Src mAb EC10 to detect the exogenously expressed chicken Src proteins (Upstate Biotechnology, Lake Placid, New York), anti-Src monoclonal antibody N2-17 to detect endogenous Src protein (a kind gift from T. Hunter, Salk Institute, California), anti-vinculin mAb, rhodamine-conjugated phalloidin (both Sigma, Poole, UK), or rhodamine-conjugated paxillin antibody (Research Diagnostics, Flanders, New Jersey). Nonconjugated antibody detection was by reaction with species-specific TRITC-conjugated antibodies (Sigma, Poole, UK). Cells were visualized using a confocal microscope (MRC 600; BioRad Labs, Hercules, California).

FAK phosphorylation

Cells infected with pBABEpuro or pBABEpuro-SrcMF were trypsinized and plated at 2.5 × 10^6 onto 100 mm culture plates in growth medium for 24 hr. The cells were then serum starved for 24 hr prior to stimulation with 25 ng/ml^{-1} PDGF. Cell extracts were prepared in 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 1 mM EGTA, 10 mM sodium pyrophosphate, 0.5 mM sodium fluoride, 1 mM PMSF, 10 μg/ml^{-1} aprotinin, and 100 μM sodium orthovanadate, and 1 mg of cell lysate incubated with 2 μg of phospho-FAK-Y925 rabbit polyclonal antibody. Generation of this antibody was as described in [38] using the peptide NDKVYENVT GLVKA(C), which corresponds to residues 921–934 in FAK, in which tyrosine 925 has been chemically phosphorylated. The immune complexes were collected with protein A sepharose and the proteins resolved by SDS-PAGE. FAK phosphorylated on tyrosine 925 was detected following incubation with anti-FAK monoclonal antibody (Transduction Laboratories, BD Biosciences, Oxford, UK) using ECL detection.

Cell migration and polarization

Cells were transfected with Src251-GFP or GFP or cotransfected with SrcY527F and GFP or SrcMF and GFP to allow detection of the transfected cells. To monitor polarization, cells were trypsinized 24 hr post-transfection and plated onto coverslips for 1 hr. Cells were then serum straved for 1 hr and mounted on Dunn chambers with PDGF 10 ng/ml^{-1} as chemoattractant [19]. For measurement of both random migration and polarization, microscopic images of the cells were acquired from a charged couple device camera (Hamamatsu C4742) and captured by Open Lab (Improvision Software, UK) every 15 min over a 5 hr period. Details of how the mean cell speed was calculated and the criteria used to determine cell polarity are given in the Supplementary materials.

Supplementary material

Further experimental details on the different methods used for the introduction of plasmids into the cells and the rationale for their use can be found in the Supplementary material online at http://images.cellpress.com/supmat/supmatin.htm. More detailed descriptions are also given for the measurement of cell migration and polarity.

Additional data are provided in which we have used the kinase inhibitor PP2 as further confirmation of the requirement of Src kinase activity for efficient remodeling of cellular adhesions in response to PDGF, and data are also presented which demonstrates that bradykinin-induced focal adhesion remodeling to produce focal complexes was also impaired in Src251-GFP-expressing cells. We also provide further evidence that Src kinase activity is required for the turnover of focal adhesions in experiments where treatment of cells expressing Src251-GFP with the ROCK inhibitor results in the stabilization of focal adhesions which are normally broken down in the absence of a Rho signal.

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