SRF activation in immediate early gene transcription

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ABSTRACT

The research described in this thesis was focused on the study of signalling pathways that lead to activation of serum response factor (SRF) and its target genes in response to diverse extracellular signals, such as serum, LPA, PDGF or TPA. SRF is able to bind to the serum response element (SRE) which is found in many, but not all immediate early gene promoters. At some promoters, like c-fos and egr-1, in conjunction with SRF, ternary complex factor (TCF) can bind to the SRE. TCF-dependent transcription is regulated by MAP kinases in response to growth factors or stress stimuli, while SRF activity is potentiated by RhoA-dependent signalling pathways in response to serum or LPA. In contrast to transiently transfected templates, an integrated SRF reporter gene is also activated by growth factors or the phorbol ester TPA. Using pharmacological inhibitors it is demonstrated that activation by growth factors and TPA was dependent on PI-3 kinase activity, while activation of SRF by serum and LPA was not. SRF activation by all stimuli was absolutely dependent on RhoA activity and largely independent of MEK activity. Cloning of LIMK, a regulator of the actin treadmilling cycle, as an SRF activator, suggested that actin dynamics are involved in regulating transcriptional activation. Studies with activators and inhibitors of actin polymerisation demonstrated that depletion of the cellular G-actin pool is necessary and sufficient for SRF activation. In contrast, alterations in actin dynamics are neither necessary nor sufficient for activation of TCF. Accordingly, activation of some SRF target genes, such as c-fos and egr-1, was actin and RhoA independent, but MAPK dependent. However, another group of genes, such as srf and vinculin, was actin and RhoA dependent, but largely independent of MEK-ERK signalling. Based on these findings, two classes of SRF target gene can be defined: one regulated by actin dynamics and a second by MEK-ERK signalling. It is proposed that two classes of SRF target gene are regulated in a mutually exclusive manner, and that the presence of TCF may control signalling specificity at SRF target promoters.
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PUBLICATIONS

Some of the data described in this thesis has been presented in the following publications:


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ABBREVIATIONS

Frequently used abbreviations are listed below; other less frequently used abbreviations and gene name acronyms are introduced as they are used.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol Acetyl Transferase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<td>Curie</td>
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<td>Dbl homology</td>
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<td>DMSO</td>
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</tr>
<tr>
<td>DNase</td>
<td>Deoxy ribonucleic acid endonuclease</td>
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<tr>
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<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis-tetra acetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell scanner</td>
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<tr>
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<td>Foetal bovine serum</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>GTP hydrolase</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)-piperazine-N'-(2-ethane sulphonic acid)</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>Symbol</td>
<td>Term</td>
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<tr>
<td>--------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>m</td>
<td>Milli-</td>
</tr>
<tr>
<td>µ</td>
<td>Micro-</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>n</td>
<td>Nano-</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonidet P40</td>
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<tr>
<td>Nucleotides:</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Adenosine</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
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<tr>
<td>G</td>
<td>Guanosine</td>
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<td>Nucleotide</td>
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<tr>
<td>dNTP</td>
<td>Deoxy-nucleotide triphosphate</td>
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<tr>
<td>ddNTP</td>
<td>Dideoxy-nucleotide triphosphate</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PI-3 K</td>
<td>Phosphatidylinositide 3 kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositide 3, 4, bisphosphate</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulator of G protein signalling</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonucleic acid endonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIE</td>
<td>Serum inducible element</td>
</tr>
<tr>
<td>SRE</td>
<td>Serum Response Element</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum Response Factor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TCF</td>
<td>Ternary complex factor</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethylenediamine</td>
</tr>
<tr>
<td>TPA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume to volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight to volume</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-Bromo-4-chloro-3-indoyl-b-D-galacto-pyranoside</td>
</tr>
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</table>
1. Introduction

1.1. Chapter Summary

This thesis describes studies of signalling pathways that lead to the activation of serum response factor (SRF), and its target genes, in response to diverse extracellular signals. Section 1.2 of this introduction outlines some of the general principles and mechanisms by which different extracellular stimuli causes activation of a range of signalling pathways. Section 1.3 describes the mechanisms involved in regulation of transcription factors based on their site of activation. I will describe in more detail immediate early genes, their promoter structure and the signalling pathways that lead to their transcriptional activation in Section 1.4. Section 1.5 deals with the mechanisms by which Rho family GTPases and changes in actin dynamics lead to SRF activation. A brief description of the actin binding drugs will be presented. Section 1.6 and 1.7 describes pharmacological inhibitors and bacterial toxins used in this study.

1.2. Principles of Signal Regulated Transcription

Transcription of immediate early genes can be activated by a number of different stimuli such as growth factors, cytokines of TGFβ or interferon families and lysophospholipids. A diverse array of the environmental stress signals, like ionising or ultraviolet irradiation, membrane depolarisation and others also induce immediate early gene activation. The mechanisms by which both types of signalling result in immediate early gene induction are thought to be similar, but signalling by extracellular ligands has been more extensively defined and will be discussed further here.

1.2.1. Activation signalling pathways by extracellular stimuli
1.2.1.1. **Lysophosphatidic acid and other bioactive lipids**

One of the major mitogen in serum is lysophosphatidic acid (LPA), which is also an intermediate in membrane component biosynthesis. LPA is produced extracellularly by platelets. Besides LPA, sphingosine-1-phosphate (S1p) and sphingosylphosphoryl-choline (SPC) are other bioactive lipids which are able to activate members of the G-protein coupled receptor superfamily. Eight genes of LPA receptor family have been cloned so far, and at least three of them encode high-affinity LPA receptors (Moolenaar, 1999). All three subfamilies signal through the α-subunits of the Gi/o, G\(_{12/13}\) and Gq proteins, although the third LPA receptor subfamily does not efficiently couple to the G\(_{12/13}\) subunits (Ishii et al., 2000). The first cDNA encoding a functional LPA receptor, now termed Edg2 was identified during a search for heptahelical receptor genes showing enriched expression in the developing cerebral cortex (Hecht et al., 1996). The previously thought to be an orphan receptor Edg1 has been reported to have high affinity to sphingosine-1-phosphate (S1p) (Lee et al., 1998). There is no single classification of LPA receptors, since the recently emerged Edg gene family has been linked to both LPA- and S1p- induced signalling. It has been proposed by Chan and colleagues nomenclature be based on a division between LPA I (for LPA receptors) and LPA II (for the S1p receptors). Heterologously expressed Edg/LPA receptors can couple and activate G proteins in both PTX-sensitive and PTX-insensitive manner to mediate calcium mobilisation, MAPK activation and RhoA dependent cytoskeletal changes (Lee et al., 1998; Windh et al., 1999; Zondag et al., 1998). In addition, serum and LPA have been implicated in transcriptional activation of the immediate early genes *c-fos* and *egr-1* and RhoA dependent activation of serum response factor (Hill et al., 1995; Reiser et al., 1998) (see below).

1.2.1.2. **Polypeptide growth factors**

In addition to LPA, polypeptide growth factors are believed to elicit much of the mitogenic activity of serum. The most extensively described growth factors present in serum are epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-I) and platelet derived growth factor (PDGF), which have been demonstrated to be sufficient to induce cell cycle progression in cultured cells (Pardee, 1989; Stiles et al., 1979). Binding of polypeptide growth factors to their receptors induces a cascade of phosphorylation events in a number of signalling pathways controlling cell
proliferation, differentiation, migration, and/or metabolic changes. Polypeptide growth factor receptors constitute a family of tyrosine kinase receptors (RTKs). RTKs are transmembrane glycoproteins, consisting of an extracellular ligand binding domain and intracellular kinase domain (for review see Heldin, 1995; Schlessinger, 2000). Activation of RTKs by ligands requires two processes: 1) enhancement of intrinsic kinase activity, and 2) creation of binding sites to recruit downstream signalling proteins. Upon ligand binding, receptor autophosphorylation at tyrosine residues in the activation loop (within the kinase domain) results in stimulation of kinase activity and generation of docking sites for proteins with modular domains that recognise phosphotyrosine in specific sequence contexts. The two well established phosphotyrosine-binding modules present within signal transduction proteins are the SH2 domain and the phosphotyrosine-binding (PTB) domain. It is well established that specificity of the SH2 domain binding is determined by the stretch of amino acid residues at position 1 to 6 C-terminal to the pTyr (Songyang et al., 1993). In contrast, specificity of PTB domain binding is thought to be determined by specific sequences 3 to 5 residues N terminal to the pTyr (Forman-Kay and Pawson, 1999; van der Geer and Pawson, 1995). There is a number of SH2 domain containing proteins acting downstream of RTKs. Some of the SH2 domain-containing proteins also possess intrinsic protein tyrosine kinase (PTK), eg. Src, or protein tyrosine phosphatases (PTP), eg. Shp2, activities (Kazlauskas et al., 1993; Kypta et al., 1990). The balance between kinase and phosphatase activities allows strict control over the duration and the amplitude of the signal induced by an extracellular ligand. In addition, the phosphorylated receptor can recruit other signalling molecules with other enzymatic activities, such as phospholipase C (PLCγ), phosphatidyl-inositol kinase (PI-3K), and RasGAP (Fantl et al., 1992; Kaplan et al., 1990; Meisenhelder et al., 1989). Another family of proteins contain only SH2 and SH3 domains without apparent enzymatic activity. They are often referred to as adapter proteins (for example Grb2, Nck, Shc) as they mediate interaction between RTKs and other signalling molecules via SH2 and SH3 domains. A well-studied example is the adapter protein Grb2 via its SH2 domain links a variety of receptors with an GTP-exchange factor Sos, which in turn activate Ras/MAP kinase signal cascade (Buday and Downward, 1993); (reviewed by Schlessinger, 1994).

In this study signalling through PDGF receptor provides a model system, well characterised both in vitro and in vivo. It has been demonstrated using add-back receptor mutants that two downstream effectors of the PDGF receptor PLCγ and PI-3 kinase are sufficient to induce DNA synthesis independently of each other (Valius and
Chapter 1: Introduction

Kazlauskas, 1993). However, recently it has been suggested that there is some crosstalk between these two pathways occurring in a time dependent manner in order to induce cell cycle progression: the initial wave of signalling requires PLCγ/MAPK, but not PI-3 kinase, whereas a secondary wave is absolutely dependent on PI-3 kinase activity (Jones and Kazlauskas, 2001); (reviewed by Jones and Kazlauskas, 2000). Therefore, it is of considerable importance to understand the relationship between these signalling pathways, gene expression and the cell cycle progression. Using the PDGF receptor mutants as a model, attempts have been made to characterise the induced gene expression pattern regulated by PDGF using northern blot or gene array technology (Fambrough et al., 1999; Montmayeur et al., 1997). There is some controversy over the results of these studies. One set of array experiments in NIH3T3 cells found that receptor lacking 5 tyrosines was still able to activate immediate early genes. In contrast, Montmayeur et al. found that in A431 cell line this receptor was inactive (Montmayeur et al., 1997). More recent studies in mouse models demonstrated that receptor mutant-induced signal is reduced, but not ablated, allowing to retain physiological function of the mutant receptor in developing embryo (Tallquist et al., 2000).

Although numerous families of RTKs have been identified, there are three general mechanisms by which different effectors are activated (for review see Schlessinger, 2000): (i) activation by tyrosine phosphorylation; (ii) activation by conformational change; and (iii) activation by membrane translocation (see Figure 1.1). For example, PDGF induced activation of PI-3 kinase depends on all three of these events. Upon ligand binding, due to intrinsic tyrosine kinase activity, receptor creates binding sites at Y740 and Y751, which binds SH2 domain of the regulatory subunit of PI-3 K, p85. Binding of p85 to the phospho-tyrosines on the PDGF receptor causes conformational change that is transmitted to the catalytic subunit of PI-3 K, p110, thereby enhancing its activity. By binding to the PDGFR, PI-3 K is translocated to the plasma membrane where its main substrate, PI(4,5)P₂, is located. Activation of PI-3 kinase leads to generation not only of PI(3,4,5)P₃, but also of PI(3,4)P₂. Interestingly, both phosphoinositides have been shown to bind the PH domain of phospholipid dependent kinase (PDK1), which is required for PKB (Akt) activation (Alessi et al., 1997). PKB and PLCγ also contain PH domains and bind to the products of the PI-3 K activity (Franke et al., 1995). It has been suggested that PLCγ requires both PDGFR-mediated phosphorylation and PI-3 K product dependent translocation to the plasma membrane for its activation (Falasca et al., 1998). Although PI-3 K and PLCγ activates completely different signalling pathways, it appears that both enzymes
Chapter 1: Introduction

are highly interdependent. For example they share the same substrate - PI(4,5)P₂. However, the specificity of different PH domains towards distinct phosphotidylinosides, in vivo, need to be determined (Lemmon et al., 1996).

Downregulation of the PI-3 kinase signalling pathway is achieved by at least two PI(3,4,5)P₃ specific phosphatases - PTEN and SHIP (Cantley and Neel, 1999). Following activation of the PDGF receptor, a negative feedback loop is induced in order to switch off the signal. The switch is mediated not only by lipid phosphatases, but also by tyrosine phosphatases, such as Shp2. Shp2 (also known as Syp or PTP1D) has been shown specifically to dephosphorylate PI-3 kinase binding sites in the PDGF receptor itself (Klinghoffer and Kazlauskas, 1995).

1.2.1.3. Phorbol esters

Phorbol esters, such as TPA (PMA), were originally shown to have potent tumour promoting activity. Later it became apparent that TPA, as well as diacylglycerol analogues, activate protein kinase C (PKC) family kinases (for review see Jaken and Parker, 2000; Parekh et al., 2000). It is well established that, in vivo, PKC is activated through phospholipase C which generates two second messengers: inositol (1,4,5) trisphosphate (IP₃) and diacylglycerol (DAG). For this reason, TPA has long been used to specifically activate the signalling pathway of PKC which leads to the activation of MAP kinases and eventually transcriptional activation of genes, such as c-fos and egr-1 (Hill and Treisman, 1995; Harada et al., 1996; Price et al., 1996). It should be born in mind that PKC comprises a family of at least 10 related isoforms divided into 3 groups (for review, see Parekh et al., 2000). The first group, termed classical or conventional PKCs (cPKC) comprise PKCa, PKCβI, PKCβII and PKCγ. The second group are novel PKCs (nPKC) and includes PKCδ, PKCη and PKCθ. The third group, atypical PKCs (aPKC), consist of PKCζ and PKCλ. A major difference in the regulation of PKC isoymes is that only cPKCs and nPKCs are sensitive to phorbol ester or DAG activation (Newton, 1997). A PKC related kinase, PKD (PKCδ), with high affinity binding for phorbol esters has been recently cloned (Valverde et al., 1994; Van Lint et al., 1995). Both TPA and PDGF have been shown to increase PKD kinase activity in PKC dependent manner (Abedi et al., 1998; Van Lint et al., 1998).

Although TPA is used as a tool to activate PKC, it has been reported that some PKC unrelated proteins are also able to bind phorbol esters and DAG via their C1
domain (reviewed by Kazanietz, 2000). These proteins include: Ras guanyl1-releasing protein (RasGRP) (Ebinu et al., 1998), α-, β- and n-chimaerin (Ahmed et al., 1990); (Kozma et al., 1996) and C. elegans Unc-13 (Maruyama and Brenner, 1991). None of these proteins contain kinase domains and only the C1 domain, which binds phorbol ester, shares homology with PKC family members. The function of non-kinase phorbol ester receptors is poorly understood (Ron and Kazanietz, 1999). RasGRP, for example, has Ras nucleotide exchange activity and shares 50% homology with Ras exchange factor Sos. Chimaerins might be involved in regulation of Rac, since they possess RacGAP activity (Ahmed et al., 1993). Therefore, it is possible that TPA, in addition to PKC, can also target small GTPases, which in turn are involved in cytoskeletal rearrangements and SRF activation. Indeed, in some cell types TPA is able to activate Rac, however it remains unclear whether or not it is a PKC dependent process.

1.2.2. Signalling by MAP kinase cascades and transcriptional activation

There are at least four different groups of mammalian MAPKs that are regulated by distinct extracellular signals: (i) extracellular signal regulated kinases (ERK1/2); (ii) Jun-amino terminal kinases (JNK1/2/3); (iii) p38 family MAP kinases (p38α/β/γ/δ); and (iii) ERK5 (for references, see reviews Chang and Karin, 2001; Davis, 2000; Kyriakis and Avruch, 2001; Nebreda and Porras, 2000; Treisman, 1996). Because a number of groups work on the same proteins, some confusion has arisen in the nomenclature of these kinases (see Table 1.1 for clarification). One of the most characterised functions of MAPK signalling is regulation of gene expression in response to extracellular stimuli. At least 3 families of MAPK has been reported to phosphorylate TCF and therefore contribute to immediate early gene induction, such as c-fos (Janknecht and Hunter, 1997; Marais et al., 1993).

MAPK modules

MAP kinases are proline-directed kinases which phosphorylate sites containing a core consensus motif Ser/Thr-Pro (S/T-P). MAPK can be activated by a variety of signalling pathways, such as those involving receptor tyrosine kinases (PDGFR), G-protein-coupled receptors (LPA), or small G proteins through PAK (Cdc42/Rac) (English et al., 1999). Upon activation, MAPK can enter the nucleus and phosphorylate transcription factors directly as well as other effector kinases. It has
been proposed that duration and strength of ERK activation can lead to different biological outputs. For example, PC12 cells undergo differentiation when ERK activity is sustained and proliferation when only transient (Marshall, 1995). To prevent inappropriate biological responses ERK activity must be tightly regulated in a living cell. MAP kinase function in signalling cascades of at least three components, each activated in series by another. Each particular MAPK cascade can be said to comprise a module where MAPK are activated by dual specificity MAPK kinases (MAPKK or MEK), which are themselves activated by MAPKK kinases (MAPKKK or MEKK), (reviewed by Treisman, 1996). For example, the ERK1/2 module consist of Raf, MEK, and MAPK (ERK) respectively. Activity of ERK requires phosphorylation by upstream kinases, which are tightly regulated by signals themselves, usually by feedback loop mechanisms. Similarly, ERKs are targets of dual specificity phosphatases, the MAP kinase phosphatases (MKPs) (Sun et al., 1993). MKP-1 displays specificity for ERK rather than JNK and p38 kinase, and has very limited intracellular distribution. Moreover, MKP-1 (3CH134) is an immediate early gene and is induced by growth factors and serum (Sun et al., 1993), suggesting a mechanism for feedback inhibition. Recently, another ERK specific phosphatase, VHR, has been identified, suggesting that phosphatases play very important roles in regulation of MAPK (Todd et al., 1999).

ERK activity is also regulated by intracellular location. Extracellular stimuli that lead to ERK activation also cause it translocation to the nucleus. The exact mechanism of nuclear-cytoplasmic shuttling is not known, however it appears that both dimerisation and phosphorylation on phospho-acceptor sites of ERK (Thr185/Tyr187) are necessary for translocation and the eventual activation of transcription factors and gene expression (Khokhlatchev et al., 1998).

Another group of protein that helps to achieve specificity and regulation of the MAP kinase cascade are the so-called scaffolding proteins. Scaffold protein Ste5 was first discovered in budding yeast S. cerrevisiae. Ste5 binds Ste11, Ste7 and Fus3/Kss1 - components of pheromone mating-response MAP kinase module (reviewed by Whitmarsh and Davis, 1998). Recently, similar scaffold proteins JIP-1 (JNK interacting protein 1), JIP-2, JIP-3 and MP1 (MEK partner 1) has been discovered in mammalian cells (Kelkar et al., 2000; Schaeffer et al., 1998; Whitmarsh et al., 1998; Yasuda et al., 1999). JIP and MP1 scaffold proteins bind to JNK and ERK signalling modules, respectively. JIP proteins has been shown to form homo- and hetero-oligomers and could provide a mechanism by which combinatorial specificity of
regulation may be conferred by formation of distinct scaffold assemblies (Yasuda et al., 1999).

1.3. Transcriptional Activation

Numerous signalling pathway affect the state of the cell by regulating transcription of certain genes or groups of genes. Although conserved through evolution, transcription activation is more complex in a higher eukaryotes and involves multimeric complexes consisting of multimeric subunits. RNA polymerase II is controlled by general transcription factors, activators or repressors and coactivators or co-repressors. General transcription factors and polymerase binds to the core promoter DNA sequences which determines the initiation site of transcription. The specificity of the gene transcription is determined by cis-elements or enhancers which are bound by activators or repressors. Usually they are situated 5' of the core promoter and can be several or thousand base pair apart. These regulatory elements determine the rate of initiation or promoter strength. Later in the text, I will refer to transcription factors as coactivators or repressors (but not general transcription factors, which are not the main subject of the thesis) and regulatory promoter elements bind them.

1.3.1. Transcription factor regulation

Transcription factors can be divided into classes according to their structures. Usually, transcription factors consist of distinct functional domains: a DNA binding and a transactivation domain. One classification is based on homology between DNA binding domain which determines specificity of binding to distinct promoter elements. For example the bZIP (CREB/ATF), Ets domain (Elk-1/SAP-1), Rel homology domain (NF-κB) and novel DNA-binding domain (STAT) proteins. However, transcription factors might share other domains which relate them by function. Regulation of transcription factors occurs by certain principles, which can apply to several groups that are unrelated by sequence homology. Transcription factors can be regulated by phosphorylation, localisation, proteolysis or a combination of these. Another way to subdivide transcription factors is in accordance with their site of activation.
1.3.1.1. Activation in the nucleus

One of the best examples of signal transmission from the plasma membrane to the nucleus, is found with the activation of the receptor tyrosine kinase receptors. Ligand binding to the receptor allows the recruitment of Ras-Raf complex to the plasma membrane followed by sequential activation of the MAPK cascade and activation of ERK (see section 1.2.2). When activated, ERK itself enters the nucleus where it phosphorylates Elk-1 on multiple serine and threonine residues in the Elk transactivation domain. Elk binds constitutively to its cognate DNA binding elements, but is inactive until phosphorylation by ERK. Thereby only upon signal-induced phosphorylation transactivation can occur at the c-fos promoter (Cruzalegui et al., 1999; Marais et al., 1993). Likewise, agents that induce the JNK/SAPK cascade leads to phosphorylation of specific sites at c-JUN amino terminus, leading to the dephosphorylation in its carbox'1-terminus, both of which result in c-JUN activation and transcription of target genes (Papavassiliou et al., 1995). Another basic-leucine-zipper (bZip) domain protein, CREB, can be phosphorylated by a number of kinases that translocate to the nucleus, including PKA, CaMKIV, Rsk and MAPKAP-K2, (reviewed by De Cesare et al., 1999). It has been shown that phosphorylated CREB binds to the co-activator protein CBP/p300, allowing CREB to recruit general transcription factors. In addition, the intrinsic histone acetyltransferase activity (HAT) of CBP/p300 facilitates transcription by directly participating in acetylation and chromatin remodelling (for review see Kouzarides, 1999).

1.3.1.2. Activation in the plasma membrane

Smad proteins belong to a novel family of signal transducers that translocate from the cytoplasm into the nucleus and form transcription complexes at the specific DNA binding sites. Smads are activated by TGFβ receptor family kinases, which are subdivided into type I and II receptors, (for review see Massague and Wotton, 2000). In contrast to receptor tyrosine kinase, the TGFβ type I and II receptors have canonical serine/threonine protein kinase domain. Smads consist of MH1 (DNA binding) and MH2 (Smad 4 interaction and transcriptional activation) domains connected by a linker region. Upon receptor mediated phosphorylation of the c-terminus the Smads (Smad2/Smad3) form a complex with co-Smads (Smad4) which then translocates to the nucleus. Normally Smad4 is continuously shuttling between nucleus and
cytoplasm, however, TGFβ-induced signals trap it in the nucleus (Pierreux et al., 2000). Nuclear translocation is the key feature of Smad family transcription factors. It has been suggested that regulation of localisation of Smads can be achieved by several mechanisms, in addition to TGFβ mediated phosphorylation; for example, ERK mediated phosphorylation in the linker region inhibits Smad1 accumulation in the nucleus (Kretzschmar et al., 1997). Once in the nucleus, Smad complexes associate with other factors, such as Fast-1, and form ternary complexes that bind to specific transcriptional control elements, termed AREs (Liu et al., 1997). However, complexes formed by Smad3 and Smad4 can also bind to c-jun promoter independently of other transcription factors (Wong et al., 1999).

Another example of transcription factors that can be activated at the plasma membrane are the STATs. The unique feature of this family of transcription factors is that they have SH2 domain, in addition to DNA binding and other domains (for review see Horvath, 2000; Ihle, 2001). SH2 domains play a crucial role in STAT activation, allowing interactions with other signalling molecules at the plasma membrane. Following ligand binding to the receptor, STATs are phosphorylated by JAKs and form STAT dimers via intermolecular SH2-phosphotyrosine interactions, and then translocate to the nucleus. Differential activation of STATs by various signals results in differential DNA target specificity. The family of STATs consist of at least 10 family members, which can both homo- or hetero-dimerise, thus potentially resulting in multiple complex types with different DNA binding specificities. In addition, STATs can bind some transcriptional response elements (ISRE), as a heterotrimeric complex, consisting of STAT1, STAT2 and p48 (Horvath et al., 1996).

1.3.1.3. Activation in the cytoplasm

One of the best studied examples of a transcription factor activated in the cytoplasm is NF-κB (nuclear factor κB). NF κB is involved not only in immune responses, but also cell proliferation and apoptosis. NF-κB binds DNA as a dimer, however, dimers can be composed of five different DNA binding subunits: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), p65 (Rel A) c-Rel and RelB (for review see Perkins, 2000). All NF-κB members have a Rel homology domain (RHD), DNA binding and IκB interaction domain. When inactive, NF-κB is maintained in an inactive form in the cytoplasm by association with IκBs. Upon stimulation (pro-inflammatory cytokines, LPS, viral infection etc.) IκB is
phosphorylated by IKK (IkB kinase) and subsequently degraded by ubiquitination and proteolytic degradation. The released NF-κB then translocates to the nucleus and activates transcription through binding appropriate DNA elements. However, there is some evidence suggesting that NF-κB can be regulated by signals in a IkB-independent manner. For example, Akt has been shown to increase NF-κB transcriptional activity (Sizemore et al., 1999). In addition, NF-κB subunits can be phosphorylated themselves, therefore altering DNA binding and transactivation abilities (Wang and Baldwin, 1998).

1.4. Immediate Early Genes

Initial research concerning immediate early gene activation started in the late seventies and early eighties, when it became apparent from biochemical studies that de novo RNA and protein synthesis was required for DNA synthesis and that mitogen treatment could induce the expression of specific mRNAs and proteins not detectable in quiescent, nonproliferating cells (for review see Herschman, 1991; Rollins and Stiles, 1989; Winkles, 1998). In consideration of these findings, several laboratories set up screens to identify the induced genes required for DNA synthesis in response to serum and growth factors. The definition of immediate-early genes was first introduced by L. Lau and D. Nathans by analogy with viral genes, which do not require de novo protein synthesis for their expression (Lau and Nathans, 1985; Linzer and Nathans, 1983). Mitogen inducible genes are also referred to as competence or primary response genes, since they are prerequisite for induction of secondary signals which are required later in the cell cycle, first described by Smith and Stiles (Smith and Stiles, 1981). By the late 1980s around 100 different genes had been identified as growth factor inducible in mouse 3T3 cells. One of those identified were c-fos (Greenberg and Ziff, 1984), c-myc (Kelly et al., 1983), and actin (Riddle et al., 1979). Since the cDNAs of a number of growth factor inducible genes have now been cloned, the characterisation of expression and transcriptional activation has begun. One of the most extensively characterised immediate early genes is c-fos.

1.4.1. c-fos promoter as model of SRE containing immediate early genes
Many, but not all, immediate early gene promoters contain SRE-like sequences within their promoters, (reviewed by Winkles, 1998). The c-fos promoter has been studied extensively as a model growth factor-regulated promoter. Its upstream region also contains several regulatory sequences, which are also found in many other immediate early gene promoters (see below), (reviewed by Treisman, 1995). Other elements commonly found within immediate early gene promoters include SIE, CRE, AP1/ATF and Sp1 sites. The architecture of these promoters are represented in Figure 1.2.

1.4.1.1. c-fos SRE

The c-fos promoter SRE was identified as an essential regulatory element for the response of the c-fos promoter to serum. SRE sequences function largely independent of their position and orientation relative to other promoter elements (Treisman, 1985). The c-fos SRE contains a 20 basepair dyad symmetry and originally was termed DSE (Dyad Symmetry Element) (Treisman, 1986). All functional SREs contain the core sequence CC(A/T)G (CArG box), which in the case of c-fos is the part of more extensive consensus (for review see Treisman, 1990). However, not all SREs contain dyad symmetries, but many of immediate-early gene promoters share the CC(A/T)G consensus (see Table 1.2 and Figure 1.3). This sequence represents the core binding site for the ubiquitously expressed nuclear protein SRF (serum response factor) (Norman et al., 1988). The SRE alone can mediate enhanced transcription when linked to heterologous promoters (Konig et al., 1989). However, a number of different proteins, in addition to SRF, bind in the vicinity of the SRE in vivo (Herrera et al., 1989). Although it is clear that SRF plays a major role in signal transduction to the SRE, it is unlikely that SRF is solely responsible for transcriptional activation in the context of endogenous gene. At the c-fos promoter SRF forms a complex with a 62 KDa protein TCF (ternary complex factor) (Shaw et al., 1989). Consensus TCF binding site is also present in a number of immediate early gene promoters (see Figure 1.3).

1.4.1.2. SRF and TCF
Serum response factor (SRF) is a ubiquitous nuclear polypeptide of predicted Mr 51 KDa, but apparent Mr 62-67 KDa observed by SDS-PAGE (Fisch et al., 1987; Norman et al., 1988; Schroter et al., 1987). SRF gene is conserved amongst many species and is constitutively expressed, although its mRNA is inducible by serum in NIH3T3 and HeLa cells (Norman et al., 1988; Sotiropoulos et al., 1999; Spencer and Misra, 1996).

It is worth to note, however, that in band shift experiments, using extracts obtained from cell lines and tissues, binding activity mostly does not appear to change following growth factor stimulation (Greenberg et al., 1987; Treisman, 1986). However, extracts from A431 cells, do display substantial increase in SRE binding activity following EGF treatment (Prywes and Roeder, 1986). In vitro studies have shown that SRF is a substrate for CK II. Although, phosphorylation of SRF at the amino-terminal CK II site causes a large increase in the rate of SRF-DNA exchange, it has very little effect on equilibrium DNA-binding affinity (Janknecht et al., 1992; Marais et al., 1992). Moreover, mutation of CK II phosphorylation sites does not impair binding. Overall, the evidence suggests that SRF is constitutively bound to SREs in a non-regulated fashion.

Ternary complex factor (TCF) belongs to subfamily of Ets-domain proteins and binds in conjunction with SRF to the c-fos promoter (Treisman, 1994). Several TCF proteins have been described, such as Elk-1, SAP-1, and ERP-1/NET/SAP-2 (Dalton and Treisman, 1992; Giovane et al., 1994; Hipskind et al., 1991). The TCF protein was initially identified as an activity that, in band shift assays, caused further retardation of the SRE:SRF complex. Biochemical studies identified this as a 62 KDa polypeptide, with no apparent affinity for SRE DNA in the absence of SRF (Shaw et al., 1989). Although in following studies it was established that the A box (Ets DNA domain) and B box (SRF interaction domain) are both required for participation in the ternary complex with SRF (Dalton and Treisman, 1992; Hassler and Richmond, 2001; Hill et al., 1993; Janknecht and Nordheim, 1992).

1.4.1.3. Evidence for signal induced transcriptional activation

Initial work by several groups studying the c-fos promoter suggested that growth factor induced signals target SRE (Treisman, 1986). This idea is supported by the presence of SREs in the promoters of other immediate early genes (Winkles, 1998). Several other lines of evidence also supported this premise: introduction of
mutations into SREs prevented SRF binding in vitro; microinjection of SRE oligonucleotides competed for positive acting factor/s required for serum activation (Berkowitz et al., 1989); an SRE is sufficient to confer serum response on a heterologous promoter (Siegfried and Ziff, 1989), but heterologous promoters controlled by mutants SREs are refractory to serum induction. Regulation of the SRE inducibility by different factors is more complex and depends on accessory factors in a given cell type and promoter context used (Christy and Nathans, 1989; Graham and Gilman, 1991; Hill and Treisman, 1995; Shaw et al., 1989). Promoter constructs with a mutation in accessory factor binding sites still retains inducibility to serum in most cases (Graham and Gilman, 1991), however, in some cases activation is somewhat reduced (Shaw et al., 1989).

Activation of SREs by growth factors is very dependent on promoter context and cell type used. A study by Siegfried and Ziff demonstrated that an SRE driving a heterologous promoter is refractory to PDGF induction in both NIH3T3 and HeLa cells, while TPA activates SRE in HeLa, but not in NIH3T3 cells (Siegfried and Ziff, 1989). In the context of the c-fos promoter, the SRE is required for efficient promoter activation following serum, growth factors and TPA (Buscher et al., 1988). Serum inducibility appears to be more sensitive to the mutations at the SRF binding site compared to TPA or growth factors, possibly due to the other elements, that confers the inducibility similar to wild type levels the response to growth factors or TPA (Siegfried and Ziff, 1989; Hill and Treisman, 1995).

1.4.1.4. **Complexity of the c-fos promoter**

The c-fos promoter contain other regulatory sequences in addition to the SRE, such as the SIE (sis-inducible element) (Hayes et al., 1987). The SIE binds cytokine- and growth factor-regulated transcription factors of the STAT family (for review, see Bromberg, 2001; Ihle, 2001). Adjoining the SRE is a as yet poorly characterised binding site for the AP1/ATF family member (see Figure 1.4 for consensus binding sites between different species). Genomic footprinting studies indicate that both the SRE and AP1 sites are constitutively occupied in vivo, whereas SIE binding activity is regulated (Herrera et al., 1989; Konig, 1991). The role of AP1 site in the c-fos promoter is not completely resolved. Deletions of this AP1 site in c-fos reporter constructs has no effect on serum, EGF or TPA induction (Fisch et al., 1987). However, transgenic mice, carrying the AP1 deletion in an integrated fos-LacZ
displays no expression of the reporter in tissues constitutively expressing c-fos (Robertson et al., 1995). In the vicinity of the c-fos TATA box there is also CRE (cAMP response element) regulated upon activation of signalling pathways (reviewed by Shaywitz and Greenberg, 1999). CREB, and the related transcription factor ATF1, bind the CRE. Although CRE alone is not sufficient to mediate growth factor induction, mutation of CREB binding site in the context of the c-fos promoter partially reduced growth factor induction of c-fos transcription (Ginty et al., 1994). It appears, however, that the CRE substantially contributes for calcium-regulated activation of the c-fos promoter (Johnson et al., 1997).

1.4.1.5. Cooperative activity between promoter elements in the c-fos promoter

Understanding of the mechanisms by which extracellular stimuli induces changes in c-fos gene expression would allow us to further model the transcriptional activities of genes, or clusters of genes, involved in physiological responses such as proliferation and differentiation. Traditional approaches of promoter analysis, using in vitro transcription and transient transfection assays with chimaeric promoter constructs, have been key techniques in identification of the DNA elements that are required for transcriptional activation. However, demonstration that a given transcription control element is necessary and sufficient to confer the response to the specific signal, does not necessarily explain the role of this element in the context of the endogenous gene. Evidence from the study of many different promoters led to the development of the cooperativity model. The model proposed by (Robertson et al., 1995) suggested an 'interdependent transcription complex' model (ITC). According to this model most of the c-fos promoter would be occupied with transcription factors regardless of stimulus. For example, the core sequence of c-fos SRE would be occupied by SRF/TCF complex (Hill et al., 1995; Shaw et al., 1989). Upon stimulation a higher degree of assembly would be achieved, leading to an increase of transcriptional activation. It has been previously established that the c-fos promoter can be induced by two distinguishable signal pathways: (i) by MAPK cascade activation, which in turn phosphorylate and activates elk-1 (Gille et al., 1992; Marais et al., 1993); (ii) by a TCF independent pathway, potentiated by GTPases of the Rho family and requiring SRF (Hill et al., 1995). Signalling pathways leading to c-fos activation are summarised in Figure 1.5. Recent findings in the Treisman laboratory
demonstrated that both RhoA and LIM kinase are involved in regulation of the actin treadmilling cycle, and are crucial for SRF regulation (Sotiropoulos et al., 1999). The relative contribution of MAPK and Rho-actin pathways will be discussed in following sections.

1.4.16. Variability of results associated with transfected or integrated reporters

The differences observed between research groups, when assaying the sensitivity of SRE response to different pathways, could potentially be attributed to different transient transfection methods used. Only a few reports have employed reporter constructs that were stably integrated into genome (Alberts et al., 1998; Buscher et al., 1988; Rivera et al., 1990; Sotiropoulos et al., 1999). Although activation of both transfected and stably integrated c-fos reporters usually coincide, however the shut off mechanism of transcription is affected (Rivera et al., 1990). In addition, some differences in activation were observed due to clonal variation and the number of integrated copies, thus, transient transfection assays still remains the choice of experimental approach.

It was proposed more than thirty years ago, that chromatin context and histone modification may influence gene expression (Allfrey et al., 1964). More recently a series of reports suggested that not only acetylation, but also phosphorylation is involved in transcriptional regulation. Reports from D. Allis and colleagues suggests that histone H3 is a physiological relevant target of the MAPK effector, RSK-2 (Sassone-Corsi et al., 1999). However, Thomson at al suggested that another MAPK family kinase - MSK is responsible for H3 phosphorylation (Thomson et al., 1999). Nevertheless, these findings reinforce the idea, initially proposed by L. Mahadevan and colleagues, that H3 phosphorylation and immediate early gene activation are related events (Barratt et al., 1994; Mahadevan et al., 1991). Recently was shown that histone H3 is rapidly phosphorylated upon serum or TPA treatment (Clayton et al., 2000); moreover it has been demonstrated that acetylation and phosphorylation of H3 coincides to the same nuclei loci (Cheung et al., 2000). Recent development in ChIP (chromatin immunoprecipitation) technology have provided direct evidence that newly phosphorylated H3 is associated with induced c-fos and c-myc expression (Chadee et al., 1999).
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One of the explanations of the differences between transfected versus integrated could be recruitment of transcriptional activators with HAT activity to the c-fos promoter. Another possibility could be involvement of H3 phosphorylation and acetylation in release from the mRNA elongation block at the c-fos gene (Fivaz et al., 2000a; Fivaz et al., 2000b). This idea could be supported by evidence that following stimulation c-fos chromatin becomes more DNase I sensitive (Feng and Villeponteau, 1992).

1.4.2. Two types of SREs

Originally the SRE was characterised in the c-fos promoter. However, SREs are also found not only in other immediate early gene promoters, but also in promoters of cytoskeletal, and muscle specific genes (for references see Table 2). Unlike the c-fos SRE, some other SREs do not have binding sites for TCF (therefore are not likely form ternary complexes), but contain the consensus CArG box sequence CC(A/T)GG (see Figure 1.3).

1.4.3. Promoters lacking well defined TCF binding sites in their SREs

As mentioned previously, SRF regulates muscle specific genes, expressed specifically in post-mitotic muscle cells, therefore representing an expression pattern completely different to the growth regulated immediate early genes (Chang et al., 2001). The exact mechanism that enables SRF to distinguish between different type of target gene remains unclear. Several mechanisms have been reported to regulate SRF activity, such as positive and negative cofactors (Durocher et al., 1997; Sartorelli et al., 1990), phosphorylation dependent DNA binding (Manak et al., 1990), alternative RNA splicing (Belaguli et al., 1999; Kemp and Metcalfe, 2000), and nuclear translocation (Camoretti-Mercado et al., 2000). Most likely, muscle specific genes are regulated in a similar manner to those in fibroblast cell lines that are independent of the TCF pathway (Hill et al., 1995; Sotiropoulos et al., 1999). Several reports studying SRF activity in muscle cells reinforce the finding that RhoA activity and the actin polymerisation is both necessary and sufficient for activation of cytoskeletal genes in fibroblasts (Mack et al., 2001; Sotiropoulos et al., 1999).
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1.4.3.1. srf gene, its promoter and regulation

SRF is a ubiquitous expressed transcription factor and was originally identified as a critical component in mediating serum-induced activation of c-fos (Norman et al., 1988). It belongs to a family termed MADS box proteins, which have a very highly conserved 90 amino acid motif involved in DNA binding and dimerisation. MADS box proteins are highly conserved throughout evolution and can be found in eukaryotes from yeast to human. For example, Saccharomyces cerevisiae proteins MCM1 and ARG80 share 65% homology with SRF. Amino acid sequence comparison of different species suggests however, that the most divergent homologs of human SRF are found in X. laevis and zebrafish (Mohun et al., 1991; Vogel and Gerster, 1999). Invertebrate homologs of SRF, such as those found in drosophila (blistered, pruned) and brine shrimp Artemia franciscana apparently have completely divergent N termini, but still these proteins can bind consensus SREs and activate SRF target genes (Casero and Sastre, 2000; Casero and Sastre, 2001). Interestingly, the shrimp and drosophila SRF homologs share 95% identity and lack CKII phosphorylation sites which are present in the homologs of evolutionarily higher species. It is not clear however, whether these proteins have different physiological function.

As described in section 1.4.1, SRF binds to the promoters of a number of immediate early genes and in contributes to their activation in response to mitogen stimulation. Interestingly, SRF binds to the promoter of srf gene itself, since it contains two consensus CC(A/T)_{n}GG (CArG box) motifs in close proximity to the transcriptional start site. SRF gene is induced by serum and polypeptide growth factors (Misra et al., 1991; Pollock and Treisman, 1991). Although the srf gene promoter contains other elements, such as a putative Ets binding motif, SP1 sites and CCAAT box, deletion and mutation analysis revealed that only SRE1, SRE2 and Sp1 are important for inducibility of the SRF reporter by serum (Spencer and Misra, 1996), (see Figure 1.6). A contribution by the Ets domain remains unclear, since, for example, following bFGF stimulation CArG box and Ets motif deletions rendered srf promoter almost uninducible, while deletion of each alone only reduced promoter activity by 50% (Spencer et al., 1999). In this particular study Spencer and colleagues suggested that bFGF-mediated induction was mainly dependent on CArG and Ets motifs, even though the Sp1 binding site was also required for full activation.
1.4.3.2. Vinculin promoter

Although vinculin transcriptional activation has not been studied in great detail and the complete genomic sequence was not available until Human Genome project was released. The detailed analysis of promoter sequences suggests that it contains consensus SRF binding motif. The vinculin gene is inducible by serum and polypeptide growth factors (Ben-Ze'ev et al., 1990). Mutation of SRF binding site abolishes serum inducibility of a vinculin reporter construct (Gemma Smith, personal communication). Comparison of mouse and human vinculin promoters sequences revealed a very high homology in CArG box and in a region adjacent to it, in addition to five putative Sp1 binding sites (see Figure 1.7; courtesy of Celera Genomics and Human Genome Project). No consensus TCF binding site is found in mouse and human promoter sequences (Moiseyeva et al., 1993). It would be of interest to compare promoters of other species, however so far the sequences are unavailable.

1.4.4. SRE that binds TCF

1.4.4.1. egr promoter and its activation

One of the most characterised immediate-early genes, in addition to c-fos, is egr-1. egr-1 is a member of an evolutionarily conserved subfamily of zinc finger proteins. The characteristic feature of the egr subfamily is three zinc finger modules organised in tandem repeat. Interestingly, egr-1 promoter, intron-exon structure and coding sequence is highly conserved in all vertebrates including zebrafish, Xenopus, mouse and human (for review see Gashler and Sukhatme, 1995). However, no egr-1 homologous protein is found in non-vertebrates, suggesting that the Egr transcription factor has specific function in higher species. Indeed, egr-1 is implicated in development and differentiation, neuronal signalling and liver regeneration (Cao et al., 1990; Panitz et al., 1998). Paradoxically, egr-1 is induced by a number of different stimuli such as growth factors, pro-inflammatory cytokines, stress etc., and thus belongs to the immediate early gene family. So far three other family members have been identified in addition to egr-1 (egr-2/krox20, egr-3 and egr-4). Little is known about the function or regulation of these other egr genes, but some studies suggest that
*egr-2* displays similar mitogen inducibility patterns to that of *egr-1*, possibly explained by promoter similarities (see below).

**egr-1 cloning and promoter analysis**

*egr-1* was identified by a number of different groups in the mid eighties whilst searching for mitogen induced genes not requiring *de novo* synthesised protein. Therefore it is attributed a number of different names: NGFI-A was cloned as a nerve growth factor inducible transcript in PC12 cells (Milbrandt, 1987); *zif268* was identified in serum stimulated BALB/c3T3 cells (Lau and Nathans, 1987); TIS8 was cloned as a phorbol ester-inducible gene (Lim et al., 1987); and *krox24* was named after homology to the Drosophila factor Kruppel (Lemaire et al., 1988). It is not surprising that *egr-1* was identified in so many different screens, since later studies have demonstrated that it one the most highly inducible immediate early genes, and, at least for some stimuli, more inducible than *c-fos*. The high inducibility could be explained by an extraordinary promoter structure containing 5 SREs. Besides, it has AP1, Sp1 and Ets consensus binding motifs. It has been demonstrated that at least four of these SREs are functional when fused to a heterologous promoter (Christy and Nathans, 1989). Multiple SREs have been shown to cooperate in increasing transcription, supporting the notion that SREs cooperate in an endogenous gene context. Moreover, three of the SREs conserved in Xenopus, zebrafish, mouse, rat and human *egr-1*, suggesting that they play an important role in promoter regulation (see Figure 1.8). It is intriguing to note that these conserved SREs (SRE1, SRE4 and SRE5) all contain consensus Ets binding sites or have an Ets binding site within close proximity, similarly to the SREs in the *c-fos* promoter, which form a ternary complex (Shaw et al., 1989). Gel shift experiments have demonstrated that SRE4 and SRE5, both from mouse and Xenopus, can form a ternary complex with SRF and elk-1 (McMahon and Monroe, 1995; Panitz et al., 1998). Indeed, although all five SREs has been shown to confer induction when fused to a heterologous promoter, deletion analysis of the *egr-1* promoter implies that SRE1, SRE4 and SRE5 are most likely to mediate serum and TPA induced signalling (Clarkson et al., 1999; Mora-Garcia and Sakamoto, 2000). This notion is supported by the site selection studies for TCF binding (Treisman et al., 1992), since the *egr-1* SRE1, SRE4 and SRE5 contain the CGGAA sequence, increasing the chance of ternary complex formation. However, further characterisation of the SRE contribution is needed, since the deletion analysis studies of the *egr-1* promoter have been performed with different cell lines and using a variety of different stimuli.
1.5. Signals That Activate SRF

1.5.1. Small G proteins and GTPase cycle

Low molecular weight G proteins are well recognised as mediators of the cell growth and actin cytoskeletal rearrangement in mammalian cells. The discovery that extracellular signals regulate these proteins confirms their role in signal transduction pathways. Studies in the late eighties and early nineties focussed on the Ras family of GTP binding proteins and receptor tyrosine kinase activation of MAPK cascades. Later it became apparent that Ras is a member of large superfamily of proteins (so far around 60 members), which have been divided into several subgroups (Marshall, 1999).

GTPases of the Ras superfamily act as molecular switches to control a wide range of essential biochemical pathways in all eukaryotic cells. GTPases can exist in an inactive (GDP-bound) or active (GTP-bound) conformation. Guanine nucleotide exchange factors (GEFs) catalyse the release of GDP, allowing GTP to bind. GEFs are also known as GDIs (GDP dissociation stimulators), because of their ability to facilitate release of GDP from GTPases. Contrary, GDIs (GDP dissociation inhibitors), inhibit release of GDP. In their active, GTP-bound form, GTPases interact with their effector proteins. The intrinsic GTPase activity, catalyse further by GTPase activating proteins (GAPs), completes the cycle and the GTPase returns to its inactive GDP-bound state. (reviewed by Bar-Sagi and Hall, 2000).

1.5.1.1. Rho family

Rho was first identified in 1985 as a small GTPase binding protein related to Ras. It has been demonstrated that Rho is the target of the Clostridium botulinum exoenzyme C3 transferase which has been shown previously to induce changes to the morphology of tissue culture cells (Paterson et al., 1990). Subsequently, Rho, and similarly regulated protein Rac, were shown to be major regulators of the actin cytoskeleton in eukaryotic cells (Ridley et al., 1992).
Rho GTPases have been found in all eukaryotic species, from yeast and worms to mammals. Different mammalian RhoA GTPases are at least 40% identical to each other at the protein level. There are three highly homologous isoforms of Rho, known as RhoA, RhoB, and RhoC, which are over 85% identical. Similarly, Rac1, Rac2 and Rac1B/Rac3 are over 88% identical. The Cdc42 gene was initially identified as a cell cycle mutant defective in yeast budding (Johnson and Pringle, 1990). Alignment of Rho family GTPases with Ras shows conservation of amino acids critically required for nucleotide binding. However, Ras does not contain insert region, which is common for most Rho family GTPases.

1.5.1.2. Regulation and function of the Rho family GTPases

Rho family GTPases are involved in variety of biological responses, including cytoskeleton organisation, transformation and transcriptional regulation and can be activated by a number of growth factor cytokines and adhesion molecules. It is, therefore, important to understand the mechanisms that regulate the function of such crucial molecules. It has been established that the nucleotide state of GTPases is the key in regulation of their activity. A number of studies have tried to address the question of how the GTPases are regulated by their respective GEFs, GDIs and GAPs. The most favoured model is that the key regulators are GEFs, whereas GDIs and GAPs play more passive roles. However, some evidence supports the idea that in principle all three classes could be equally as important (Symons and Settleman, 2000).

GEFs

GEFs catalyse the conversion of Rho family GTPases from an inactive GDP-bound state to an active GTP-bound state. Many GEFs contain two specific domains: DH (DBL-homology) domain, required for catalytic activity, and PH domain, which has been implicated in diverse functions, including subcellular localisation. In addition, some exchange factor contains motifs found in other signalling molecules, such as SH2, SH3 (vav), PDZ, and similar to zing finger motif (lfc). It is assumed that the diversity of motifs defines specificity towards different GTPases. Most of the GEFs have oncogenic activity, and not surprisingly, a number these genes have cloned from oncogenically transformed cells. The best characterised GEFs are vav, sos and dbl. Specificity towards different GTPases determined biochemically. Unfortunately,
the in vitro data does not always correspond to the in vivo conditions. For example, vav was originally thought of as an exchange factor for Ras (Gulbins et al., 1994), but has since turned out to be a GEF for the Rho family. Although some GEFs act as exchange factors for several GTPases, usually they show specificity for the same subfamily, for example dbl (RhoA, Rac and Cdc42) (Olson et al., 1996). Several lines of evidence suggested that Ras and Rho GTPases are coordinately regulated at GEF levels via DH (dbl homology) and PH (plekstrin homology) domains. Bar-Sagi and colleagues suggested that Sos stimulates Ras activity, which in turn through the PI-3 kinase activates RacGEF vav, leading to Rac activation (Nimnual et al., 1998). In addition, recently src family kinases has been implicated in PDGF-induced vav activation ultimately leading to transcriptonal activation of the c-myc (Chiariello et al., 2001).

RhoGAPs

The Rho family proteins have high spontaneous GTPase activity when compared to Ras, however this activity can be increased further by RhoGAPs (GTPase activating proteins). All RhoGAP related proteins share a 140 amino acid motif, which is called the rhoGAP domain. The RhoGAP family is not as extensively studied as the RhoGEFs, although a number GTPase enhancing proteins has been described: p50rhoGAP, p180rhoGAP, p122rhoGAP, graf, N-Chimerin, Bcr, Abr, myr5 and others (Amano et al., 2000; Kaibuchi et al., 1999; Van Aelst and D'Souza-Schorey, 1997). Some GAPs, similarly to GEFs, contain other signalling modules domains, which are implicated in regulation of these proteins. Interestingly, some, like bcr have DH and PH motifs, which are the common feature of GEFs. So far little is known about GAP regulation. One of the mechanisms suggested is by targeting them to the plasma membrane, where Rho proteins interact with their effectors.

RhoGDIs

The first identified GDI was a ubiquitously expressed protein that interacted with GDP-bound Rho (Fukumoto et al., 1990). Later it was demonstrated that RhoGDI is also able to bind both Rac and Cdc42. Two other GDI proteins have been cloned, D4-GDI and GDI3. The Rho GDIs have been implicated in translocation of the GTPases between the cytosol membrane. It has been demonstrated that before rhoGEF can active Rho, GDI needs first to be released. GDI traps Rho in the cytosol in it in inactive state, however, following stimulation GDI is released, Rho translocates to the membrane where Rho is activated. Recently the model has been
suggested, by which members of the Ezrin/Moesin family might also be involved in activation of Rho, possibly by sequestering Rho GDI activity (Takahashi et al., 1997).

1.5.1.3. **Effectors of Rho family GTPases - regulation and function**

At least 30 downstream effectors of Rho, Rac, and Cdc42 have been identified so far, mostly using two hybrid screens and affinity chromatography (for review, see Bishop and Hall, 2000). Most of these interact with Rho family proteins through the so-called effector loop region. Analysis of single amino acid substitutions throughout this region have proved very useful to pinpoint specific effectors and functions for Rho family proteins (Joneson et al., 1996a; Joneson et al., 1996b; Lamarche et al., 1996; Sahai et al., 1998; Westwick et al., 1997). The list of effectors that bind Rho family proteins is presented in Table 1.3. Three major groups of Rho family effectors could be distinguished: serine-threonine kinases, lipid kinases (and lipases), and cellular scaffold proteins. In addition, it has been established that in the most cases Rho downstream targets are different to those of Rac/Cdc42 (see Table 1.3).

**Rho Targets**

Rho targets usually are classified into two classes, based on the sequence similarity of their Rho binding regions, although not all can be classified in this way. The first group has a REM motif (Rho effector motif) and, the second, a RKH motif (ROCK, kinectin homology region). It is not surprising, then, that effectors from different classes recognise different regions of Rho (Fujisawa et al., 1998). However, some of effectors, such as p140Dia or PLD do not have either of a REM or RKH motif, and consequently bind other regions of Rho (Bae et al., 1998).

**Rac/Cdc42 targets**

A number of Rac and Cdc42 targets are serine–threonine kinases with a CRIB motive (Cdc42/Rac interactive binding), such as PAK, MEKK, MLK, MRCK (for review see Bishop and Hall, 2000). PAK and MEKK have been implicated in regulation of the JNK family kinases, which in turn regulate gene transcription (Fanger et al., 1997b; Minden et al., 1994). Another group of effectors consist of scaffold proteins, such as WASP, N-WASP, POR-1, and Posh, which have been implicated in actin organisation. Many, but not all Rac/Cdc42 effectors are activated via their CRIB motive, which, while necessary, may not always be sufficient, as for
example with WASP. The mechanism of action of these proteins is not completely understood. Some of the effectors, like PAK, MRCK, MEKK are activated both by Cdc42 and Rac, and some are specific to a single GTPase, for example, WASP (for Cdc42) and Por1 (for Rac). However, further analyses are needed to define specificity of different effectors of in vivo.

1.5.1.4. Rho family GTPases and actin cytoskeleton

Two major targets of Rho, ROCK (Rho associate kinase) and mDia (diaphanous related formin homology domain protein) are required for Rho induced stress fibre formation and formation of focal adhesions. Activation of ROCK has been shown to be necessary, although not sufficient for stress fibre formation (Ishizaki et al., 1996; Ishizaki et al., 1997; Matsui et al., 1998). A specific pharmacological inhibitor of ROCK, Y27632, prevents stress fibre formation. mDia contributes to stress fibre formation by inducing actin polymerisation. A number of ROCK substrate have been described, but mDia targets yet poorly characterised (Watanabe et al., 1999). There is evidence that the actin binding protein, profilin, is involved in actin polymerisation through interaction with diaphanous proteins (Narumiya et al., 1997; Watanabe et al., 1997). It has been reported recently that LIM kinase is a downstream target of ROCK. LIM kinase has two LIM, PDZ and a kinase domain. LIM kinase phosphorylates cofilin at residue Ser3, and that reduces its binding affinity for the pointed end of actin, therefore promoting actin polymerisation (Agnew et al., 1995). The exact mechanism of LIM kinase activation is not known, although ROCK, as well as Rac effector, PAK1, phosphorylates LIM kinase, suggesting that Rho contribute to actin polymerisation via two pathways involving ROCK and mDia, respectively (Edwards et al., 1999; Maekawa et al., 1999; Ohashi et al., 2000).

1.5.1.5. Rho and transcriptional activation

It has been well established that the Rho family GTPases are involved in transcriptional regulation. Constitutively active forms of Rho are able to activate JNK family kinases, and hence downstream targets transcription factors, such as c-jun, ATF2 and SAP-1 are activated (Coso et al., 1995; Janknecht and Hunter, 1997). A number of downstream targets of GTPases have been reported to activate JNK,
including MEKK1 (MEKK4), MLK3, and PAK (for review, see Fanger et al., 1997a). It has been well established that Rho family GTPases activates SRF (Hill et al., 1995). It has been demonstrated that Rho A is both necessary and sufficient to activate the transfected SRF reporter gene. However A.Alberts and colleagues have shown that RhoA is not sufficient to activate an integrated SRF reporter gene, which additionally requires signals, capable to induce histone acetylation (Alberts et al., 1998) (see section 1.4.1.6). In addition, Rho family GTPases have been implicated in activation of NF-κB transcription factors. It has been shown that Rho proteins mediate phosphorylation of IκB, allowing NF-κB to translocate to the nucleus (Perona et al., 1997; Sulciner et al., 1996).

1.5.2. The Actin cycle

The cytoskeletal organisation of actin filaments, has a well recognised role in maintaining the structure of cells. However, it is beginning to emerge that cytoskeletal organisation is a very complex and dynamic process, with an influence on almost every function of the cell. Regulation of actin polymerisation is a key event in allowing actin structures to form and/or be reorganised, and thus, a number of proteins are involved in this regulation (for review and references see Pollard et al., 2000). The simplest model proposed involves dynamic cycling between two forms of actin in the cell, monomeric (G-actin) and filamentous (F-actin). Monomeric actin subunits (ATP-bound) can polymerise from one end of the filament (barbed end), allowing the filament to grow (see Figure 1.9). Conversely, ADP-bound subunits can dissociate from the other end (pointed end), allowing filament to disassemble. The barbed end is also called the fast growing end, and pointed end, the slow growing end (Chen et al., 2000). Regulation of F-actin formation can be achieved in at least in two ways: regulation of the activity of severing proteins, such as cofilin and gelsolin, and binding of stabilising proteins, such as tropomyosin. Rho family protein-induced regulation of actin stability can be explained by phosphorylation of cofilin. It has been reported that ROCK and PAK kinases activates LIM kinase, which in turn inactivates cofilin by phosphorylation (Edwards et al., 1999; Maekawa et al., 1999). When phosphorylated cofilin is displaced from actin monomers allowing them polymerise from the barbed end (Bamburg et al., 1999; Pollard et al., 2000). The severing of actin filaments by gelsolin can be regulated by extracellular stimuli, that modulate free calcium ion concentration, since gelsolin activity is calcium dependent. Regulation of gelsolin
activity by LPA has been reported, suggesting that serpentine receptors can modulate actin polymerisation (Meerschaert et al., 1998).

In addition to stabilising filamentous actin, Cdc42 and Rac family GTPases have been implicated in initiation of new filament formation through the Arp2/3 complex (Machesky, 1997). Biochemical and electronmicroscopic data suggest that Arp2/3 complex localises to regions of lamellipodial protrusions and nucleates new branches of actin filaments (Machesky et al., 1997; Svitkina and Borisy, 1999). Consequently, it has been demonstrated that N-WASP and Scar1 promote Arp2/3 nucleation activity (Machesky and Insall, 1998; for review, see Machesky and Gould, 1999; Machesky and Insall, 1999). Moreover, the WASP family protein family Ena/VASP has been demonstrated to recruit actin monomers and promote filament elongation by bridging monomers and filaments. Ena/VASP proteins contain the conserved amino-terminal domain EVH1 (Ena/VASP homology) and proline-rich sequences that bind to profilin and to SH3 domains (Machesky and Schliwa, 2000; Prehoda et al., 1999; Symons et al., 1996). Based on these and other findings, the following model for signal induced regulation of actin polymerisation was proposed. Growth factor activation of receptor tyrosine kinases creates phosphorylation sites for docking proteins like grb2 and NCK, which then recruit WASP (or WASP family members) to the plasma membrane, which in turn activates the Arp2/3 complex and promote actin polymerisation. In addition, the relative activities of capping proteins are also regulated by signalling intermediates. The rate that knew filament elongation depends on Ena/VASP proteins and profilin. Established filaments are severed and depolymerised by cofilin. Conversely, positive signals leading to LIM kinase activation inactivate cofilin's severing ability, hence promoting polymerisation. (reviewed by Machesky, 2000; Machesky and Schliwa, 2000); (see Figure 1.9).

1.5.2.1. Actin binding drugs

Jasplakinolide

Jasplakinolide is a cyclic peptide from marine sponge Jaspis johnstoni (Bubb et al., 1994). It has been demonstrated that Jasplakinolide stabilises filamentous actin in vitro, however in vivo data suggests that Jasplakinolide disrupts actin filaments and induces actin polymerisation in the amorphous phase (Bubb et al., 2000). This apparent paradox could be explained by the fact that Jasplakinolide induces nucleation of actin filaments, thus, resulting in an induction of actin monomer (G-actin).
polymerisation. It has been suggested that the disrupting effects on stress fibres and depletion of G-actin could happen by a two step mechanism: first, inducing the release of actin from its sequestering protein (β-thymosin or others), second, by nucleation of filament assembly, leading to a state where the levels of G-actin is limiting to maintain normal stress fibre turnover. Other reports support the evidence that Jasplakinolide leads to depletion of G-actin in a time and concentration dependent manner. The amount of the drug needed to titrate G-actin depends on the cell type used or state of the cell. For instance, serum starved cells are more sensitive to actin binding drugs than growing ones. Indeed, it is recognised, that serum starved fibroblasts have reduced levels of stress fibres, therefore, possibly containing a larger pre-existing pool of polymerisation-competent actin. However, the assessment of polymerisation-competent actin levels is difficult due to its distribution, nucleotide content, posttranslational modifications and/or sequestration by actin binding proteins, such as β-thymosin and profilin (Cao et al., 1993; Goldschmidt-Clermont et al., 1992).

Latrunculins

Latrunculin was isolated from Red Sea sponge Necombata magnifica, and identified as an inhibitor of actin polymerisation (Spector et al., 1983). Latrunculin binds to monomeric actin with a ratio 1:1, with a dissociation constant 0.2 μM (Coue et al., 1987). Analysis of the crystal structure has revealed that latrunculin binds to the nucleotide binding cleft of actin (Morton et al., 2000), and therefore affects the nucleotide exchange rate of actin (Ayscough et al., 1997). Latrunculin has been reported to lower β-thymosin binding to actin by 10 fold, without affecting the complex of profilin and actin in vitro. In addition, latrunculin did not affect binding of DNAs which is otherwise used as a measure of G-actin in cell culture experiments (Yarmola et al., 2000). However, the in vivo effects of latrunculin binding to actin, with respect to other actin binding proteins, remains poorly understood. Both Latrunculin A and Latrunculin B have similar effects on the actin cytoskeleton, however, they act with slightly different binding affinities.

Cytochalasin D

Although Cytochalasin D have been used for a number of years as an agent capable of disrupting the actin cytoskeleton, the reports concerning Cytochalasin D binding to actin in vivo are very limiting (for review see Cooper, 1987). Cytocha lasin D binds to the barbed end of actin filaments, inhibiting both association and dissociation of subunits at the end. Affinity of Cytochalasin D for the barbed ends
is high ($K_d = 2nM$), which is by 3 orders of magnitude lower when usually used in the experimental systems ($K_d = 2\mu M$). Capping barbed actin filaments is the only known function of Cytochalasin D in vivo. In vitro, however, Cytochalasin D has been reported to bind both actin monomers and dimers as well as promoting ATP hydrolysis (Brenner and Korn, 1981). Some reports suggest that cytochalasins might bind to the a subunit in the interior of an actin filament and thus break the filament suggesting that Cytochalasin D possesses severing activities (Hartwig and Stossel, 1979). Despite the complexity of Cytochalasin D action, no reports suggest that it binds other molecules then actin. It is clear that Cytochalasin D disrupts supramolecular organisation of actin filaments, but not necessarily depolymerise actin filaments like latrunculins.

**Swinholide A**

Swinholide A, another actin binding drug, has also been discovered whilst searching for marine natural products with biological activity. Swinholide A (isolated from marine sponge *Theonella swinhoei*) is a 44-carbon ring dimeric dilactone macrolide. This drug is highly cytotoxic, although the mechanism of cytotoxicity is unknown (Bubb *et al.*, 1995). Swinholide A binds co-operatively to two actin subunits with a $K_a \times 10^{-13} M^2$. In addition to sequestering non-polymerised actin subunits, Swinholide A increases the number of filament ends by severing F-actin (Bubb and Spector, 1998). Swinholide A is a very powerful disrupter of the actin cytoskeleton: a concentration of 10nM is sufficient to cause substantial changes to cytoskeletal arrangements within 30 minutes. In contrast to cytochalasins, the effects of Swinholide A on actin are very specific, and appears to only sequester actin as a dimers and sever F-actin. Thus, this is a promising reagent for use in the study of actin dynamics and cytoskeletal rearrangements in the future (Bubb and Spector, 1998).

### 1.5.2.2. The actin cycle and SRF activation

It has been previously demonstrated that Rho A activity is absolutely necessary for serum- and LPA-induced SRF activation in transfection assays (Hill *et al.*, 1995). Subsequently, it has been demonstrated that Rho signalling impinges upon the actin treadmill cycle via two downstream effectors, mDia1 and ROCK. Like Rho, mDia is very a strong activator of SRF (Sotiropoulos *et al.*, 1999; Tominaga *et al.*, 2000). The
exact mechanism by which this occurs is not clear, however, it has been demonstrated that Dia1 can interact with the actin binding protein profilin (Watanabe et al., 1999). Another powerful activator of SRF, LIMK has been identified in a screen looking for potential activators (Sotiropoulos et al., 1999).

**LIMK activation of SRF**

Several lines of evidence suggest that LIMK activate SRF through the actin pathway. First, the actin binding protein cofilin is the only known physiological target of LIMK. Second, mutation of phosphorylation site Ser3 of cofilin inhibit SRF activation. Third, the actin binding drug latrunculin blocks SRF activation by LIMK (Sotiropoulos et al., 1999). It has been reported that LIMK activity can be modulated by phosphorylation of Thr508, in its activation loop, by ROCK (Ohashi et al., 2000) or by PAK (Edwards et al., 1999). This suggests that both RhoA and Rac could contribute to regulation of LIM kinase activity through their downstream effectors, and thereby activate SRF. The relative contribution of each GTPase in SRF activation would be dependent on different signalling pathways and/or different cellular context.

**Activation of SRF by Dia**

Interestingly, in NIH 3T3 cells, a constitutively active form of mDia1 strongly activates the SRF reporter gene (Copeland and Treisman, 2001). In contrast, another effector of RhoA, ROCK, is only a very weak activator of SRF, even though the constitutively active ROCKA3 is C3 sensitive. In addition, the specific Rho inhibitor, Y27632, does not significantly inhibit the SRF reporter activation after serum stimulation (Sahai et al., 1999). These data strongly suggest that Dia is the main downstream effector of RhoA leading to SRF activation in NIH 3T3 cells. This is consistent with the fact that dominant negative form of LIMK (which is regulated by ROCK) does not substantially inhibit SRF activation in NIH3T3c cells (Sotiropoulos et al., 1999). Conversely, in PC12 cells LIM kinase is a major effector of Rho GTPases leading to SRF activation, since cofilin S3A is able to inhibit signals activating SRF (Geneste et al., 2001).

**Actin binding drugs and SRF activation**

It has been reported previously that latrunculin has an adverse effect on expression of certain cytoskeletal genes, such as vinculin and actin (Ben-Ze'ev et al., 1990; Bershadsky et al., 1995; Lyubimova et al., 1999). It has also been shown that
phalloidin treatment changes the levels of actin (Serpinskaya et al., 1990). The promoters of these genes have subsequently been shown to contain consensus SRF binding sites (Mohun et al., 1987; Moiseyeva et al., 1993). Activation of an SRF reporter gene by the actin binding drug Cytochalasin D has been demonstrated in the Treisman laboratory. Interestingly, Cytochalasin D action was demonstrated to be independent (downstream) of RhoA (C. Hill and R. Treisman, personal communication ; Sotiropoulos et al., 1999 ).

1.6. Chemical Compounds – Advantages and Limitations

Protein kinase inhibitor drugs have been extensively used both, in vitro kinase assays, and in tissue culture-based assays. They have proved to be very powerful tools in analysing protein kinases and their substrates involved in various signalling pathways. Some inhibitors, following intensive and rigorous biochemical tests, were tested in clinical trials as a therapeutic agents in the treatment of disease, such as cancer (Laird et al., 2000). Most inhibitors were designed to block kinase activity by directly preventing entry to the ATP binding site of various protein kinases. Although hundreds of inhibitors are available, most are broad spectrum inhibitors, and only a few, such as PD98059, U0126, Y27623 and others, provide specificity to kinase subfamilies (Davies et al., 2000); ( for review, see Cohen, 1999).

1.6.1. Inhibitors of the Ras–ERK pathway

PD098059 was originally identified as a non-competitive inhibitor of MEK, a intrinsic kinase in the Ras-ERK pathway. It has no effect on upstream activator c-Raf, nor on the ERK enzymatic activity in vitro. It has been suggested by D.Alessi and colleagues that, by binding to MEK, PD 98059 blocks it's ability to be activated by c-Raf (Alessi et al., 1995). Consequently, the signals that activate MAPK through c-Raf and MEK are blocked by pretreatment with PD98059. Such as is the case for signalling by growth factor, G-protein coupled receptors, N-formyl peptide receptors, the T cell receptor complex and others.

Another MAPK signalling pathway inhibitor, U0126, has recently been identified (Favata et al., 1998). U0126 inhibits activation of MEK, but not other
related kinases, such as MEKK, ERK, JNK, MKK4 or MKK6 (Davies et al., 2000). U0126 exerts very similar blocking effects compared to when used in tissue culture. Although it was initially thought that U0126 and PD098059 inhibit MEK by different mechanisms, recent data suggests that both act in a similar manner, possibly even binding to the same site of MEK (Davies et al., 2000). It has been demonstrated, however, that U0126 has proved to be a more potent inhibitor than PD098059. For example, IC$_{50}$ for MEK in Swiss3T3 cells is 70nM concentration, which is almost two orders of magnitude lower than that for PD98059 (Davies et al., 2000). Nevertheless, although PD98059 is a less potent inhibitor, it appears to be somewhat more specific then U0126. All recent reports suggest that both MEK inhibitors are specific in blocking Ras-MAPK signalling pathway. When used in parallel, PD098059 and U0126 complement each other, therefore are very useful tools for inhibiting MAPK signalling pathway.

1.6.2. PI-3 kinase inhibitors

The two most common used PI-3 kinase inhibitors are Wortmannin and LY294002. Wortmannin is a natural compound and was originally discovered as a substance from *Penicillium wortmannii* capable of inhibiting the respiratory burst in neutrophils (Baggioni et al., 1987). LY294002 is a synthetic compound based on the structure of fvdvanoid (Vlahos et al., 1994). Although LY294002 is approximately 500 fold less potent than Wortmannin, it is more commonly used due to its higher specificity for PI-3 kinases and its stability in aqueous solution. Recently crystal structures of both inhibitors binding to PI-3K have been determined. Both LY294002 and Wortmannin are competitive inhibitors of ATP binding. Wortmannin, however, is an irreversible inhibitor and LY294002 is not. Wortmannin covalently and irreversibly modifies Lys833 in the ATP binding pocket of PI-3 kinase, while LY294002 only makes hydrogen bonds with Lys833, through its morpholino ring (Walker et al., 2000). Both inhibitors are thought to be relatively specific for the PI-3 kinase family. However, inhibition of CKII by LY294002 has been recently reported (Davies et al., 2000). Results, obtained using PI-3K inhibitors must be interpreted with caution, since it has been shown that it can inhibit other phospholipid kinases, (reviewed by Fruman et al., 1998; Toker, 2000).
1.6.3. PKC inhibitors

Ro31880 has been used as a specific inhibitor of conventional protein kinase C (PKCα, PKCβ and PKCγ), although has no effect on atypical PKCs (ε and ζ). However, recent reports by Alessi et al. has shown that this compound also inhibits a number of other kinase in vitro (Alessi, 1997). This recent study compared six bisindolylmaleimide (BIS) derivatives, in addition to Ro31880 and Go6976, in inhibition of different kinases in vitro. Most of the BIS derivatives strongly inhibited four unrelated kinases: MAPKAPK1/RSK, MSK1, S6 kinase and GSK3β. No significant inhibition was observed towards MAPK, JNK or p38 family kinases (Davies et al., 2000). These data support the notion that, although inhibitors may display specificity towards certain specific to certain family members within a large family of kinases, they can still potentially inhibit kinases of unrelated types. Thus, experiments using PKC inhibitors should be rigorously controlled for specificity and results interpreted with caution.

1.6.4. ROCK inhibitor

The Y27632 compound, containing a pyridine moiety, has been shown to have an effect on smooth muscle contractility and to inhibit the RhoA downstream effector, ROCK (Uehata et al., 1997). Photo-crosslinking studies confirm that ROCK is a molecular target for Y27632. Y27632, and related compound Y32885, inhibit the Rho-kinase as a competitive inhibitor of ATP binding. Specificity of Y27632 towards different kinases has been determined in vitro (Davies et al., 2000). This study revealed that Y27632 is a very specific inhibitor, with a single exception. In vitro kinase data showed that PRK2 kinase activity was inhibited as strongly as that of ROCK (IC50=600nM and 800nM, respectively). The specificity of the ROCK inhibitor toward these kinases in vivo remains to be determined.

Although discovered just four years ago, Y27632 has proved very useful in many different studies. The role of ROCK in transformation and transcriptional regulation has been analysed (Sahai et al., 1999). It has also contributed to the identification and characterisation of ROCK downstream targets, such as LIM kinase, adductin, ERM family proteins, and MLC (Maekawa et al., 1999; Matsui et al., 1998; Ohashi et al., 2000; Tran Quang et al., 2000).

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1.7. Bacterial Toxins and Their Effects

A large number of bacterial toxins are known to cause covalent modifications to eukaryotic GTPases, resulting in drastic changes to their biological and biochemical properties (reviewed by Aktories, 1997; Busch and Aktories, 2000).

1.7.1. Toxins inhibiting Rho family GTPases

Clostridium botulinum C3 ADP-ribosyltransferase is the prototype of a family of C3-like transferases. C3 was originally isolated during screening for high producer strains of Clostridium botulinum C2 toxin, a transferase that ADP-ribosylates actin. The various isoforms of the C3 transferase have been described. The C3-related transferases produced by Clostridium limosum, Staphylococcus aureus and Bacillus cereus shares a high degree of homology (Just et al., 1992).

All C3-like transferases ribosylate the three closely related isoforms of Rho (RhoA, RhoB, and RhoC), while other members of the Rho family are poor substrates. By ribosylating at residue Asn41, C3 renders Rho protein biologically inactive, without affecting GTP or GDP binding, or intrinsic GAP-stimulated GTPase activity (Aktories, 1997).

The primary effect of C3 on cells is the loss of actin stress fibres and integrin adhesion plaques, while actin dependent membrane ruffling and filopodia, which are regulated by Rac and Cdc42, respectively, are unaffected (Ridley et al., 1992). Due to the high specificity of C3 to Rho family proteins, this inhibitor is widely used as a tool to study Rho function. Although C3 can be taken up by mammalian cells through non-specific mechanisms, very high concentrations (more than 10 mg/ml) and long incubation times (24-48 hours) are required for C3 effects to be visualised. Thus this technique is very rarely used. More commonly, expression constructs are transfected or microinjected. More recently a chimaeric C3 toxin has been constructed, consisting of the C3 fused to an active component of the binary C2 toxin (Barth et al., 1998b). Using the latter method of delivery, C3 is active in cells within 3-5 hours at concentrations bellow 1 µg/ml (Barth et al., 1998a).

1.7.2. C2 Toxin inhibits actin polymerisation.
Chapter 1: Introduction

The C2 toxin consists of two parts. The enzymatic component C2I, an ADP-ribosyltransferase that modifies G-actin, and the binding component C2II, which mediates cell entry of the toxin (Aktories et al., 1986; Barth et al., 1998b). The assembly of both components is required at the surface of the cell for toxin activity (Eckhardt et al., 2000). Subsequent to assembly, the C2II and C2I complex is taken up by receptor-mediated endocytosis and is released in the cytoplasm where it ADP-ribosylates actin at R177, causing inhibition of actin polymerisation. In addition, ADP-ribosylated actin acts like a capping protein to block polymerisation of any unmodified actin at the barbed end of F-actin (Wegner and Aktories, 1988) or actin-gelsolin complexes (Wille et al., 1992). This sequence of events eventually leads to depolymerisation of actin filaments, a breaking down of the actin cytoskeleton and rounding up of cells.

1.7.3. Large clostridial cytotoxins

The prototypes of this toxin family are Clostridium difficile toxins A and B, which are the major virulence factors involved in diarrhoea and pseudomembranous colitis. Other members of the family include lethal and haemorrhagic toxins from Clostridium sordelli and the α-toxin from Clostridium novyi. All clostridium toxins are cytotoxic and cause destruction of the actin cytoskeleton evidently by 'rounding up' of affected cells. The underlying mechanism of the toxin's induced cytotoxic effects involves glucosylation of the members of Rho family GTPases.

Clostridium difficile toxins A and B catalyse the monoglucosylation of the Rho GTPases, using UDP-glucose as a co-substrate (Just et al., 1995a; Just et al., 1995b). All Rho subfamily members, including Rho, Rac, and Cdc42, are substrates for monoglucosylation by toxins A and B. The lethal toxin from Clostridium sordelli, glucosylates Rac and Cdc42, but not Rho, however Ras family proteins, including Ras, Rap, and Ral, are also substrates of this toxin. All Clostridium toxins modify Rho at Thr37 (Rac and Cdc42 at Thr35). Toxin B (or toxin A)-induced glucosylation of Rho results in a decrease in the affinity to nucleotides, and inhibits p50rhoGAP-stimulated GTPase activity, therefore preventing activation of downstream effectors (Sehr et al., 1998). Treatment of tissue culture cells toxin A and B results in complete loss of stress fibres, the subcortical actin ring, lamellipodia, and filopodia, leading to rounding up of the cells and eventually complete breakdown of the actin cytoskeleton (Ottlinger and Lin, 1988).
1.7.4. CNF1 and CNF2 activate the Rho family GTPases

CNF1 (cytotoxic necrosis factor) was originally isolated from *E.Coli* found in patients with enteritis. CNF1 treatment of cells leads to striking morphological changes, such as transient filopodia and membrane ruffles. It has been established that Rho GTPases are the targets of both CNF1 and CNF2. Biochemical studies have revealed that CNF1 causes deamidation of Gln63, which is essential for both the intrinsic and GAP-stimulated GTPase activity of RhoA (Schmidt *et al*., 1997). Recent findings suggest that Rac and Cdc42 are also targets of CNF1, resulting in deamidation of Gln61 (Lerm *et al*., 1999a; Lerm *et al*., 1999b). It is tempting to speculate that treatment of cells with CNF toxins, resulting in RhoA activation, would be sufficient to activate SRF and result in transcription of SRF target genes.
Figure 1.1. Activation of signalling proteins in response to RTK activation. (i) Activation by tyrosine phosphorylation. Binding of SH2 domains of PLCγ to pTyr sites facilitates tyrosine phosphorylation of PLCγ. This is mediated in part by binding of the PH domain to PIP3. Activation of PLCγ leads to hydrolysis of PIP2 and generation of two second messengers. (ii) Activation by conformational change. Binding of the SH2 domains of p85, the regulatory subunit of PI-3K to pTyr sites on activated receptor stimulates catalytic domain (p110) and generates PIP3. (iii) Activation of Akt by membrane translocation. PIP3 generated by PI-3K serves as a binding site for PH domains of Akt and PDK1. Membrane translocation is accompanied by release of autoinhibition leading to activation of PDK1 and Akt. Full activation of Akt requires phosphorylation by PDK1.
Figure 1.2. SRF target gene promoters. Many of the promoters, which have SRF binding sites, including *c-fos* contain Ets, AP1 and CRE binding motives. The response elements are represented by colour coded circles. The promoter structures represented are based on mouse sequence (except vinculin). The *c-fos* SIE promoter element is shown in purple.
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**Figure 1.3. Conservation between human and mouse CArG and ETS motifs in SRF target genes.** Mouse and human CArG and Ets motifs from the promoters of selected SRF target genes are aligned; mouse sequences are represented in black, human in blue, respectively. Consensus CArG and Ets motifs are represented in red. Mismatches are shown in lower case letters.
Figure 1.4. Alignment of c-fos SRE and its flanking sequences. The stretch of the c-fos promoter from different species is shown: Ets binding motif (red); CArG box (green) and AP1/ATF binding site (yellow).
Figure 1.5. Signalling pathways leading to the c-fos promoter. Many transcription factors bind the c-fos promoter including STAT (grey); Elk-1, which is a member of the TCF family (red); SRF (green); AP1/ATF (yellow) and CREB (light grey). A number of stimuli activate multiple transcription factors via a range of signalling pathways, including JAK-STAT, MAPK and Rho-actin mediated pathways. The c-fos SRE can be activated by two distinguishable signal pathways: (i) by MAPK cascade activation via TCF, and (ii) by Rho-actin pathway via SRF.
Figure 1.6. Alignment of *srf* promoters. Alignment between human and mouse *srf* promoter sequences are shown; human sequences are represented in blue, mouse in black, respectively. SREs are represented in green boxes; Sp1 binding motif in grey; putative Ets binding motif in red. Mismatches are shown in capital letters.
Figure 1.7. Alignment of vinculin promoters. Alignment between human and mouse vinculin promoter sequences are shown: SRE (green), SP1 binding motifs (grey) and AP1 binding site (yellow). Human sequences are represented in blue, human in blue, respectively. Mismatches are shown in capital letters.
Figure 1.8. Alignment of egr-1 promoters. A. Schematic comparison between different species. B. Alignment between human and mouse sequences. Human sequences are represented in blue, mouse in black, respectively. Mismatches are shown in capital letters.
Figure 1.9. Regulation of the actin treadmilling cycle by GTPases, PAK, and LIMK. ATP-bound actin monomers can polymerise at the barbed end of actin filaments. ATP hydrolysis occurs when the actin is polymerised and ADP-bound actin can depolymerise from the pointed end. Multiple mechanisms exist to regulate polymerisation of actin (see the section 1.7.4). Cofilin can bind the pointed end of actin filaments and promote depolimerisation. Phosphorylation of cofilin by LIMK blocks its ability to promote actin depolimerisation. Profilin acts as an exchange factor for ADP-bound G-actin, which can polymerise at the barbed end. Extracellular signals regulate the system via control of Rho GTPase and LIMK activity.
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Table 1.1 Nomenclature of mammalian MAPK pathway components.
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<td>c-fos</td>
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Table 1.2. Immediate early genes containing SRE-like regulatory sequences
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**Table 1.3 Potential effector proteins for Rho, Rac and Cdc42**
2. Activation of the SRF-dependent Reporters

2.1. Chapter Summary

In this chapter transcriptional activation of the SRF-dependent reporter gene was examined following activation by different extracellular stimuli. Comparative analyses of both integrated and extrachromosomal (transfected or microinjected) templates are presented. A variety of methods have been used to test the inducibility of the SRF reporter gene, including lipofectamine transfection followed by RNase protection assay, luciferase assay, CAT assay or western blotting. In addition, the microinjection technique was used followed by indirect immunofluorescence. The data presented reveals that only foetal calf serum (serum) and LPA stimulation activate the SRF reporter gene irrespective of its extrachromosomal or integrated state. In contrast, following PDGF and TPA stimulation, the SRF-controlled reporter gene is efficiently activated only in stable (integrated) transformants.

2.2. Induction of the Integrated Reporter

2.2.1. Activation of the integrated SRF reporter measured at the RNA Level

It has been previously reported that an SRF-dependent reporter can be activated by serum and LPA, and is refractory to receptor tyrosine kinases and phorbol esters (Hill et al., 1995). It has also been shown that the small GTPase, RhoA, is necessary and sufficient to activate SRF-controlled reporter genes. I decided to establish whether a chromosomally integrated reporter would behave in a similar manner compared to the transfected reporter. A NIH3T3-derived cell line carrying a stably integrated SRF-controlled reporter gene, 3D.AFos, was used. The 3D.AFos reporter comprises the human c-fos transcription unit controlled by a chimeric promoter containing a cytoskeletal actin TATA region (minimal promoter) and three upstream SRF binding sites. I took advantage of the RNase protection assay, which allows monitoring of both the kinetics and magnitude of induction of mRNAs of
interest. To extend the data obtained by transfection experiments (Hill et al., 1995), I used other stimuli, such as the polypeptide factor PDGF-BB and the phorbol ester TPA, in addition to serum and LPA. In contrast to serum and LPA, which induced activation of both integrated and transiently transfected reporters, PDGF and TPA strongly activated integrated reporters, but not transiently transfected ones (see Figure 2.1). The peak of RNA accumulation induced from an integrated reporter by serum LPA and TPA, occurred at 60 minutes, contrasting to PDGF induction which peaked around 30 minutes and declined rapidly thereafter. The magnitude of induction at 30 minutes was comparable for all stimuli used. To make sure that observed differences were not single clone artefacts, arising from integration positional effects, I examined two additional clones (1-6 and 2-9). As illustrated in Figure 2.2, both clones were induced by PDGF, however, TPA-induced SRF reporter activity in clone 2-9 was somewhat weaker than in other clones, possibly due to a lower copy number of the integrated reporter.

2.2.2. Activation of the SRF reporter measured at the protein level

2.3.2.1. Analysis of the SRF reporter activation by Western blotting

To confirm the somewhat unexpected behaviour of integrated reporter clones measured by RNase protection analysis I tested whether the reporter is expressed at the protein level. Several single clones were tested by western blotting using anti HA-tag antibody As expected (see Figure 2.3A), all clones that were inducible at RNA level, produced 56 kDa 3D.AFosHA protein. Thus, at least 4 independent clones expressed from the reporter at the protein level. To compare protein accumulation levels and kinetics, I performed analogous experiment to those using RNase protection assay. Cells were starved and then stimulated with serum, LPA, PDGF or TPA and tested for the expression of the HA-tagged protein at different time points (see Figure 2.3B). Protein accumulation was first detectable after 30 minutes, and rapidly increased thereafter. As in the case of RNA the levels of serum-, LPA- and TPA-induced SRF reporter activity were comparable, although in PDGF-treated cells the induction of the 3D.AFosHA reporter protein was lower. These data show that the integrated SRF reporter induction can be readily detected both at protein and RNA level following serum, LPA, PDGF or TPA stimulation.
2.3.2.2. Analysis of the SRF reporter activation by immunofluorescence

To test whether reporter activity was uniform throughout the cell population, I used a single cell based technique employing immunofluorescence. This technique allows comparison of the activity of the reporter gene between single cells as well as the whole population of cells following different stimuli. However, one disadvantage of this technique is that it allows only an approximation of the levels of expressed reporter. In this experiment, the cells were seeded on glass coverslips, starved for 24 hours in 0.5% FBS and then stimulated with serum, LPA, PDGF and TPA for 60 minutes, fixed and tested for the presence of the SRF reporter expression using anti-HA and fluorescence conjugated antibodies. As shown in Figure 2.4, serum induced SRF reporter expression in almost all cells and the level of activity was relatively homogenous within the cell population. A similar pattern was observed after LPA stimulation, although the signal was much weaker, consistent with that observed by western blotting (see Figure 2.3). A different distribution of the signal was observed after PDGF and TPA stimulation: approximately half of the cells exhibited high reporter activity, yet some cells showed very weak immunofluorescence. The lower mRNA and protein measurements observed previously in PDGF- and TPA-treated cells could possibly be explained by the fact that only a fraction of the cells respond to the stimulation. These data suggest that results obtained using total cell lysates or total RNA represents an average signal from the cell population.

2.2.3. Other reporter systems

To test whether activation of the stably integrated SRF reporter gene by growth factors and TPA is not restricted to the 3D.AFos reporter, additional SRF-dependent reporters were tested. A NIH3T3 cell line was previously constructed carrying an integrated reporter gene in which expression of the cell surface markers CD2 and CD8 are controlled by SRF-dependent transcription (cells were kindly provided by A. Alberts). To assess the ability of different extracellular stimuli to induce marker expression, cells were starved for 24 hours and then stimulated with serum, LPA, PDGF, TPA or Cytochalasin D for 16 hours to allow surface marker accumulation at the plasma membrane. The level of reporter gene expression was tested using
monoclonal anti-CD8-FITC or anti-CD2-TRITC conjugated antibodies and then measuring relative immunofluorescence (Figure 2.5). This assay allows a quantitative measurement of the level of marker expression and the distribution of expression levels within the cell population. The SRF-regulated genes were induced by these stimuli to differing extents. As previously observed in transfection assays, the serum response was very strong (Hill et al., 1995), while LPA gave a smaller but significant response. As observed for the 3D.AFosHA stably integrated reporter, PDGF and TPA also showed significant activation of this reporter (compare Figure 2.3 and Figure 2.5). Taken together these results suggest that activation of SRF-controlled reporters by PDGF and TPA requires reporter integration into genomic DNA.

2.3. Induction of Transfected (Extrachromosomal) Reporter

2.3.1. Activation of the transfected 3D.AFos reporter measured at the RNA level

Although 3D.AFos reporter has been well characterised in transfection experiments (Hill and Treisman, 1995), I sought to re-examine the inducibility of the SRF reporter by different stimuli using the Lipofectamine transfection technique. In addition, it was tested whether activation of the SRF reporter gene depends on the amount of the reporter DNA template transfected. Different amounts of reporter were transfected: 0.1μg, 0.3μg and 1.0μg, cells were starved in 0.5% serum for 24 hours and then stimulated with various agents. As shown in Figure 2.6, the SRF reporter was activated at high level by serum and LPA, barely detectable level by PDGF, while no detectable level activation was observed by TPA. In addition, the level of the reporter activity varied depending on the amount transfected. Higher amounts of DNA resulted in a greater signal, although fold induction was approximately the same in all cases, since background expression was also increased when using a higher amount of DNA template. This data shows that, in contrast to serum and LPA, TPA does not result in activation of the transfected SRF reporter in any conditions tested. However, PDGF treatment resulted in small, but reproducible induction of the reporter. Taken together these data suggest that activity of the transfected reporter using the Lipofectamine transfection technique does not largely depend on the amount of DNA template used. Although I did not directly compare Lipofectamine and the previously used reagent for transfection DEAE-Dextran, the results obtained suggest that different transfection
methods do not affect the activation characteristics of the transfected SRF reporter gene.

2.3.2. Activation of the transfected SRF-controlled reporter measured by enzymatic assays

2.3.2.1. CAT assays

It has been reported recently that SRF-dependent reporters can be activated by some RTKs and TPA in the HeLa cell line (Spencer et al., 1999; Wang et al., 1998). The previously described 3D.ACAT reporter was used in order to test whether this SRF reporter is activated by growth factors in NIH3T3 cells. Therefore cells were transfected by the lipofectamine method with an appropriate amount of reporter and tested for their ability to respond to serum, Cytochalasin D or three different concentrations of PDGF. No significant activation was observed following PDGF stimulation at any concentration when compared to serum or Cytochalasin D stimulation (Figure 2.7). In addition, analogous experiments were performed using the calcium phosphate transfection method to ensure that the results were not dependent on the transfection method used. This experiment essentially gave the same result as observed with lipofectamine transfection (data not shown). Taken together these data demonstrate that the 3D.ACAT reporter, when transiently transfected, is refractory to PDGF, and this result is independent of transfection conditions.

2.3.2.2. Luciferase assay

Although CAT reporters have been extensively used in the last decade, more recently other reporter systems have been developed. One of the reporter systems used in recent years is the luciferase reporter. Luciferase reporters have been shown to be more sensitive and have a greater linear range when compared to CAT reporters. Therefore I tested the inducibility of the luciferase based reporter, 3DA.Luc (kindly provided by O. Geneste), by various stimuli. First, the amount of transfected DNA template was titrated in order to obtain maximum stimulation of the reporter activity over background levels. Different amounts of DNA template was transfected (0.1μg
Chapter 2: Results

and 0.3μg per 6 cm dish). Cells were starved and then stimulated for 6 hours, lysed and luciferase activity measured (see Figure 2.8A). Serum stimulation resulted in approximately 50 fold induction, LPA - 30 and PDGF - 5 fold induction, TPA did not show significant inducibility of the reporter over background levels (2 fold induction). Similarly to results observed with RNase protection assays, the relative fold induction does not greatly depend on the amount of DNA template transfected. These data show that SRF-dependent luciferase reporter is almost completely insensitive to TPA stimulation.

One of the reasons why PDGF induction of the reporter might be much smaller then serum is the transient nature of the PDGF-induced signal. Therefore the accumulation of luciferase activity was examined in a time dependent manner. Cells were transfected with optimal amount of template, starved and then stimulated with serum, LPA or PDGF for 2, 4, 6 and 12 hours. Cells were harvested and luciferase activity was determined. As shown in Figure 2.8B, induction of reporter activity was detectable after 1 hour and was increasing thereafter. Two hours following stimulation, serum and PDGF activation was 3 and 5 fold higher, respectively. However, serum-induced activity continued to increase into the prolonged time points, and reached a 30 fold induction level, while PDGF-induced reporter activity did not change after the 2-hour time point. These data suggest that transient PDGF signalling does not allow accumulation of the reporter, and thus leads to much lower, although detectable, inducibility.

2.3.3. Activation of the microinjected SRF-controlled reporter

Another way to deliver the reporter into the cell is to directly inject the DNA template into the cell nucleus. This technique allows the monitoring of reporter activity within hours of transfection, in contrast to traditional transfection methods with which reporter activity is measured one or two days after transfection. Clearly, microinjection is a substantially different method of DNA delivery to the cell, therefore I tested whether this would result in different activation characteristics for the reporter when compared to conventional lipofectamine transfection techniques. Cells were seeded on glass coverslips, starved for 24 hours and then injected with the reporter alone or co-injected with a coactivator expression plasmid as a positive control. As expected, serum addition caused the induction of the SRF reporter, however, in contrast to some transfection experiments, PDGF treatment also resulted
in significant reporter expression (see Figure 2.9). However, only 60-70% of the cells responded to PDGF, and the relative reporter level was still lower than that achieved with serum. These data are in agreement with the transfection experiments of the 3D.ALuc reporter described in Figure 2.8A. Taken together, these data suggest that SRF-controlled reporter activation by PDGF only registers in more sensitive assays, such as microinjection, RNase protection or luciferase assay.

2.3.4. The kinetics of activation of the transiently transfected SRF-dependent reporters

In order to examine more rigorously the differences between integrated and transfected SRF-controlled reporter gene, the kinetics of activation of transfected reporters were determined. In this experiment, cells were transfected with 0.5 μg of template per 6 cm dish, starved for 24 hours in 0.5 % serum and then stimulated for appropriate times. RNA was prepared and hybridised with an antisense reporter probe, as previously described. The transfected reporter showed a robust response, starting at 15 minutes with an increase of transcript levels in a time dependent manner, following both serum and LPA stimulation (Figure 2.10). In these optimised conditions, PDGF-induced SRF reporter activity was detectable, however TPA-induced activation of the reporter was still at background levels. These data suggest that by using sensitive techniques, such as the RNase protection assay, PDGF-induced SRF activity can be detected, although only at a very weak level when compared to serum or LPA. In contrast, TPA-induced SRF reporter activation (non-integrated) was virtually undetectable at all conditions tested.
Figure 2.1. Activation of the stably integrated SRF reporter gene by different stimuli. A. Serum-deprived SRE.FosHA cells were stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 50 ng/ml TPA or 25 ng/ml PDGF for the times indicated on the figure. Transcripts of the reporter and control gene, GAPDH, were analysed by RNase protection; nuclease resistant fragments derived from the two RNAs are indicated in the figure.

B. At peak accumulation relative transcript levels expressed as a percentage of those induced by serum at 60 minutes are shown (mean +/- SEM, 3 independent experiments).
Figure 2.2. Activation of two independent stably integrated SRF reporter clones. 

A. Two independent clones were serum deprived and then stimulated for 30 minutes with 15% Fetal Calf Serum (S), 10μM LPA (L), 25 ng/ml PDGF (P), 50 ng/ml TPA (T) or 2μM Cytochalasin D (C) as indicated in the figure. 3D.AFos reporter and GAPDH transcripts were analysed by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 2.3. Induction of 3D.AFosHA reporter at protein level. A. Serum-deprived SRE.FosHA cells were stimulated for 60 minutes with 15% Fetal Calf Serum (S), 10μM LPA (L), 25 ng/ml PDGF (P), 50 ng/ml TPA (T), 2μM Cytochalasin D (C) or 0.5μM Jasplakinolide as indicated in the figure. Whole cell lysates were prepared, fractionated by SDS-PAGE and transferred to PVDF membrane before immunoblotting for 3D.AFosHA protein with anti-HA antibody. B. Serum-deprived SRE.FosHA cells were stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 50 ng/ml TPA or 25 ng/ml PDGF for the times indicated on the figure. Whole cell lysates were prepared, fractionated by SDS-PAGE and transferred to PVDF membrane before immunoblotting for 3D.AFosHA protein with anti-HA antibody.
Figure 2.4 Analysis of 3D.AFosHA reporter activation by immuno-fluorescence. SRE.FosHA cells were seeded on coverslips, serum starved for 24 hours and then stimulated for 60 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF, 50 ng/ml TPA or 2μM Cytochalasin D (Cyt D) prior to fixation and staining for expression of the 3D.AFosHA reporter with HA antibody (red). Cell nuclei were stained with hoechst (blue).
Figure 2.5 3D.A-CD8 marker gene expression analysis by FACS. 3D.A-CD2/CD8 cells were serum starved for 24 hours and then stimulated for 16 hours with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF, 50 ng/ml TPA or 2μM Cytochalasin D (Cyt D) prior to staining with anti CD8-FITC conjugated antibody. The levels of CD8 expression was detected by FACS.
Figure 2.6. Activation of transiently transfected 3D.AFosHA reporter gene. A. NIH3T3 cells were transfected with different amounts of the SRF reporter gene template as indicated in the figure. Cells were maintained in 0.5% FBS for 24 hours and then stimulated for 30 minutes with 15% Fetal Calf Serum (S), 10μM LPA (L), 5 ng/ml PDGF (P1), 25 ng/ml PDGF (P2), 50 ng/ml TPA (T) or 2μM Cytochalasin D (C). 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. At peak accumulation relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 2.7. Activation of transiently transfected 3D.ACAT reporter gene. NIH3T3 were transfected (Lipofectamine) with 3D.ACat reporter plasmid, maintained in 0.5% FBS for 24 hours and then stimulated for 8 hours with 15% Fetal Calf Serum (SER), 2μM Cytochalasin D (Cyt D) and 1, 5 or 25 ng/ml PDGF as indicated in the figure. Cells were harvested and analysed for CAT activity.
Figure 2.8. Activation of transiently transfected 3D.ALuc reporter gene. A. NIH3T3 were transfected (Lipofectamine) with 3D.ALuc reporter plasmid, maintained in 0.5% FBS for 24 hours and then stimulated for different amounts of time with 15% Fetal Calf Serum (SER) or 25 ng/ml PDGF as indicated in the figure. Relative luciferase activity was normalised to the amount of protein. B. NIH3T3 were transfected (Lipofectamine) with 0.1µg or 0.3µg 3D.ALuc reporter plasmid, maintained in 0.5% FBS for 24 hours and then stimulated for 6 hours with 15% Fetal Calf Serum (S), 10µM LPA (L), 25 ng/ml PDGF (P) or 50 ng/ml TPA (T). Relative luciferase activity was normalised to the amount of protein.
**Figure 2.9. Activation of injected 3D.AFosHA reporter gene.** NIH3T3 cells were seeded on coverslips, serum starved for 24 hours and then microinjected with 3D.AFosHA reporter plasmid and GFP. Four hour later cells were stimulated for 60 minutes with 15% Fetal Calf Serum (SER) or 25 ng/ml PDGF, fixed and probed with anti-HA antibody to measure the 3D.AFos HA reporter expression.
Figure 2.10. Activation kinetics of transiently transfected SRF reporter gene. A. NIH3T3 cells were transfected with 0.5μg of 3D.AFosHA reporter plasmid, starved for 24 hours and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for times indicated in the figure. 3D.AFos reporter and GAPDH transcripts were analysed by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
3. Dissection of the Signalling Pathways to SRF

3.1. Chapter Summary

In order to examine signalling pathways leading to SRF activation a range of signalling specific pharmacological inhibitors were used, such as PD98059, U0126, Wortmannin, SB203580, BIM, and the src family kinase inhibitor, PP1. Inhibitors were tested for their ability to prevent activation of the SRF-dependent reporter by different stimuli. Control experiments were performed in order to ensure the specificity of inhibitors applied. Activation by RTK's and TPA was demonstrated to be dependent on PI-3 kinase activity, while serum and LPA activation of SRF were not. All stimuli-induced activations, with the exception of TPA, were largely independent of MEK activity. Similarly, results obtained with the protein kinase C inhibitor, suggesting that serum, LPA and PDGF activate SRF largely independently of the PKC-MAPK signalling pathway, while TPA is absolutely dependent on it. Inhibitor studies revealed that other kinases, including p38 kinase, mTOR, src family kinases and ROCK do not substantially participate in SRF activation.

3.2. The Role of MEK-ERK Pathway in SRF Activation

Since observing that SRF-integrated reporters could be activated by PDGF and TPA, which are very potent activators of MAPK signalling cascades, I sought to examine whether activation of the SRF-dependent reporter gene was MAP kinase dependent. To this end, two different pharmacological inhibitors were used, PD98059 and U0126.

3.2.1. Specificity of the MEK inhibitors U0126 and PD98059

In order to use the inhibitors in transcriptional activation assays, it is of importance to determine the minimal concentration required for complete inhibition of
the kinase of interest, without affecting other signalling pathways. Therefore the activity of the U0126 and PD98059 inhibitors were measured by monitoring the phosphorylation state of endogenous ERK using phospho-specific antibodies. A pilot experiment tested whether 25 μM of PD98059 abolished phosphorylation of MAPK following PDGF stimulation. Cells were treated with inhibitor and then stimulated for 2, 5, and 15 minutes. At the 2-minute time point phosphorylation of ERK was almost blocked, however normal phosphorylation levels were restored after 15 minutes (Figure 3.1). To investigate this in more detail, I used another unrelated MEK inhibitor U0126. Cells were pre-treated for 30 minutes with 10μM, 25μM or 50μM of U0126 and then stimulated with serum, LPA, PDGF and TPA. Cells were lysed at 5 and 15 minutes following stimulation and MAP kinase phosphorylation monitored. As shown in Figure 3.2, 10μM concentration of the U0126 inhibitor was sufficient to completely inhibit ERK phosphorylation by all stimuli, except serum. Serum-induced phosphorylation of ERK was abolished at 25 μM concentration. It is conceivable that serum induces a higher level of phosphorylation, which requires a higher inhibitor concentration to block the activity of ERK. Alternatively, it is possible that the serum-induced ERK phosphorylation utilises additional pathways to MEK1, which are only blocked by the higher concentration of the inhibitor.

To investigate this partial inhibition phenomenon in more detail, I examined the kinetics of inhibition using the 10μM concentration of U0126 inhibitor. In this experiment, cells were pre-treated with U0126 inhibitor for 30 minutes and then stimulated for different times (see Figure 3.3). Although all stimuli tested are potent activators of the MAPK signalling pathway, the kinetics of ERK phosphorylation was different depending on the stimuli used. Serum and TPA induced phosphorylation that was prolonged and observed for up to 2 hours after stimulation. The PDGF induced signal was very strong at 5 and 15 minutes and decreased gradually thereafter. LPA activity induced transiently and was barely detectable at 15 and 30 minutes. As determined by western blotting with phospho-ERK antibody, 10 μM U0126 inhibitor was sufficient to block LPA, PDGF and TPA induced ERK phosphorylation. However, the kinetics of the inhibition observed after serum stimulation were slightly different. At the five minute time point there was no phosphorylation signal, but at later time points a slight increase was observed, which declined after 30 minutes. This data suggest that 10μM of U0126 inhibitor significantly inhibits ERK activity following LPA, PDGF or TPA stimulation, however a small residual activation persists in the case of serum stimulation.
In order to test whether the same concentration of U0126 inhibitor blocks transcriptional activation by TCF, I used a luciferase reporter system responsive to the activity of a downstream target of MAP kinase, Elk-1. Cells were transfected with an Elk-1-dependent reporter, comprising 2 LexA binding sites upstream of a minimal promoter driving the luciferase gene, together with an expression vector encoding a LexA/Elk-1 fusion protein, as previously described (Marais et al., 1993). Cells were pre-treated with 10μM U0126 and then stimulated for 8 hours with various agents. As shown in Figure 3.4, U0126 completely inhibited activation of the Elk reporter gene by any of the stimuli tested. Similar results were obtained using PD98059 inhibitor. These data suggest that U0126 specifically inhibit both ERK activation and transcriptional activation by the ERK target, Elk-1.

3.2.2. U0126 does not affect PI-3 kinase activity

Since the optimal concentration for inhibition for MAP kinase was determined, I examined drug specificity and whether this concentration would not inhibit other signalling pathways, such as the PI-3 kinase pathway. To test this, an indirect assay that indicates PI-3 kinase activity was used. It has been previously shown that phosphatidinositol products are absolutely necessary to activate the PI-3 K downstream target, Akt/PKB (Toker, 2000). Therefore the measurement of Akt phosphorylation as a readout for PI-3 K activity is widely accepted. Cells were starved, pre-treated with 10μM U0126 and then stimulated with serum, LPA, PDGF or TPA. High levels of Akt activation were observed after serum and PDGF treatment. However, Akt is phosphorylated at much lower levels after LPA treatment and phosphorylation is not detected after TPA treatment. Pre-treatment with U0126 did not abolish serum-, LPA- or PDGF-induced phosphorylation of Akt (see Figure 3.5). Thus the MEK inhibitor U0126 does not block induced PI-3 K activity at this concentration. Similar results were obtained using another MEK inhibitor PD98059 (data not shown). Taken together, these data suggest that serum, LPA and PDGF induce at least two different pathways and the U0126 inhibitor specifically blocks one of them without affecting the other.

3.2.3. Serum and LPA pathways to SRF are mostly ERK independent
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To examine the dependence of the SRF-controlled reporter gene activation on the Ras-MEK-ERK signalling pathway, RNase protection experiments were performed. A cell line carrying an integrated 3D.AFos construct was tested for the ability to induce the SRF reporter gene after serum, LPA, PDGF or TPA treatment, and its sensitivity to the inhibition of MEK by U0126. Cells were starved for 24 hours, pre-treated with 10μM U0126 and then stimulated with serum, LPA, PDGF or TPA. Serum- and LPA-induced transcription were reduced by 40-50%, whereas activation by PDGF, by 60-65%; only induction by TPA was reduced to background levels (Figure 3.6). This data demonstrates that serum- and LPA-induced SRF reporter activation was mostly ERK independent, whilst PDGF activation was partially ERK sensitive. TPA-induced activation was completely sensitive to blockage of MEK activity. To confirm the data obtained with U0126, and to validate partial inhibition effects on 3D.AFos induction, analogous experiments were performed using another MAPK signalling pathway inhibitor PD 98059 (Figure 3.7). No significant differences from the results obtained with the two different inhibitors were observed, although reduction of PDGF-induced signal was more pronounced with U0126 inhibitor, possibly due to the more potent inhibition of the MAPK signalling cascade. Taken together these data suggest that at least two types of signals lead to SRF activation, one of which is ERK dependent, and the other, ERK independent.

3.3. The Involvement PI-3 Kinase in SRF Activation

3.3.1. Specificity of the PI-3 kinase inhibitors

The in vitro specificity of the most commonly used inhibitors of PI-3 kinases towards a number of signalling molecules has recently been determined (Davies et al., 2000). Although tissue culture experiments usually require a greater concentration of inhibitor to achieve the same Ki. In addition, the chemical concentrations of organic compounds do not always correspond to the active concentration, and varies depending on the batch number. I therefore tested whether the recommended concentration of inhibitor fully inhibits PI-3 K activity using the phospho-Akt assay as described in section 3.1.2. In this experiment cells were pre-treated with 20 μM of LY294002 inhibitor for 30 minutes and then stimulated with serum, LPA, PDGF or TPA for various durations (see Figure 3.8, upper panels). As determined by the
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phospho-Akt antibody, the induced activity of PI-3 kinase following serum, LPA or PDGF treatment was abolished when cells were pre-treated with LY294002 inhibitor. Interestingly, LY294002 pre-treatment also inhibited background Akt phosphorylation in starved cells, suggesting that this inhibitor reduces not only induced, but also basal PI-3 K activity. TPA stimulation did not alter the phosphorylation level of Akt. These data suggest that PI-3 K activity is increased following serum, LPA and PDGF, but not TPA stimulation and that pre-treatment with 20 μM of LY294002 is sufficient to block this activation.

To test whether 20μM of the LY294002 inhibitor affects other signalling molecules due to non-specific effects, I tested whether ERK kinase phosphorylation was compromised. For this control experiment, cells were pre-treated with 20μM LY294002 and tested for the ability to induce ERK activation following addition of various stimuli, as assessed by ERK phosphorylation. (Figure 3.8, lower panels). No significant inhibition of ERK activation was detected at 3 separate time points. Thus, these data show that LY 294002 inhibitor specifically blocks PI-3 K, but not the MEK-ERK signalling pathway.

3.3.2. The PI-3 kinase pathway is required for PDGF- and TPA-induced activation of SRF

PI-3 kinase has been implicated in SRF activation in transfection experiments in HeLa cells (Wang et al., 1998). Therefore, I tested whether activation of the SRF reporter was dependent on PI-3 K activity. For this reason cells were pre-treated with 20μM LY 294002 and stimulated for 30 minutes with serum, LPA, PDGF or TPA. Activity of the stably integrated SRF-controlled reporter was determined using RNase protection assay (Figure 3.9). Both the PDGF- and TPA-induced reporter activity was substantially inhibited with pre-treatment of PI-3 K inhibitor LY294002, however, serum- and LPA-induced activity was not significantly affected by this inhibitor. These data suggest that SRF can be activated by two distinct signalling pathways, one of which requires PI-3 K activity. To confirm these findings, another structurally different inhibitor of PI-3 K, Wortmannin, was used. Wortmannin pre-treatment results in the same pattern of inhibition of the SRF reporter gene as was observed with LY294002 (see Figure 3.10). However, LPA-induced SRF activity was slightly more sensitive to wortmannin then to LY294002. To confirm that PI3-K is not involved in serum- and LPA-induced SRF reporter gene activation, the effects of LY294002 on
the kinetics of the SRF reporter activation were examined following serum and LPA treatment. For this reason SRF reporter gene activity was tested at 30, 60 and 120-minute time points following addition of serum and LPA (Figure 3.11). No significant inhibition of SRF activation was observed at the three time points tested. Taken together these data show that PI-3 K activity is dispensable for serum- and LPA-, but, in contrast, is required for PDGF- and TPA-induced SRF reporter gene activation.

3.4. The Role of PKC in Signalling to SRF

Since I observed that activation of the 3D.AFos reporter is sensitive to the ERK signalling pathway inhibitors, I tested the possible involvement of upstream components of the ERK signalling pathway, such as protein kinase C (PKC). For this reason, the relatively specific PKC inhibitor GF109203X (BIM) was used. Cells were starved, pre-treated with inhibitor and then stimulated for 30 minutes with different stimuli. The activity of the SRF reporter was measured by RNase protection as above. The effect of the inhibition is presented in Figure 3.12A. Activation of the 3DA.Fos reporter following serum and LPA stimulation was PKC independent, while the PDGF response was affected by 30%, and TPA stimulation was essentially blocked. To corroborate the data obtained with GF109203X inhibitor, I performed additional experiments to test whether some TPA-dependent PKC isoforms are involved in signalling to SRF. For this reason cells were starved in 0.5 % FBS, pre-treated with TPA, in order to downregulate PKCs, and then stimulated with various agents. SRF reporter activity was determined as above (Figure 3.12B). The inhibition levels were substantially the same, both in GF109203X or TPA pre-treated cells. Activation of the SRF reporter was insensitive to PKC inhibition following serum stimulation, while LPA and PDGF were partially sensitive, and TPA was blocked. The percentage of activation inhibition of the SRF reporter gene was similar to those observed using MAPK inhibitors (compare with Figure 3.6 and 3.7). These data suggest that serum and LPA activate SRF reporter independently of the PKC-MAPK pathway, while PDGF is only partially dependent, and TPA is absolutely dependent on this pathway.

3.5. The Role of p38 Kinase in SRF Activation
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It has previously been reported that another MAPK family member, p38 kinase, is involved in signalling to SRF target genes, \textit{c-fos} and \textit{junB} (Hazzalin \textit{et al.}, 1997; Price \textit{et al.}, 1996). However, the contribution to the SRF reporter gene activation by p38 kinase has not been assessed. Therefore I examined whether blocking this kinase with the relatively specific inhibitor SB203580 would affect the SRF reporter gene activation. SRE.FosHA cells were starved, left untreated or pre-treated with 20\textmu M of SB203580 and then stimulated for 30 minutes with serum, LPA, PDGF or TPA. Activity of the SRF reporter gene was determined by RNase protection. The percentage of inhibition by SB203580 is presented in Figure 3.13. In contrast to MEK inhibition, SB203580 did not affect activation of the SRF reporter activity by all stimuli tested. In fact, some enhancement of SRF reporter gene activation was observed when SB203580 was used. Similar potentiation phenomenon was observed with transfected and endogenous \textit{c-fos} activation after serum and TPA stimulation (Price \textit{et al.}, 1996). This data suggests that p38 kinase is not essential for SRF activation.

3.6. S6 Kinase Is Not Required for SRF Activation

It has been previously reported that full activation of S6 kinase requires both mTOR (mammalian target of rapamycin) and PI-3 K activities (Pullen \textit{et al.}, 1998). Having found that PDGF- and TPA-induced activation of the SRF reporter gene are dependent on the PI-3 K activity, I decided to test the involvement one of the downstream targets of PI-3 K, S6 kinase. To test whether S6 kinase is involved in SRF signalling the relatively specific inhibitor of mTOR, rapamycin, was used. Cells were starved, pre-treated with 20nM rapamycin and then stimulated with serum, LPA, PDGF or TPA. Activity of the SRF-dependent reporter was determined by RNase protection as above. Rapamycin pre-treatment did not inhibit SRF reporter gene activation by any of the stimuli tested (see Figure 3.14). Thus, these data suggest that S6 kinase is not involved in SRF activation by the stimuli under investigation. Since both PDGF- and TPA-induced SRF activation is dependent on PI-3 K activity, these data imply that another PI-3 K-dependent component must exist which is required for SRF activation by these stimuli.
3.7. The src Family Kinases and SRF Activation

One of the downstream targets of LPA and PDGF signalling are the src family of kinases. These kinases have been implicated in cell cycle progression (Roche et al., 1996), activation of the downstream target of the RhoA effector mDia (Tominaga et al., 2000), as well as SRF activation by Tec/BMX kinases (Mao et al., 1998). Therefore I examined whether src family kinases are involved in the activation of an integrated SRF-controlled reporter gene following serum, LPA, PDGF or TPA stimulation. To test this one of the possible strategies would be to use src family knockout cells. However, due to the functional redundancy of different members of the src family kinases, and the unavailability of the triple knockout cell line (src, yes, fyn), which has recently been developed (Klinghoffer et al., 1999), I was limited to use the relatively specific src family kinase inhibitor PP1.

3.7.1. Specificity of the src kinase inhibitor PP1

The specificity of PP1 towards several src family kinases has been tested, but serine-threonine family kinases were not tested (Hanke et al., 1996). I therefore first tested whether PP1 affects activation of other kinases, such as ERK or Akt, following serum and PDGF stimulation. Cells were pre-treated with 0.1μM, 1.0μM or 10μM PP1 and subsequently stimulated with serum or PDGF for 2, 5 and 15 minutes. The activity of ERK was estimated with anti phospho-ERK antibody by western blotting (see Figure 3.15A, upper panels). At 0.1 or 1.0μM concentration, ERK phosphorylation was not impaired after either stimuli, although 10μM concentration blocked PDGF-, but not serum-induced ERK activation. Similar results were obtained when the membranes were re-probed with the phospho-Akt antibody, in order to estimate PI-3 kinase activity (Figure 3.14A, lower panels). These data suggests that 10μM PP1, although not blocking some specific serum-induced signalling pathway, however completely blocks PDGF receptor function. Since the PDGF receptor is a tyrosine kinase itself, it is possible that at 10μM PP1 impairs PDGF receptor kinase activity, thereby blocking the activation of downstream targets of the PDGF receptor. To test this possibility I examined autophosphorylation state of PDGF receptors at different PP1 concentrations. In this experiment, cells were left untreated or pre-treated with 0.1μM, 1.0μM or 10μM of PP1 concentration and then stimulated for 2 and 15 minutes with PDGF (Figure 3.14B). Autophosphorylation of PDGF receptor was
estimated with anti phospho-tyrosine antibody. It was observed that 10μM PP1 blocked PDGF receptor kinase activity, although 0.1μM or 1.0μM did not. Taken together these data suggest that PP1 is a relatively specific inhibitor that could be used at 0.1μM or 1.0μM concentrations. Using higher concentration of PP1 appears to result in some non-specific inhibition and would require addition controls for interpretation of results.

3.7.2. Signals to SRF are mostly src independent

Results described in section 3.7.1 suggest that PP1 appears to be a relatively specific inhibitor, at least at lower concentrations. Therefore I investigated whether src family kinases are involved in SRF activation using PP1. Three different concentrations of inhibitor were used to allow interpretation of the results obtained. Cells were pre-treated with 0.1μM, 1.0μM or 10μM PP1, and then stimulated for 30 minutes with various agents. SRF reporter activity was measured by RNase protection as described in section 2.2. As shown in Figure 3.16, even a 10 μM concentration of PP1 did not impair SRF induction following serum stimulation. However, no PDGF-induced SRF reporter gene activation was observed when 10μM PP1 was used, most probably since PDGF receptor kinase activity is impaired at this concentration of inhibitor. At 1μM concentration of PP1 inhibitor there was no significant reduction in induction of the SRF reporter gene activity following all stimuli tested. Taken together these data suggest that src family kinases are not necessary, at least for serum- and LPA-induced, SRF reporter activity. In the case of PDGF, it remains possible that src kinases are required for efficient activation of the SRF reporter gene, although, it is most likely that src contributes to the PDGF receptor complex activation itself (Paukku et al., 2000).

3.8. ROCK Is Not Required for SRF Activation

It has been recently shown that one of the Rho effectors, ROCK (Rho-kinase), is involved in actin rearrangement and activation of SRF following serum stimulation in transfection and microinjection assays (Sahai et al., 1999). I have extended these observations for the integrated SRF-controlled reporter cell line using additional stimuli. A well characterised ROCK inhibitor Y-27632 was used. At a concentration
of 10μM this inhibitor impairs ROCK kinase activity and has no effect on MAP kinase or JNK signalling (Sahai et al., 1999). RNase protection assay was used to examine whether ROCK inhibitor treatment impairs SRF-controlled reporter gene activity following various stimuli. In this experiment, cells were pre-treated for 30 minutes with 10μM of Y-27632 and then stimulated for 30 minutes with serum, LPA, PDGF or TPA. Following Y-27632 treatment LPA- and TPA-induced SRF reporter activity was slightly reduced, in contrast to serum- or PDGF-induced SRF activity, which was almost unaffected (see Figure 3.17). This finding is in agreement with the transfection data previously described (Sahai et al., 1999). Taken together these data suggest that although ROCK kinase is not absolutely necessary for SRF activation it may effect the efficiency of signal transmission for some pathways.
Figure 3.1. **PD98059 effects ERK phosphorylation.** Serum-deprived NIH3T3 cells were pretreated for 30 minutes with 25µM PD98059 and then stimulated with 25 ng/ml PDGF for times indicated. Whole cell lysates were prepared, fractionated by SDS-PAGE and transferred to PVDF membrane before immunoblotting for: diphospho-ERK and total ERK.

Figure 3.2. **U0126 inhibits ERK phosphorylation.** Serum-deprived NIH3T3 cells were pretreated for 30 minutes with 25µM PD98059 or different concentrations of U0126 and then stimulated with 15% Fetal Calf Serum (SER), 10µM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for the times indicated on the figure. Whole cell lysates were prepared, fractionated by SDS-PAGE and transferred to PVDF membrane before immunoblotting for: diphospho-ERK and total ERK.
Figure 3.3. U0126 inhibits ERK activation by the stimuli tested. Serum-deprived NIH3T3 cells were pretreated for 30 minutes with 10μM U0126 and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for times indicated on the figure. Whole cell lysates were prepared, fractionated by SDS-PAGE and transferred to PVDF membrane before immunoblotting for: diphospho-ERK and total ERK.
Figure 3.4. TCF Elk-1 activation is inhibited by U0126. NIH3T3 cells were transfected with an expression plasmid encoding the chimeric transactivator NLexElkC together with a Lex operator-controlled luciferase reporter gene, maintained in 0.5% FCS for 24 hr, and then stimulated following 30 minutes pretreatment with 10μM U0126 as indicated. Data are normalised to the serum response shown as 100; error bars indicate SEM from 3 independent transfections, where not shown, the SEM was always less than 20% of the experimental value.
Figure 3.5. U0126 does not affect PI-3K activation by the stimuli tested. Serum-deprived cells were pretreated for 30 minutes with 20μM LY29402 and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for times indicated. Whole cell lysates were prepared, fractionated by SDS-PAGE and transferred to PVDF membrane before immunoblotting for: Akt phospho-Ser473 and total Akt.
Figure 3.6. SRF-linked signalling pathways dependence on MEK. Effect of the MEK inhibitor U0126 on SRF reporter gene activity. A. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with 10µM U0126 and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10µM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Transcript levels at 30 minutes expressed as a percentage of those in untreated cells are summarised (mean+/−SEM, 3 independent experiments).
Figure 3.7. SRF-linked signalling pathways dependence on MEK. Effect of the MEK inhibitor PD98059 on SRF reporter gene activity. A. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with 25μM PD98059 and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. 3D.AFos reporter and GAPDH transcripts were analysed by RNase protection. B. Transcript levels at 30 minutes expressed as a percentage of those in untreated cells are summarised (mean +/- SEM, 3 independent experiments).
Figure 3.8. LY294002 does not affect ERK activation, but blocks Akt activation. Serum-deprived NIH3T3 cells were pretreated for 30 minutes with 20µM LY294002 and then stimulated with 15% Fetal Calf Serum (SER), 10µM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for times indicated on the figure. Whole cell lysates were prepared, fractionated by SDS-PAGE and transferred to PVDF membrane before immunoblotting for: Akt phospho-Ser473 and total Akt (top panels) and diphospho-ERK and total ERK (bottom panels).
A. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with 20μM LY294002 and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection.

B. Transcript levels at 30 minutes expressed as a percentage of those in untreated cells are summarised (mean±/SEM, 3 independent experiments).

**Figure 3.9. SRF-linked signalling pathways dependence on PI-3 kinase. Effect of the PI-3 kinase inhibitor LY294002 on SRF reporter gene activity.**
Figure 3.10. SRF-linked signalling pathways dependence on PI-3 kinase. Effect of the PI-3 kinase inhibitor wortmannin on SRF reporter gene activity. A. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with 100µM wortmannin and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10µM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. 3D.AFos reporter and GAPDH transcripts were analysed by RNase protection. B. Transcript levels at 30 minutes expressed as a percentage of those in untreated cells are shown.
Figure 3.11. Effect of the PI-3 kinase inhibitor LY294002 on the kinetics of SRF reporter gene activation. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with 20μM LY294002 and then stimulated for 30, 60 or 120 minutes with 15% Fetal Calf Serum (SER) or 10μM LPA. 3D.AFos reporter and GAPDH transcripts were analysed by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 3.12. SRF-linked signalling pathways dependence on PKC. A. Effect of PKC inhibitor GF109203X on SRF reporter gene activity. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with 10μM GF109203X and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 minutes. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. Transcript levels at 30 minutes expressed as a percentage of those in untreated cells are shown.

B. Effect of TPA pretreatment on SRF reporter gene activity. Serum-deprived SRE.FosHA cells were pretreated for 8 hours with 5ng/ml TPA and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 minutes. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. Transcript levels at 30 minutes expressed as a percentage of those in untreated cells are shown.
Figure 3.13. Effect of p38 kinase inhibitor SB203580 on SRF reporter gene activity. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with 20μM SB203580 and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 minutes. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. Transcript levels at 30 minutes expressed as a percentage of those in untreated cells are shown.
Figure 3.14. SRF-linked signalling pathways dependence on mTOR kinase. Effect of Rapamycin on SRF reporter gene activity. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with 10μM Rapamycin and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 minutes. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels at 30 minutes expressed as a percentage of those in untreated cells are shown (mean±/range, 2 independent experiments).
Figure 3.15. Specificity of src kinase inhibitor PP1. Serum-deprived NIH3T3 cells were pretreated for 30 minutes with different concentrations of PP1 and then stimulated with 15% Fetal Calf Serum (A) or 25 ng/ml PDGF (B) for the times indicated on the figure. Cell lysates were prepared, fractionated by SDS-PAGE and transferred to PVDF membrane before immunoblotting for: phospho-Akt, diphospho-ERK and total ERK. (C) Autophosphorylation state of PDGF receptor following PDGF treatment was examined with phospho-tyrosine antibody (PY20).
Figure 3.16. SRF-linked signalling pathways dependence on Src family kinases. Effect of Src kinase inhibitor PP1 on SRF reporter gene activity. A. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with different amounts of the Src inhibitor PP1 as indicated in the figure and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 minutes. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 3.17. Effect of Src kinase inhibitor PP1 on c-fos activation. A. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with different amount of Src inhibitor PP1 as indicated in the figure and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 minutes. c-fos and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 3.18. Effect of ROCK inhibitor Y27632 on SRF reporter gene activity. A. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with 10μM Y27632 and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
4. Two Classes of SRF Target Genes

4.1. Chapter Summary

The cloning of LIM kinase, a regulator of the actin treadmilling cycle, as an SRF activator, suggested that actin dynamics are involved in the transcriptional activation of SRF (Sotiropoulos et al., 1999). This chapter describes studies concerning actin dynamics, RhoA, PI-3 K, and MEK signalling pathways and their contribution to activation of the SRF reporter and endogenous SRF target genes. The use of inhibitors of actin polymerisation and Rho specific toxins has revealed that induction of the SRF reporter gene by all stimuli is dependent on actin dynamics. Regulation of srf and vinculin (vcl) genes were similar to that of the SRF reporter, in that activation by all stimuli was Rho-dependent and required actin dynamics, but was largely independent of MEK activity. In contrast, activation of c-fos and egr-1 occurred independently of RhoA and actin polymerisation, but was almost completely dependent on MEK activation. Based on these findings, two classes of SRF target gene are defined. It is proposed that (i) there are two types of SRF target gene which are regulated in a mutually exclusive manner, and that (ii) the presence of a TCF binding site in close proximity to the SRF binding site may control the signalling specificity to SRF target promoters.

4.2. Actin Polymerisation and Induction of SRF Target Genes

4.2.1. Latrunculin B and its effects on the actin cytoskeleton

I first tested the effect of Latrunculin B treatment on SRF activation by extracellular stimuli such as serum, LPA, PDGF or TPA. Cells were pre-treated with 0.5μM Latrunculin B and then stimulated with various agents. The effect of Latrunculin B became apparent after 15 minutes, and within 30 minutes all cells had 'rounded up' and lost stress fibres. To ensure that latrunculin has completely disrupted
the actin cytoskeleton, I extended the time of treatment to 60 minutes. Further experiments involving latrunculin pre-treatment of the cells were carried out using this longer period. Cells treated with 0.5μM Latrunculin B are shown in Figure 4.1.

4.2.2. Actin polymerisation is not required for ERK activation

Since it is apparent that the effect of Latrunculin B on actin filaments is drastic and concentration dependent, I tested its specificity and whether it also affects other signalling pathways, such as the activity of ERK or PI-3 kinase. Activation of these pathways was assayed by phospho-ERK and phospho-Akt antibodies as described in section 3.2. Cells were starved, pre-treated with 0.5 μM Latrunculin B and then stimulated with serum, LPA, PDGF or TPA for the times indicated (see Figure 4.2). Lysates were resolved on a SDS-PAGE, transferred to PVDF membrane and probed with either phospho-Akt or phospho-ERK antibodies. Latrunculin had no apparent effect on the strength or kinetics of activation of ERK at 3 different time points following all stimuli. This data suggests that an intact actin cytoskeleton is not required for ERK activation, and is consistent with previously reported data demonstrating EGF-induced ERK activity is not significantly affected by Latrunculin A pre-treatment (Aplin and Juliano, 1999). Similar results were observed with examination of Akt phosphorylation, ie. There was no significant change in phosphorylation of Akt in latrunculin treated cells. Taken together this suggests that, although Latrunculin B completely disrupts the actin cytoskeleton, it has no effect on the integrity of a subset of signalling pathways.

To examine whether the transcriptional machinery is functional following Latrunculin B treatment, I used the luciferase reporter system based on the activity of a downstream target of MAP kinase, Elk-1. Cells were transfected with the lex reporter gene together with an expression vector encoding a LexA/Elk-1 fusion protein, as previously described (Marais et al., 1993). Cells were pre-treated with 0.5 μM Latrunculin B and then stimulated for 8 hours with each of the different stimuli. As shown in Figure 4.3, Latrunculin B pre-treatment does not significantly inhibit activation of the Elk-dependent reporter by any of the stimuli tested. This data suggests that Latrunculin B does not effect transcriptional activation by Elk, which is consistent with the finding that ERK activity does not depend on actin polymerisation.
4.2.3. Actin polymerisation is required for SRF activation

It has been previously reported that extracellular signals to SRF are dependent on RhoA activity (Hill et al., 1995). RhoA activity is also required for stress fibre formation, but this activity appears to be separate from SRF activation, since RhoA effector loop mutants demonstrate that these activities can be dissociated (Sahai et al., 1998). It should be noted that stress fibre formation is a two step process requiring actin polymerisation and actin bundling (Watanabe et al., 1999). To examine the involvement of actin polymerisation in SRF activation directly, I used a cell line containing an SRF-controlled reporter gene as described previously. For this experiment, cells were pre-treated for 1 hour with two different concentrations of Latrunculin B and then stimulated with for 30 minutes with serum, LPA, PDGF or TPA. Latrunculin pre-treatment completely abolished the induction of SRF-controlled reporter gene by serum, LPA, PDGF or TPA (see Figure 4.4). Surprisingly, inducibility of the SRF reporter gene was always reduced to background levels, suggesting that actin polymerisation induced by extracellular stimuli is absolutely necessary for the transcription response of SRF. Some residual SRF reporter activity persisted when cells were pre-treated with lower concentrations of Latrunculin B in the case of serum stimulation. This could possibly be attributed to the ability of serum to partially inactivate the drug, and is consistent with the observation that cells recover a stress fibre phenotype 2-3 hours after serum addition.

To ensure that the Latrunculin B effects were specific to actin, I also used the specific actin modifying C2 toxin from C. botulinum (Aktories et al., 1986). This toxin specifically ADP-rybosylates actin at residue R177, and thereby prevents actin polymerisation (Vandekerckhove, 1988). For this reason cells were pre-treated with exogenous C2 toxin for 3 or 5 hours and then stimulated with serum for 30 and 180 minutes. RNA was harvested and the induced level of SRF-controlled reporter gene was determined by RNase protection (Figure 4.5). C2 toxin pre-treatment inhibited SRF activation in a time dependent manner: 5h hours treatment was sufficient to affect approximately 90% of the cells, and therefore a 90 % inhibition of SRF reporter activation could be interpreted as complete inhibition (similar results were obtained in two independent experiments). Taken together, these data suggest that actin polymerisation is directly involved in signalling to SRF and that Latrunculin B effects on SRF activation, described in Figure 4.4, are due to the specific effect on the actin cytoskeleton.
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4.2.4. Activation of a subset of SRF target genes is dependent on actin polymerisation

4.2.4.1. Actin polymerisation is required for \textit{srf} and \textit{vcl} expression

Since finding that SRF reporter gene activation is dependent on actin polymerisation, I wanted to establish whether the same pathway is required to activate endogenous SRF target genes. Therefore I tested the inducibility of the SRE containing genes \textit{srf} and \textit{vcl}, whose promoters do not contain defined TCF binding sites within their SREs (Moiseyeva et al., 1993; Norman et al., 1988). I examined whether \textit{srf} and \textit{vinculin} genes are activated following serum, PDGF or TPA stimulation, and whether this activation is dependent on actin polymerisation. NIH3T3 cells were starved, pre-treated with 0.5\(\mu\)M Latrunculin B and then stimulated for 30, 60 and 120 minutes with various stimuli. The probes were designed to correspond to nucleotides 1201-1444 and 1128-1134 for \textit{vinculin} and \textit{srf} genes, respectively (see Materials and Methods). RNA levels were analysed by RNase protection assay as described in section 2.1. Both \textit{srf} and \textit{vcl} genes are expressed at relatively high levels in starved cells (see Figure 4.6), probably due to the stability of their RNA when compared to other immediate early genes, like c-fos, whose expression is almost undetectable in starved cells. Following stimulation, an increase in message level was observed in a time dependent manner for both \textit{vinculin} and \textit{srf} genes, although the kinetics of induction for both genes tested was somewhat different. \textit{Vinculin} RNA levels were basically unchanged at the 30-minute time point, rising to 2 fold induction over the basal level at 60 minutes, and reaching 4-5 fold induction at 120 minutes following serum stimulation (see Figure 4.6). Similar expression patterns were observed for \textit{srf} induction, albeit with a message increase at the 30-minute time point compared to \textit{vinculin} (Figure 4.7). PDGF and TPA induction, although reproducible, was never greater than two folds over basal levels (similar data was obtained in at least 4 independent experiments). Although a consistent 2 folds increase was observed, it is difficult to precisely estimate the level of induction over the high basal level due to the stability of these mRNAs. However, even an achievement of a 2 folds increase in RNA level in such a short period of time would require a substantial change in transcriptional rate. In contrast to the well described c-fos or c-jun, the \textit{vcl} and \textit{srf}
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genes exhibit high expression levels in serum deprived NIH3T3 cells. Nevertheless srf and vcl are inducible at the transcriptional level following serum, growth factor or TPA stimulation. This observation is consistent with previously reported data that srf, vinculin, and actin fall into a group of immediate early genes.

As observed with the SRF reporter gene 3D.AFos, pre-treatment with Latrunculin B completely blocked srf and vinculin induction by extracellular stimuli at all tested time points (see Figure 4.6 and Figure 4.7). These data suggest that all stimuli-induced transcription of vinculin and srf genes requires actin polymerisation.

4.2.4.2. Developing intronic RNase protection probes for srf and vcl

As describe in previous section, exogenous stimuli increase srf and vcl gene expression at the mRNA level. However, the high basal levels and stabilities of the srf and vcl mRNAs allow only strong and prolonged transcriptional changes to be reliably measured by quantification of mRNA and preclude the use of mRNA level as a measure of transcription rate. Although run-on assays, measuring transcription rate, have been well established and successfully used for more than a decade, I was eager to establish an assay, which would allow measurement of the transcription rate using total RNA. I therefore developed an RNase protection assay that allows simultaneous measurement of the level of both mRNA and unspliced precursor transcript of each gene. For this reason I designed the riboprobes spanning the srf exon 5-intron 5 and vcl exon 3-intron 3 borders, respectively (see Figure 4.8).

4.2.4.3. Induction kinetics of srf and vinculin precursor and mRNA by serum stimulation

In order to test the ability to detect both precursor and spliced mRNA, the following pilot experiment was performed. Cells were starved and then stimulated with serum for different amounts of time ranging from 15 to 90 minutes. The RNase protection assay was performed using intronic srf (exon 5-intron 5) and vcl (exon 3-intron 3) probes, respectively. As represented in Figure 4.9A and 4.9B, serum stimulation resulted in a rapid and transient induction of the srf and vcl precursor messages. In unstimulated cells precursor levels were almost undetectable, suggesting that little active transcription occurs when cells are maintained in 0.5% serum. As
expected, gradual accumulation of both \textit{srf} and \textit{vcl} mRNA occurred throughout a 2-hour period following serum stimulation. However, the \textit{vinculin} precursor was not detected until 30 minutes, in contrast to the \textit{srf} precursor, which was detectable at the 15-minute time point (compare 15 and 30 minute points in Figure 4.9A/B). Consistent with this, a slight delay of \textit{vinculin} mRNA accumulation was observed when compared to \textit{srf}. At the 30 minute time point \textit{vinculin} mRNA levels were almost unchanged, although \textit{srf} mRNA levels had reached two fold induction over the basal level (see Figure 4.9C/D for quantification of the data). Despite this small delay of \textit{vinculin} precursor and RNA induction, these two genes displayed similar characteristics in magnitude and kinetics of induction following serum stimulation (see discussion). Taken together this data strongly suggests that \textit{vinculin} and \textit{srf} gene transcription is strongly inducible by serum, and have very similar induction characteristics compared to other immediate early genes. These data are in agreement with other reports, which previously defined these SRF target genes as immediate early gene class members (Moiseyeva \textit{et al}., 1993; Norman \textit{et al}., 1988).

4.2.4.4. \textit{Induction of the srf and vinculin precursors by different stimuli}

Having established a sensitive technique for examining \textit{srf} and \textit{vcl} transcription rate levels, I determined the kinetics and induction levels of these genes by different stimuli. NIH3T3 cells were starved for 36 hours and then stimulated for 15, 30, 45, 60, 90 and 120 minutes with LPA, PDGF or TPA. RNA was analysed as described in previous sections. All stimuli induced both \textit{srf} and \textit{vcl} precursor RNAs, peaking between 30 and 45 minutes (see Figure 4.10A and Figure 4.11A). Relative induction levels of precursor RNAs are represented in Figure 4.10B and 4.11B, respectively. LPA and TPA activated \textit{srf} and \textit{vcl} transcription with similar kinetics to serum stimulation, while PDGF-induced activation was much more transient, reverting to pre-stimulation levels after 60 minutes. Noticeably, a similar delay in \textit{vcl} transcriptional induction, observed previously after serum stimulation, was also apparent following all stimuli used. Taken together, these data establish that the \textit{srf} and \textit{vinculin} genes are rapidly and transiently induced at the transcriptional level, by all stimuli under investigation. In addition, the kinetics, as well as the magnitude, of \textit{srf} and \textit{vcl} precursor accumulation, apart from the delayed appearance of the \textit{vcl} precursor, resemble those of the SRF reporter gene. The nature of the observed delay in appearance of \textit{vcl} precursor is not clear. It is possible that differences in splicing
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kinetics of the srf and vcl results in difference of RNA accumulation, since the vcl gene consist of 22 exons compared to 7 exons in srf gene. Another possibility could be the requirement of a newly synthesised protein for the efficient transcriptional activation of vinculin. However, both possibilities are unlikely, given our own experiments (see Chapter 5) and previously reported data using the Swiss3T3 cell line (Bellas et al., 1991).

4.2.5. Activation of SRF target genes with well defined TCF binding sites within SREs are not dependent on actin polymerisation

I sought to examine the dependence on actin polymerisation of other immediate early genes that contain TCF binding sites within SREs of their promoters. Two of the best characterised immediate early genes are c-fos (Treisman, 1986) and egr-1 (Sukhatme et al., 1988). Both of these genes are inducible to a great extent by serum and growth factors, therefore they represent good models to test inhibition effects. Cells were starved, pre-treated with 0.2µM or 0.5µM Latrunculin B and then stimulated for 30 minutes with serum, LPA, PDGF or TPA. RNA levels were determined by RNase protection assay, using probes specific to the endogenous mouse c-fos gene. Surprisingly, I found that induction of the endogenous c-fos gene, in contrast to srf and vinculin, was not significantly affected by pre-treatment with latrunculin B (Figure 4.12). Although some variation in sensitivity was observed, depending on the stimuli used (see discussion). In addition, I examined c-fos expression at the protein level by western blotting, and again no significant inhibition of c-fos expression was observed (data not shown). Taken together, these results suggest that actin polymerisation is dispensable for c-fos induction, both at the RNA and the protein level.

In contrast to c-fos, the egr-1 promoter contains five SREs, two of which do not contain well defined binding sites for TCF. I tested whether induction of egr-1 exhibited the same insensitivity to Latrunculin B as c-fos. Cells were pre-treated, as above, and then stimulated with serum or PDGF for 30, 60 and 120 minutes. To detect egr-1 transcript levels the RNase protection assay was used with an egr-1 RNA probe spanning the 5'-flanking region and part of exon I (see Materials and Methods). Both serum and PDGF induced egr-1 to a similar extent, peaking at 60 minutes and declining thereafter. Interestingly, Latrunculin B did not affect either kinetics or the
magnitude of egr-1 induction following serum or PDGF treatment (see Figure 4.13). Taken together these data suggest that there are at least two SRF target genes, which contain a well-defined TCF binding site at their SREs and are activated independently of actin dynamics.

To substantiate this finding I again used the C2 toxin (see Chapter 3), which inhibits actin polymerisation similarly to Latrunculin B. Although both Latrunculin B and C2 toxin lead to actin filament disruption, the mechanism of activity of both drugs is different, therefore it was important to determine whether both drugs have the same effect. As C2 toxin inhibits actin polymerisation by its enzymatic function the effect on the morphology of the cells was time dependent, I determined the time and active concentration of the toxin required to have an observable effect for the whole population of cells. For this reason cells were pre-treated with C2 toxin for two different lengths of times (3 or 5 hours) and then stimulated for 30 and 120 minutes (Figure 4.14). c-fos levels were determined by RNase protection as above. Cells pre-treated for either 3 or 5 hours exhibited very similar results. In both samples the c-fos induction level by serum was largely independent of C2 toxin pre-treatment. These data suggest that c-fos and egr-1 induction, in contrast to the SRF-controlled reporter, does not depend on actin polymerisation.

4.3. RhoA Activity and SRF Target Genes

4.3.1. The activity of the stably integrated SRF reporter is dependent on Rho family GTPases

4.3.1.1. C2-C3 toxin blocks activation of the SRF reporter gene

SRF activation by serum and LPA requires functional RhoA in transient transfection assays (Hill et al., 1995), however it is not clear whether PDGF- or TPA-induced SRF activation is dependent on RhoA activity. Interestingly, it has been reported that PDGF and TPA causes a reduction in RhoA-GTP loading in fibroblasts (Sander et al., 1999), (Robert Grosse, personal communication). I, therefore tested whether extracellular stimuli-induced SRF activation is dependent on RhoA. For this experiment, a cell line carrying a stably integrated SRF-controlled reporter gene,
3D.AFosHA, was utilised. Cells were starved, treated with chimaeric C2-C3 toxin for 5 hours, and then stimulated with serum, LPA, PDGF, TPA or Cytochalasin D. C2-C3 toxin pre-treatment caused inactivation of RhoA and rounding up of the treated cells, as previously described (see Figure 4.15) (Barth et al., 1998a). Induction of the SRF reporter gene was determined by RNase protection. Activation of SRF by all extracellular stimuli was completely blocked by C2-C3 toxin pre-treatment (see Figure 4.16). In contrast, activation of the reporter by Cytochalasin D, which alters actin dynamics, by interacting directly with actin, was only partially affected. These data suggest that extracellular stimuli-induced SRF activation is RhoA dependent. It is tempting to speculate that although PDGF and TPA do not activate RhoA themselves, they may require basal RhoA activity to allow transduction of the signal to SRF.

4.3.1.2. Toxin B blocks activation of the SRF reporter gene

In order to corroborate the results obtained with C2-C3 toxin, I used toxin B from Clostridium difficile, which specifically inhibits the Rho family GTPases (Rho, Rac, and Cdc42) by glucosylation (Just et al., 1994). To test the effects of toxin B treatment, cells were pre-treated for one hour with the toxin, as it could be observed at this point that almost 100% of the cells had rounded up (see Figure 4.17), and then stimulated with various agents for 30 or 120 minutes (Figure 4.18). 3D.AFos expression levels were determined by RNase protection as described above. Toxin B pre-treatment completely blocked SRF reporter gene induction by serum, LPA, PDGF and TPA, but had no effect on the cells stimulated with the actin interacting agents, Cytochalasin D or Jasplakinolide (see Chapter 5, Figure 5.8 and 5.9). As observed with C2-C3 toxin, induction of the SRF reporter gene was blocked nearly to background levels, suggesting that Rho family GTPases are absolutely necessary for signal-induced SRF activation, but are not required for SRF activation induced by actin binding drugs (see Chapter 5 and discussion).

4.3.2. Srf and vcl transcriptional activation is Rho dependent

Since it is well established that activation of the SRF-controlled reporter gene is RhoA dependent, I decided to determine whether activation of endogenous SRF target genes, such as srf and vcl, also depends on the Rho family GTPases. To test this,
I used two different toxins: C2-C3 toxin and Toxin B, as described in section 4.3.1. First, I tested the effect C3 on both *srf* and *vcl* precursor accumulation. Cells were starved, either left untreated or treated with exogenous C2-C3 toxin, and then stimulated with different agents. RNA was isolated and probed either with *srf* or *vcl* intronic probes, as previously described (see Figure 4.19A and Figure 4.20A). C2-C3 toxin absolutely abolished induction of *srf* and *vcl* precursor RNA following serum, LPA, PDGF or TPA stimulation, whereas cells stimulated by Cytochalasin D were only partially affected by toxin pre-treatment. Relative comparisons of the inhibition levels are represented in Figure 4.19B and 4.20B, respectively. Similar results were obtained when cells were treated with toxin B, which inactivates RhoA, Rac and Cdc42 (see Figures 4.21 and 4.22). These data demonstrate the absolute requirement for Rho family GTPases in the activation of *srf* and *vcl* transcription. Interestingly, the same inhibition of transcription by these toxins was observed using the SRF reporter gene, in contrast to the other subset of SRF target genes, such as *c-fos* and *egr-1*, which showed no great sensitivity to C3 or toxin B pre-treatment. Thus only those SRF target genes whose activation is critically dependent on actin polymerisation exhibit a requirement for Rho GTPase activity.

### 4.3.3. Rho activity and SRF target genes that contain TCF binding site

#### 4.3.3.1. Effects of C3 toxin on *c-fos* and *egr-1* gene induction

It has been previously shown, in transfection experiments, that SRF target genes containing a well defined TCF binding site at the SRE, such as *c-fos*, are sensitive to RhoA inhibition by the expression of C3 exoenzyme (Hill and Treisman, 1995; Kumagai *et al.*, 1993). Having observed that in certain circumstances transfected reporter genes do not follow the same induction patterns following stimulation by different agents, I therefore examined the requirement of Rho family GTPases in the activation of endogenous genes compared to transfected ones. Most of the data describing the involvement of Rho family GTPases in immediate early gene induction has been obtained using transient transfection assays. In this type of experiment, two plasmids are co-transfected, one of which is the reporter gene of interest, and the other one encoding C3 ribosyl transferase. Specificity of C3
transferase from *C. botulinum* towards Rho has been well described (Paterson *et al.*, 1990). However, reports concerning Rho involvement in endogenous gene activation are limited (Alberts *et al.*, 1998; Beltman *et al.*, 1999). This is due to technical problems, arising from the requirement to deliver the C3 toxin plasmid to a large proportion of the cell population - which ideally should be close to 100%. Alternatively, the microinjection technique can be used, where an expression of the endogenous gene can be monitored by indirect immunofluorescence in a single cell, after injection of C3 or another inhibitory construct. However, in this type of assay, quantification of fluorescent signal is difficult.

To overcome the need for very high plasmid transfection efficiency, I used a purified chimaeric toxin C2-C3, applied exogenously without transfection, which enters cells and ADP-ribosylates and therefore inactivates RhoA (Barth *et al.*, 1998a). To test this procedure, cells were starved, treated with the C2-C3 exogenous toxin for 5 hours, causing all populations the cells to round up (*Figure 4.15*), and then stimulated with serum, LPA, PDGF or TPA for 30 minutes. The levels of endogenous *c-fos* and *egr-1* induction were measured simultaneously by RNase protection as previously described (see *Figure 4.23A*). The activation of *c-fos* following serum stimulation was not affected by pre-treatment with C2-C3 toxin, although inducibility by LPA, PDGF and TPA was somewhat reduced. Interestingly, *egr-1* inducibility was not affected at all. Quantification of this experiment is shown in *Figure 4.23B/C*. The results examining endogenous *c-fos* gene sensitivity to C2-C3, are not in exact agreement with previously reported data, using C3 and transfected Fos templates (Hill *et al.*, 1995). In order to confirm these results, I used toxin B from *Clostridium difficile* using similar conditions as those described in the previous section. In this experiment, cells were pre-treated for one hour with toxin B, and then stimulated with various agents for 30 or 120 minutes (*Figure 4.24A*). The results obtained with toxin B were very similar to those obtained using C2-C3 toxins (see *Figure 4.24B/C* for quantification of the data). Toxin B treatment caused around 50% reduction in serum- and PDGF-induced *c-fos* transcription, but substantially reduced that by LPA; TPA induction was not significantly affected. Again, similarly as observed with C2-C3, *egr-1* activation was not significantly affected by toxin B, except in the case of LPA treatment.

Taken together these data suggest that transcription of endogenous *c-fos* does not absolutely require active RhoA following serum stimulation, although stimulation by LPA, PDGF and TPA is partially sensitive to inhibition of RhoA. Activation of endogenous *egr-1* by all stimuli tested, with the exception of LPA, appears to be
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RhoA independent. Interestingly, the effects on gene induction levels obtained with C3 toxin and toxin B are reminiscent of the effects observed with Latrunculin B. These data suggest that RhoA input on the actin cytoskeleton is not necessary to activate *c-fos* and *egr-I*. However, RhoA activity might be required for efficient signal transmission, possibly by contributing to the activation of another signalling pathway, such as Ras-MEK-ERK.

4.3.3.2. C3 toxin inhibits, but does not block activation of transfected Fos templates

Given that endogenous *c-fos* induction is less sensitive to C3 treatment, I decided to re-confirm data obtained previously in the laboratory, using the Dextran-DEAE transfection technique (Hill *et al.*, 1995). NIH3T3 cells were co-transfected with the Fos reporter or SRF-controlled 3D.AFos reporter and the C3 expressing plasmid together with a reference plasmid, α-globin. The SRF-controlled reporter was used as a positive control to ensure, that in the conditions used, C3 efficiently inhibits RhoA activity. Cells were transfected with or without a C3 encoding plasmid and then stimulated with serum, LPA, PDGF or TPA for 30 minutes. In addition, Cytochalasin D was used as a control to make sure that C3 did not exert non-specific effects on other signalling pathways, since activation of 3D.AFos reporter by Cytochalasin D is known to be insensitive to C3 pre-treatment (Sotiropoulos *et al.*, 1999). RNase protection was performed with Fos WT probe, allowing to distinguish between Fos and 3D.AFos templates. RNA levels and transfection efficiency was monitored with an α-globin reference plasmid (see Figure 4.25 and the figure legend). In the presence of C3, the induction of 3D.AFos reporter was nearly undetectable following the addition of all stimuli, except Cytochalasin D. In contrast, in the presence of C3, the Fos reporter construct was still inducible by serum, PDGF or TPA, although at reduced levels (see Figure 4.26). It is worth mentioning that inducibility of the Fos reporter gene by LPA was reduced almost to background levels, similarly to inhibition observed for endogenous *c-fos* case using C2-C3 toxin, as described in section 4.3.3.1. Taken together this data suggests that transfected Fos templates are more sensitive to RhoA inhibition compared to the endogenous *c-fos* gene. Thus, when transfected, sensitivity of the SRF-controlled reporter gene and Fos gene to C3 differs dramatically: 3D.AFos are absolutely dependent on RhoA activity, and Fos requires Rho A activity for maximum inducibility (see discussion).
4.4. MAPK and c-fos Activation

It has been previously demonstrated using PD98059 inhibitor that c-fos transcriptional activation critically depends on the MAPK pathway (Price et al., 1996). During the course of this work I extensively used the U0126 inhibitor, therefore, I first tested whether 10 μM of U0126 would affect c-fos inducibility with a view to compare how the same concentrations of U0126 affects other SRF target genes. In this experiment starved cells were pre-treated with 10 μM U0126, then stimulated with serum, LPA, PDGF or TPA for 30 minutes. RNA levels were then determined by RNase protection assay. 10μM U0126 pre-treatment inhibited c-fos by 85 % following serum and essentially abolished induction levels following LPA, PDGF or TPA stimulation (see Figure 4.27). The same concentration of inhibitor did not substantially inhibit activation of the SRF-controlled reporter, 3D.AFos, as described in Chapter 3 (see Figure 3.6). These data strongly imply that the c-fos and the 3D.AFos reporter exhibit different sensitivities to the ERK signalling pathway. Based on these findings and those determined previously, I decided to directly compare SRF target gene sensitivity to actin dynamics and ERK signalling pathways.

4.5. SRF Target Genes Differ in Their Sensitivity to Rho-Actin Dynamics and ERK Signalling Pathway

To directly compare the effects of ERK and actin signalling pathways on the induction of SRF target genes, SRE.FosHA cells were pre-treated with either Latrunculin B or U0126 inhibitor, or in combination, before stimulating with a number of various agents. Gene expression or RNA precursor levels were tested by RNase protection for c-fos, egr-1, srf, vcl and JunB transcription. As expected, pre-treatment with Latrunculin B effectively blocked activation of srf and vcl transcription (see Figure 4.28A/B). In contrast, activation of c-fos, egr-1 and JunB was strikingly less sensitive to the inhibitor, egr-1 transcription was essentially unaffected, while c-fos and junB induction were reduced by only 30-40% (see Figure 4.29 and Figure 4.31). A small residual activation of these genes by serum in U0126 treated cells (see bellow) was also sensitive to Latrunculin B treatment. Next I evaluated the
contribution of MEK-ERK signalling to the transcriptional activation of the same panel of genes, using the U0126 inhibitor. The inhibitor reduced activation of \textit{srf} and \textit{vcl} genes by serum, LPA and PDGF by 30-50\%, with \textit{srf} being slightly less sensitive than \textit{vcl} (see Figure 4.28A/B and Figure 4.28C/D); only in the case of TPA did U0126 block induction. In contrast, U0126 treatment effectively prevented induction of c-fos, \textit{egr-1} and JunB transcription by all of the stimuli under investigation, although residual \textit{c-fos} induction by serum was still detectable. Interestingly, Latrunculin B and U0126 effects on activation of the SRF-controlled reporter gene are very similar to those observed in \textit{srf} and \textit{vcl} precursor RNA induction (compare Figure 4.28A/B and Figure 4.30).

Since previously I had observed differences between transfected and integrated templates, I tested whether transfected \textit{fos} templates exert the same sensitivity with respect to treatment of Latrunculin B and U0126. As shown in Figure 4.32, transfected Fos behaves in a similar manner to the endogenous gene: activation of \textit{c-fos} was not sensitive to Latrunculin B treatment, but was however completely inhibited with U0126 inhibitor. Thus, with respect of these treatments, the behaviour of the \textit{srf} and \textit{vcl} genes resembles that of the SRF reporter, whereas those of \textit{c-fos}, \textit{egr-1} and \textit{junB} are similar to that of the TCF reporter.

Taken together these data show that at least two classes of SRF target genes can be distinguished on the basis of their relative sensitivity to Rho-actin and MEK-ERK signalling pathways.

4.6. PI3-kinase Activity Is Dispensable for Serum and LPA, But Required for PDGF and TPA Induced \textit{srf} and \textit{vinculin} Gene Activation

I also examined the effect of the PI-3 K inhibitors LY294002 and wortmannin on activation of the various target genes, because this treatment also differentially effects SRF and TCF reporters. RNase protection assay was employed as described previously. Serum starved cells were pre-treated with LY294002 or wortmannin, and then stimulated with serum, LPA, PDGF or TPA. Transcriptional activation of the \textit{srf} and \textit{vcl} genes exerted different sensitivity to the PI-3 K inhibitors, LY294002 or wortmannin; induction by PDGF and TPA was effectively blocked and induction by LPA reduced some 50\%, whereas serum induction was either slightly impaired (\textit{srf}) or not affected (\textit{vcl}) (see Figure 4.33 and Figure 4.34). Similar effects of the PI-3 K inhibitors were observed when using the SRF reporter gene, 3D.AFos (see Chapter 3):
serum- and LPA-induced reporter gene activation was essentially unimpaired, whereas PDGF- and TPA-induced SRF activation was substantially inhibited. In contrast, activation of \textit{fos} and \textit{egr-1} transcription by all stimuli was insensitive to LY294002, with \textit{fos} transcription actually showing a slight enhancement of activation (*Figure* 4.35). As with MEK-ERK and actin signalling, PI-3 K thus makes qualitatively distinct contributions to \textit{srf} and \textit{vcl}, compared with \textit{fos} and \textit{egr-1}. These data suggest that PI-3 K pathway is essential for \textit{srf} and \textit{vcl}, but not for \textit{fos} and \textit{egr-1}, activation by PDGF and TPA. It is tempting to suggest that these stimuli require PI-3 K activity to impinge on the actin treadmilling cycle, eventually leading to transcriptional activation of SRF.
Figure 4.1. Latrunculin B disrupts the actin cytoskeleton. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 0.5\(\mu\)M Latrunculin B (Lat B) prior to addition of various stimuli. Phase-contrast images of the cells are shown.
### Figure 4.2 Latrunculin B does not inhibit Akt or ERK activation by stimuli tested.

Serum-deprived NIH3T3 cells were pretreated for 60 minutes with 10μM U0126 and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for times indicated on the figure. The cell lysates were pre-cleared by centrifugation, fractionated by SDS-PAGE and transferred to PVDF membrane before immunoblotting for: phospho-Akt, total Akt and diphospho-ERK.
Figure 4.3. TCF Elk-1 activation is not dependent on actin polymerisation. NIH3T3 cells were transfected with an expression plasmid encoding the chimeric transactivator NLexElkC together with the Lex operator-controlled luciferase reporter gene, maintained in 0.5% PCS for 24 hr, then stimulated following 30 minutes pretreatment with 0.5 μM Latrunculin B as indicated. Data are normalised to the serum response as 100; error bars indicate SEM from 3 independent transfections, where not shown, the SEM was always less than 20% of the experimental value.
Figure 4.4. Activation of SRF by all stimuli requires actin polymerisation. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with different amount of Latrunculin B (Lat B) as indicated in the figure and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.5. Inhibition of SRF reporter activity by C2 toxin. A. Serum-deprived SRE.FosHA cells were pretreated for 3 or 5 hours with C2 toxin and then stimulated for 30 or 120 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels are expressed as a percentage of those induced by serum at 30 minutes.
Figure 4.6. Endogenous srf gene activation requires actin polymerisation. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 0.5μM Latrunculin B (Lat B) and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for times indicated on the figure. srf and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels are expressed fold induction.
Figure 4.7. Endogenous vcl gene activation requires actin polymerisation. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 0.5μM Latrunculin B (Lat B) and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for times indicated on the figure. Vinculin (vcl) and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as fold induction are shown.
Figure 4.8. Schematic representation of *srf* and *vcl* intronic RNase protection probes. RNA probes are represented in blue; protected fragments in red.
Figure 4.9. Induction of srf and vcl (vinculin) precursor RNAs. NIH3T3 cells were maintained in 0.5% FBS for 36 hours and then stimulated with 15% Fetal Calf Serum (SER) for the times indicated on the figure. Transcripts levels were quantified by RNase protection. A. Nuclease resistant fragments derived from precursor srf (pre-srf), srf and GAPDH are indicated. B. Nuclease resistant fragments derived from precursor vcl (pre-vcl), vcl and GAPDH are indicate. C. Relative pre-srf and srf transcript levels expressed as fold induction. D. Relative pre-vcl and vcl transcript levels expressed as a fold induction.
Figure 4.10. Transient kinetics of \textit{srf} gene activation in NIH3T3 cells. NIH3T3 cells maintained in 0.5% FBS for 36 hours and then stimulated with 10\textmu M LPA, 25 ng/ml PDGF or 50 ng/ml TPA for the times indicated on the figure. Transcripts levels were quantified by RNase protection. \textbf{A.} Nuclease resistant fragments derived from precursor \textit{srf} (pre-\textit{srf}), \textit{srf} and GAPDH are indicated on the figure. \textbf{B.} Relative pre-\textit{srf} transcript levels expressed as a fold induction are shown.
Figure 4.11. Transient kinetics of vcl (vinculin) gene activation in NIH3T3 cells. NIH3T3 cells maintained in 0.5% FBS for 36 hours and then stimulated with 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for the times indicated on the figure. Transcripts levels were quantified by RNase protection. A. Nuclease resistant fragments derived from precursor vcl (pre-vcl), vcl and GAPDH are indicated on the figure. B. Relative pre-vcl transcript levels expressed as a fold induction are shown.
Figure 4.12. Actin polymerisation is not required for c-fos activation. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 0.2μM and 0.5μM Latrunculin B (Lat B) and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 minutes as indicated on the figure. c-fos and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels are expressed as a percentage of those induced by serum at 30 minutes.
Figure 4.13. Actin polymerisation is not required for egr1 activation. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 0.5μM Latrunculin B (Lat B) and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for the times indicated on the figure. Egr1 and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 60 minutes are shown.
Figure 4.14. C2 toxin does not block c-fos activation. A. Serum-deprived SRE.FosHA cells were pretreated for 3 or 5 hours with C2 toxin and then stimulated for 30 or 120 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. c-fos and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.15. C2-C3 toxin treatment causes the loss of stress fibres and rounding up of cells. Serum-deprived SRE.FosHA cells were pretreated for 5 hours with C2-C3 toxin as described in the materials and methods section. Phase-contrast images of the cells are show.
Figure 4.16. Activation of the SRF reporter is Rho dependent. A. Serum-deprived SRE.FosHA cells were pretreated for 5 hours with C2-C3 toxin and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. 3DA.Fos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Control + Toxin B

Figure 4.17. Toxin B treatment causes the loss of stress fibres and rounding up of cells. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 5ng/ml Toxin B as described in the materials and methods section. Phase-contrast images of the cells before the addition of stimuli are shown.
Figure 4.18. Inhibition of SRF reporter activity by Toxin B. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 5ng/ml Toxin B and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 and 120 minutes as indicated on the figure. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.19. Activation of the srf gene is dependent on Rho activity. A. Serum-deprived SRE.FosHA cells were pretreated for 5 hours with C2-C3 toxin and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from precursor srf (pre-srf) and GAPDH are indicated. B. Relative pre-srf transcript levels expressed as a fold induction are shown.
Figure 4.20. Activation of vcl (vinculin) gene is dependent on Rho activity. A. Serum-deprived SRE.FosHA cells were pretreated for 5 hours with C2-C3 toxin and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from precursor vcl (pre-vcl) and GAPDH are indicated. B. Relative pre-vcl transcript levels expressed as a fold induction are shown.
Figure 4.21. Activation of *vcl* (vinculin) gene is dependent on Rho activity. A. Serum-deprived SRE.FosHA cells were pretreated for 5 hours with C2-C3 toxin and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from precursor *vcl* (pre-*vcl*) and GAPDH are indicated. B. Relative pre-*vcl* transcript levels expressed as a fold induction are shown.
Figure 4.22. Inhibition of vcl (vinculin) gene induction by Toxin B. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 5ng/ml Toxin B and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 and 120 minutes as indicated on the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from precursor vcl (pre-vcl) and GAPDH are indicated. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.23. Activation of *c-fos* and *egr-1* genes is not dependent on Rho activity. A. Serum-deprived SRE.FosHA cells were pretreated for 5 hours with C2-C3 toxin and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. *c-fos*, *egr-1* and GAPDH transcripts were quantified by RNase protection. Relative transcript levels of *c-fos* (B) and *egr-1* (C), expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.24A. Toxin B effects on c-fos and egr-1 activation. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 5ng/ml Toxin B and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 and 120 minutes as indicated on the figure. c-fos, egr-1 and GAPDH transcripts were quantified by RNase protection.
Figure 4.24B/C. Toxin B effects on c-fos and egr-1 activation. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 5ng/ml Toxin B and then stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for 30 and 120 minutes as indicated on the figure. c-fos, egr-1 and GAPDH transcripts were quantified by RNase protection. Relative transcript levels of c-fos (B) and egr-1 (C), expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.25. Transiently transfected SRF reporter activity is dependent on Rho.
A. NIH3T3 were transfected with 3D.AFos reporter, C3 expression construct and α-globin reference plasmid. Cells were maintained in 0.5% FBS for 24 hours and then stimulated for 30 minutes with 15% Fetal Calf Serum (S), 10μM LPA (L), 25 ng/ml PDGF (P), 50 ng/ml TPA (T) or 2μM Cytochalasin D (C). 3D.AFos reporter and α-globin transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.26. Activation of a transiently transfected Fos gene is not dependent on Rho activity. A. NIH3T3 were transfected with fos gene, C3 expression construct and α-globin reference plasmid. Cells were maintained in 0.5% FBS for 24 hours and then stimulated for 30 minutes with 15% Fetal Calf Serum (S), 10µM LPA (L), 25 ng/ml PDGF (P), 50 ng/ml TPA (T) or 2µM Cytochalasin D (C). Fos and α-globin transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.27. The activation of c-fos transcription is dependent on MEK. A. Serum-deprived SRE.FosHA cells were pretreated for 30 minutes with 10μM U0126 and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. c-fos and GAPDH transcripts were quantified by RNase protection. B. Transcript levels at 30 minutes expressed as a percentage of those in untreated cells are shown (mean+/− range, 2 independent experiments).
Figure 4.28A/B. SRF target genes are differentially dependent on actin polymerisation and MEK activity. Serum-deprived SRE.FosHA cells were pretreated with 10μM U0126 (U), 0.5μM Latrunculin B (L) or both (U/LB) and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from precursor pre-srf (A), srf (A), pre-vcl (B) and GAPDH are indicated.
Figure 4.28C/D. SRF target genes are differentially dependent on actin polymerisation and MEK activity. A./B. Serum-deprived SRE.FosHA cells were pretreated with 10μM U0126 (U), 0.5μM Latrunculin B (L) or both (U/LB) and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure 4.28A/B. Srf and vcl activation was reduced to background levels after LB treatment. Transcript levels of U0126 treated cells expressed as a percentage of those in untreated cells are shown (mean+/−range, 2 independent experiments) pre-srf (C) and pre-vcl (D).
Figure 4.29. SRF target genes differential dependence on actin polymerisation and MEK activity. A. Serum-deprived SRE.FosHA cells were pretreated with 10μM U0126 (U), 0.5μM Latrunculin B (L) or both (U/LB) and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. Egr-1, c-fos and GAPDH transcripts levels were quantified by RNase protection. B/C. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.30. SRF reporter is inhibited by the G-actin sequestering drug Latrunculin B, but not by U0126. A. Serum-deprived SRE.FosHA cells were pretreated with 10μM U0126 (U), 0.5μM Latrunculin B (L) or both (U/LB) and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Transcript levels at 30 minutes expressed as a percentage of those in untreated cells are shown. Reporter activity in LB treated cells was reduced to background levels. U0126 pretreatment resulted in only partial inhibition of the SRF reporter (for quantification see also Figure 3.6).
Figure 4.31. JunB differential dependence on actin polymerisation and MEK activity. A. Serum-deprived SRE.FosHA cells were pretreated with 10μM U0126 (U), 0.5μM Latrunculin B (L) or both (U/LB) and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. JunB and GAPDH transcripts levels were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.32. Sensitivity of transient transfected Fos gene to the inhibition of actin polymerisation and MEK activity. A. NIH3T3 were transfected with 0.5μg Fos (F711) reporter gene and carrier plasmid MLVβ128. Cells maintained in 0.5% FBS for 24 hours, pretreated with 10μM U0126 (U), 0.5μM Latrunculin B (L) or both (U/LB) and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 4.33. Differential dependence on PI-3 kinase for srf activation. A. Serum-deprived cells were pretreated for 30 minutes with 20μM LY294002 (LY) or 0.2μM wortmannin (W) and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated on the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from precursor pre-srf, srf and GAPDH are indicated. B. Transcript levels of pre-srf at 30 minutes expressed as a percentage of those in untreated cells are shown (mean+-/-range, 2 independent experiments).
Figure 4.34. Differential dependence on PI-3 kinase for vcl activation. A. Serum-deprived cells were pretreated for 30 minutes with 20µM LY294002 (LY) or 0.2µM wortmannin (W) and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10µM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from precursor pre-vcl and GAPDH are indicated. B. Transcript levels of pre-vcl at 30 minutes expressed as a percentage of those in untreated cells are shown (mean+/range, 2 independent experiments).
Figure 4.35. *c-fos* and *egr-1* activation do not depend on PI-3 K activity. A. Serum-deprived cells were pretreated for 30 minutes with 20µM LY294002 (LY) and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER), 10µM LPA, 25 ng/ml PDGF or 50 ng/ml TPA as indicated in the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from precursor *egr1*, *c-fos* and GAPDH are indicated. B/C. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
5. Actin Dynamics and SRF Target Genes

5.1. Chapter Summary

In this chapter I present the effects of changes in actin dynamics to an SRF reporter gene and endogenous SRF target genes. Conventional RNase protection assays were employed for SRF reporter, c-fos and egr-1. A novel approach, based on RNase protection assays using intronic probes was employed for measuring srf and vinculin (vcl) transcriptional activation. The data presented reveals that the actin binding drugs induce a subset of SRF target genes. Changes in G-actin levels result in transient transcriptional activation of srf and vcl. In contrast, another subset of SRF target genes, including c-fos, is not induced by any of the actin binding drugs tested. It has been demonstrated that changes in actin dynamics are sufficient to activate srf and vcl genes and that this activation is not dependent on new protein synthesis.

5.2. Actin Binding Drugs Activate SRF

5.2.1. Actin binding drugs that stabilise F-actin activate SRF

Recent findings in the laboratory by A. Sotiropoulos suggest that LIMK activates SRF by increasing F-actin levels in the cell (Sotiropoulos et al., 1999). Therefore, the inducibility of a stably integrated SRF reporter gene by different actin binding drugs was tested. First, I tested the kinetics of the SRF reporter gene activation after treatment with Jasplakinolide, an actin binding drug which has been reported to increase F-actin levels (Bubb et al., 1994). In this experiment, cells were starved, and then stimulated with Jasplakinolide for different amounts of time. RNA was collected and analysed by RNase protection, as described in previous chapters (see Figure 5.1). Jasplakinolide strongly activated transcription of the SRF reporter gene with kinetics similar to those of serum stimulation. This data suggests that level of F-actin affects SRF activity. This is consistent with data demonstrating that
overexpression of the WASP family proteins or constitutively active Dia also strongly activate the SRF reporter gene (Sotiropoulos et al., 1999). Thus, in addition to LIMK, actin binding drugs and proteins that promote F-actin accumulation, are sufficient to activate SRF. It is proposed that Jasplakinolide activates transcription via direct interaction with actin (see also below and section 5.3.1).

5.2.2. SRF responds to G-actin levels

Experiments described in previous sections demonstrate that increased F-actin levels are associated with increased SRF activity, however, they do not address the question whether SRF senses increased F-actin level, an increased F-/G-actin ratio, or a decreased G-actin level. To address this issue two other actin binding drugs, Cytochalasin D and Swinholide A, were used. Cytochalasin D caps actin filaments, stimulates ATP hydrolysis and, thereby promotes disassembly of F-actin. Swinholide A also destabilises F-actin, however it sequesters G-actin as dimers (Bubb et al., 1995). Therefore, Cytochalasin D and Swinholide A cause opposite effects as compared to Jasplakinolide which promotes actin accumulation (Sampath and Pollard, 1991). In this experiment, SRE.FosHA cells were starved and then stimulated with 2μM Cytochalasin D or 0.1μM Swinholide A for 30, 60, 90, 120, and 180 minutes. 3D.AFos reporter levels were determined by RNase protection assay. Both Cytochalasin D and Swinholide A strongly activated the SRF reporter genes with prolonged activation kinetics similar to those of Jasplakinolide (see Figure 5.2). These data strongly suggest that it is not an increase of F-actin, but rather a decrease in G-actin, which leads to transcriptional activation of SRF. The fast kinetics of SRF activation by both compounds is consistent with participation of actin itself in regulation of SRF activity, possibly by interaction in regulatory molecule.

5.3. Actin Binding Drugs Compete for Actin Binding Sites to Activate SRF

5.3.1. Latrunculin B affects on Jasplakinolide- and Cytochalasin D-induced SRF activation
As described in Chapter 4, Latrunculin B completely blocks serum-induced activation of the SRF-controlled reporter gene. I wanted to determine whether Latrunculin B, which binds G actin and severes actin filaments, would block activation of SRF induced by Jasplakinolide, which has an opposite effect on the actin cytoskeleton by stabilising actin filaments. For this reason, cells were pretreated with 0.2μM or 0.5μM Latrunculin B for 60 minutes and then stimulated with Jasplakinolide for 30 minutes. BDA.Fos reporter gene induction levels were determined by RNase protection assay (Figure 5.3). 0.2μM Latrunculin B inhibited the SRF reporter gene induction by 80%, while 0.5μM completely blocked the induction by Jasplakinolide. This data suggests that inhibition of SRF activation by Latrunculin B is concentration dependent and that these two drugs compete for binding sites on an actin molecule. In addition, it was tested whether Cytochalasin D-induced SRF activation is blocked by pretreatment with Latrunculin B. In this experiment, cells were pretreated with 0.2, 0.5, 1.0 or 1.5μM Latrunculin B and then stimulated with 2μM of Cytochalasin D. Similarly to Jasplakinolide, Cytochalasin D-induced SRF activation was inhibited by Latrunculin B (Figure 5.4). A 1.5μM concentration of Latrunculin B was required to fully block the SRF reporter gene activation by Cytochalasin D, compared to 0.5μM required to block serum-induced SRF reporter activation. Taken together these data suggest that Latrunculin B is able to compete with Cytochalasin D and Jasplakinolide for actin binding, and thus block SRF activation. This may occur through titration of an actin interacting partner molecule that is involved in SRF regulation.

5.3.2. Latrunculin B retards, but does not block Jasplakinolide-induced SRF activation

Since Latrunculin B blocked Jasplakinolide induced SRF activation at the 30 minute time point in a concentration dependent manner, it was hypothesised that Jasplakinolide-induced SRF activation would be less sensitive to inhibition by Latrunculin B at later time points following Jasplakinolide addition. To test this, cells were starved, pretreated with 0.5μM Latrunculin B and then stimulated with serum or Jasplakinolide for 30, 60, 120 and 180 minutes. 3D.AFos transcript levels were determined by RNase protection. Jasplakinolide strongly activated transcription of the SRF reporter gene, although with somewhat slower and more prolonged kinetics than serum (Figure 5.5). As observed previously, Jasplakinolide-induced activation of the SRF reporter gene was substantially inhibited at the 30 minute time point, however by
60 minutes the inducibility of the SRF reporter started to recover, reaching a complete recovery at 120 minutes. In contrast serum-induced reporter activation was blocked all time points tested. This data suggests that the Jasplakinolide and Latrunculin B competition for actin binding is not only a concentration but also a time dependent process. This is consistent with the view that the two drugs bind reversibly to different types of actin target (see discussion).

5.4. Activation of Endogenous SRF Targets Genes by Actin Binding Drugs

5.4.1. Actin binding drugs activate srf and vcl (vinculin) endogenous genes

Previous studies have suggested that expression of two endogenous SRF target genes, cytoskeletal actin and vinculin, responds to actin expression levels (Bershadsky et al., 1995) (Reuner et al., 1996). Having established that actin binding drugs activate the SRF reporter gene, I determined whether actin binding drugs would also activate endogenous SRF target genes. For this reason, NIH3T3 cells were starved and then stimulated with 0.5µM Jasplakinolide, 2µM Cytochalasin D or 0.1µM Swinholide A for 30, 60, 90 or 120 minutes. The srf and vcl gene induction levels were determined by RNase protection (Figure 5.6 and Figure 5.7). Both genes were induced by all actin binding drugs tested. The kinetics of induction of both srf and vcl were a fraction delayed compared to serum-induced activation of these genes. That is consistent with the fact that the actin binding drugs have a cumulative effect on transcription, since they require a time window to achieve equilibrium of binding between the actin binding drug and actin itself. The level of induction was similar to that observed with serum, reaching 2-4 fold induction at the 120 minutes time point. The low level of induction levels of srf and vcl are due to the relatively high basal levels of these genes in starved cells. A significant amount of RNA synthesis is required to achieve, for example, a two fold induction over background level. In the case of the SRF reporter gene, 3D.AFos, even small changes in RNA synthesis are readily detectable due to the very low level of the activity of the reporter gene in starved cells (compare Figure 5.1, 5.2 with 5.6, 5.7). Taken together, these data suggest that actin binding drugs efficiently activate a subset of endogenous SRF target genes.
5.4.2. Actin binding drugs efficiently induce transcription of \textit{srf} and \textit{vcl} in a Rho independent manner

As described in Chapter 4, \textit{srf} and \textit{vcl} RNA synthesis is induced at 15 and 30 minutes respectively following serum stimulation, as judged by precursor RNA levels. Examining the effect of actin binding drugs on the induction of \textit{srf} and \textit{vcl} mRNA levels, it was observed that the induction of the SRF reporter gene by these drugs is delayed compared to serum stimulation. To clarify whether this delay occurs due to late transcription initiation, or slow mRNA accumulation, I re-examine the induction of \textit{srf} and \textit{vcl} using intronic \textit{srf} and vinculin probes (described in Chapter 4, Figure 4.19). In addition, I examined whether activation of \textit{srf} and \textit{vcl} by the actin binding drugs occurs in a Rho-independent manner. This would support the notion that the actin binding drugs activate SRF directly due to their effect on the actin treadmilling cycle. In this experiment, cells were starved pretreated with toxin B, and then stimulated for 30 or 120 minutes with Jasplakinolide or Cytochalasin D. Cells were lysed and RNA levels were analysed by RNase protection assay using intronic \textit{srf} and \textit{vcl} probes (see Figure 5.8 and Figure 5.9). Both \textit{srf} and \textit{vcl} RNA precursors were induced at 30 minutes. In contrast to serum induction, \textit{vcl} and \textit{srf} precursor RNA were detectable at 120 minutes by induction with the drugs. This observation is consistent with the data obtained using the SRF-controlled reporter gene 3D.AFos, where similar kinetic patterns were observed when cells were treated with actin binding drugs (see Figure 5.1 and 5.2). This data suggests that Jasplakinolide and Cytochalasin D induce \textit{srf} and \textit{vcl} genes with very similar, although a fraction prolonged kinetics compared to serum and other extracellular stimuli. Inhibition of Rho family GTPases did not have any substantial effects on transcriptional activation of \textit{srf} and \textit{vcl}. This data suggests that the actin binding drugs activate transcription due to direct effects on actin dynamics, rather than indirect activation of other signals.

5.4.3. Actin binding drugs do not activate the \textit{c-fos} promoter

It has been previously reported that RhoA is both necessary and sufficient to activate the \textit{c-fos} promoter (Alberts \textit{et al.}, 1998; Hill \textit{et al.}, 1995). In addition, it has been shown that the Rho effector Diaphanous takes part in actin polymerisation and therefore contributes to SRF activation (Tominaga \textit{et al.}, 2000; Watanabe \textit{et al.}, 1999). I therefore wanted to establish whether alterations in actin dynamics caused by
the actin binding drugs are sufficient to activate c-fos. For this reason, the ability of several actin binding drugs were tested to activate the endogenous c-fos gene. NIH3T3 cells were starved and then stimulated with Cytochalasin D, Jasplakinolide or Swinholide A for 30, 60 or 120 minutes. c-fos induction was determined by the RNase protection assay (see Figure 5.10). Surprisingly, no c-fos induction was observed when cells were treated with Jasplakinolide or Swinholide A. Cytochalasin D-induced c-fos activation was somewhat 2-3 fold above background, which is negligible compared to the serum-induced levels of c-fos transcription which reach up to 100 fold (see, for example, Figure 4.29). Previous observations demonstrate that Latrunculin B is unable to block c-fos activation (see Chapter 4, section 4.2.5). These findings taken together demonstrate that actin dynamics are neither necessary nor sufficient to activate the endogenous c-fos gene.

As previously described, RhoA family GTPases are capable of activating transcription from transfected or injected templates, therefore the possibility remained that actin dynamics contribute to activation of transfected Fos templates. However, as described in Chapter 4 (see Figure 4.26), transiently transfected fos templates were refractory to Cytochalasin D stimulation. In addition, transiently transfected Fos template was also refractory to stimulation by the actin binding drug, Jasplakinolide (K.Murai, personal communication). These data demonstrate that actin dynamics contribute little to either endogenous or transfected fos gene transcriptional activation.

5.5. Differences between the Kinetics of the srf and vcl Gene Induction

It was observed that, although srf and vinculin genes are induced by the same stimuli and are sensitive to the same inhibitors, there are some differences in the kinetics of induction. As described in Chapter 4, vinculin induction is delayed both at mRNA and precursor levels when compared to srf. This delay of induction is observed with all extracellular stimuli under investigation, suggesting that it is not restricted to a specific signalling pathway. In addition, a similar phenomena was observed in a Swiss3T3 cell line (described in section 5.5.3). I envisaged two possibilities: (i) activation of the vinculin promoter requires a newly synthesised protein, and therefore vinculin would not be classified as an immediate early gene, which is not in agreement with previously reported data (Ben-Ze'ev et al., 1990); (ii) the late transcriptional activation of vinculin is a feature of the vinculin promoter itself. Two different protein synthesis inhibitors were used to determine whether activation of the vcl gene is direct.
Chapter 5: Results

In addition, by using different actin binding drugs I investigated the precise kinetics of vcl and srf gene transcriptional activation in order to demonstrate that direct intervention with the actin treadmill cycle leads to the same delay in activation of the vcl gene.

5.5.1. Activation of srf and vcl genes do not require new protein synthesis

To test whether the activation of srf and vinculin is direct I used two different protein synthesis inhibitors, cycloheximide and anisomycin. NIH3T3 cells were starved, pretreated or left untreated for 30 minutes with 10 µg/ml of cycloheximide or 25 µg/ml anisomycin, and then stimulated with 15% FBS for 15, 30, 60 or 120 minutes. srf and vcl precursor and mRNA levels were determined by RNase protection assay as described in previous sections (see Figure 5.11A and 5.11B). As a positive control, I used the same RNA samples to determined whether cycloheximide and anisomycin effect c-fos expression (see Figure 5.11C) which has been previously described (Hazzalin et al., 1998; Treisman, 1985). Similarly, as observed previously, vcl precursor was not detectable at 15 minutes, although srf precursor was readily detectable. Consistent with this, mRNA levels of vcl were not induced until 60 minutes, in contrast to srf, where a 2 fold induction was observed at the 30 minute time point. Both srf and vcl precursor RNA peaked at the 30 minute time point, declining thereafter (for quantification of the data, see Figure 5.12(A-E)). Pretreatment with anisomycin and cycloheximide resulted in very similar effects. Neither of the drugs blocked induction of srf or vcl precursors. In contrast, the decline in the level of was abolished until 120 minutes after stimulation. Consistent with this, both srf and vcl mRNA levels were superinduced, possibly, as a result of the prolonged transcription. As reported previously (Wilson and Treisman, 1988), c-fos induction was not downregulated upon cycloheximide treatment, and superinduction of the c-fos message was seen at 120 minutes (see Figure 5.11C). Taken together these data directly demonstrate that both srf and vcl transcriptional activation do not require new protein synthesis, and thus support the case that both vcl and srf are members of the immediate early gene family. Evidence from the precursor kinetics suggests that superinduction does not occur through stabilisation of the message, but due to prolonged transcriptional activation.
5.5.2. Actin binding drugs do not rescue the delay of vinculin induction

As described in Chapter 4, srf and vcl genes are induced by various actin binding drugs. However, examination of srf and vcl mRNA levels did not reveal the kinetics of transcriptional activation. Therefore, I determined the kinetics of transcriptional activation by different actin binding drugs using intronic riboprobes. This would allow the determination of the precise differences in the activation of srf and vcl genes, especially concerning the delay of vcl induction. For this reason NIH3T3 cells were serum deprived for 36 hours, and then stimulated with 15% FBS, 0.5μM Jasplakinolide, 2μM Cytochalasin D or 0.2μM Swinholide A. RNA was harvested and analysed for precursor and mRNA levels of srf (Figure 5.13A) and vinculin (Figure 5.13B), respectively. Serum stimulation was used as a reference in order to compare relative inducibility by different actin binding drugs. srf and vcl precursors were induced by Jasplakinolide, Cytochalasin D or Swinholide A, peaking at 30 minute point and declining during a 2 hour period. However, at the 15 minute time point only the srf precursor was induced by all three drugs as well as by serum, in contrast to vcl precursor, whose levels were unchanged (see Figure 5.14 for quantification of the data). Jasplakinolide and Cytochalasin D efficiently induced srf and vcl mRNA, reaching maximum levels between 60 and 120 minutes. Taken together these data suggest that the kinetics of srf and vcl transcription by actin drugs resembles that of serum, where a slightly prolonged persistence of precursor RNA is seen for vcl, as judged by intronic precursor levels. The delay of vinculin induction observed with all 3 differently acting drugs indicate that the delay is a feature of the vinculin promoter itself, and does not depend on the signalling pathway used for its activation. Although, it remains possible that the kinetics of vcl precursor induction does not directly represent transcription rate and might be effected by some splicing events. However, it is very unlikely, since the splicing machinery should not interfere with the induction of the precursor transcript.

5.5.3. Delay in vinculin induction is not restricted to NIH3T3 cell line

To confirm the observed vcl delayed kinetics seen in an NIH3T3 cell line, I decided to use another fibroblast cell line, Swiss3T3. It has been previously reported that vinculin gene is activated in Swiss3T3 cells by growth factors, but not by TPA (Bellas et al., 1991). For this reason Swiss3T3 cells were maintained for 48 hours in
0.5% FBS and then stimulated with serum, LPA, PDGF or TPA for different amounts of time. Precursor vcl and vcl mRNA levels were determined by RNase protection as previously described. Interestingly, serum- and LPA-induced both vcl precursor and vcl mRNA were not detectable until 60 minutes. PDGF- and TPA- stimulation resulted in a more rapid vcl precursor accumulation which was detectable at 30 minutes (see Figure 5.15A). Similarly as observed in NIH3T3 cells, vcl mRNA induction was not detected until 60 minutes. Both serum and LPA induced vcl 20-40 fold, which is substantially higher than the induction seen in NIH3T3 cells. PDGF and TPA induced vcl at much lower levels than serum, reaching somewhat 4 fold activation (see Figure 5.15B for quantification of the data). This data shows that the kinetics of vcl precursor and vcl mRNA activation is similar in both NIH3T3 and Swiss3T3 cell lines. The delayed induction of vcl by four stimuli suggests that the mechanism of vcl gene induction differs from other SRF target genes, such as srf itself. Thus, further experiments are required to investigate this phenomenon in more detail.
Figure 5.1. The SRF reporter gene responds to F-actin levels. A. Serum-deprived SRE.FosHA cells were stimulated with 0.5μM Jasplakinolide for the times indicated on the figure. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by Jasplakinolide at 60 minutes.
Figure 5.2. The SRF reporter responds G-actin levels. A. Serum-deprived SRE.FosHA cells were stimulated with 2μM Cytochalasin D and 0.1μM Swinholide A for the times indicated on the figure. 3D.AFos reporter and GAPDH transcript levels were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by Jasplakinolide at 60 minutes.
Figure 5.3. Latrunculin B competes with Jasplakinolide to induce SRF. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with 0.2\mu M and 0.5\mu M Latrunculin B (Lat B) and then stimulated with 0.5\mu M Jasplakinolide for 30 or 60 minutes as indicated on the figure. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by Jasplakinolide at 60 minutes.
Figure 5.4. Latrunculin B blocks Cytochalasin D-induced SRF activation. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with different concentration of Latrunculin B as indicated in the figure and then stimulated for 30 minutes with 15% Fetal Calf Serum (SER) or 2μM Cytochalasin D. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 5.5. Latrunculin B blocks serum-, but not Jasplakinolide-induced SRF activation. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with and 0.5μM Latrunculin B (Lat B) and then stimulated with 15% Fetal Calf Serum (SER) or 0.5μM Jasplakinolide for the times indicated on the figure. 3D.AFos reporter and GAPDH transcripts were quantified by RNase protection. B. Relative transcript levels expressed as a percentage of those induced by serum at 60 minutes are shown.
Figure 5.6. Activation of srf by actin binding drugs. A. Serum-deprived SRE.FosHA cells were stimulated with 0.5μM Jasplakinolide, 0.1μM Swinholide A or 2μM Cytochalasin D for the times indicated on the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from srf and GAPDH are indicated. B. Relative srf transcript levels expressed as a fold induction.
Figure 5.7. Activation of *vcl* by actin-binding drugs. A. Serum-deprived SRE.FosHA cells were stimulated with 0.5μM Jasplakinolide, 0.1μM Swinholide A or 2μM Cytochalasin D for the times indicated on the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from *vcl* and GAPDH are indicated. B. Relative *vcl* transcript levels expressed as a fold induction.
Figure 5.8. Transient induction of srf transcription induced by actin binding drugs is Rho independent. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with toxin B and then stimulated with 2μM Cytochalasin D or 0.5μM Jasplakinolide for 30 and 120 minutes as indicated in the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from precursor srf (pre-srf), srf and GAPDH are indicated. B. Relative srf and pre-srf transcript levels expressed as a fold induction are shown.
Figure 5.9. Transient induction of vcl transcription induced by actin binding drugs is Rho independent. A. Serum-deprived SRE.FosHA cells were pretreated for 60 minutes with toxin B and then stimulated with 2μM Cytochalasin D or 0.5μM Jasplakinolide for 30 and 120 minutes as indicated in the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from vcl precursor (pre-vcl), vcl and GAPDH are indicated. B. Relative vcl and pre-vcl transcript levels expressed as a fold induction are shown.
Figure 5.10. c-fos is refractory to actin binding drug treatment. A. Serum-deprived SRE.FosHA cells were stimulated with 0.5μM Jasplakinolide, 0.1μM Swinholide A or 2μM Cytochalasin D for the times indicated on the figure. Transcripts levels were quantified by RNase protection. Nuclease resistant fragments derived from c-fos and GAPDH are indicated. B. Relative c-fos transcript levels expressed as a fold induction are shown.
Figure 5.11. Serum-induced SRF target gene transcriptional activation is independent of new protein synthesis. Serum-deprived NIH3T3 cells were pretreated for 30 minutes with 10 μg/ml cycloheximide or 25 μg/ml and then stimulated with 15% Fetal Calf Serum (SER) for the times indicated on the figure. Transcript levels were quantified by RNase protection. Nuclease resistant fragments derived from pre-srf and srf (A); pre-vcl and vcl (B); c-fos (C) together with GAPDH are indicated.
Figure 5.12A/B. Serum-induced SRF target gene transcriptional activation is independent of new protein synthesis. Serum-deprived NIH3T3 cells were pretreated for 30 minutes with 10 μg/ml cycloheximide or 25 μg/ml and then stimulated with 15% Fetal Calf Serum (SER) for the times indicated on the figure 5.11. Transcript levels were quantified by RNase protection. Relative pre-srf (A) and srf (B) transcript levels expressed as a fold induction are shown.
Figure 5.12C/D. Serum-induced SRF target gene transcriptional activation is independent of new protein synthesis. Serum-deprived NIH3T3 cells were pretreated for 30 minutes with 10 µg/ml cycloheximide or 25 µg/ml and then stimulated with 15% Fetal Calf Serum (SER) for the times indicated on the figure 5.11. Transcript levels were quantified by RNase protection. Relative pre-vcl (C) and vcl (D) transcript levels expressed as a fold induction are shown.
Figure 5.12E. Serum-induced SRF target gene transcriptional activation is independent of new protein synthesis. Serum-deprived NIH3T3 cells were pretreated for 30 minutes with 10μg/ml cycloheximide or 25 μg/ml and then stimulated with 15% Fetal Calf Serum (SER) for the times indicated on the figure 5.11. Transcript levels were quantified by RNase protection. Relative c-fos (E) transcript levels expressed as a percentage of those induced by serum at 30 minutes are shown.
Figure 5.13A/B. Kinetics of srf and vcl transcriptional activation by actin binding drugs. Serum-deprived NIH3T3 cells were stimulated with 15% Fetal Calf Serum (SER), 0.5μM Jasplakinolide, 2μM Cytochalasin D and 0.2μM Swinholide A for the times indicated on the figure. Transcript levels were quantified by RNase protection. Nuclease resistant fragments derived from pre-srf and srf (A); pre-vcl and vcl (B); and GAPDH are indicated.
Figure 5.14A/B. Kinetics of srf and vcl transcriptional activation by actin binding drugs. Serum-deprived NIH3T3 cells were stimulated with 15% Fetal Calf Serum (SER), 0.5μM Jasplakinolide, 2μM Cytochalasin D and 0.2μM Swinholide A for the times indicated on the figure 5.13. Transcript levels were quantified by RNase protection. Relative pre-srf (A) and srf (B) transcript levels expressed as a fold induction are shown.
Figure 5.14C/D. Kinetics of srf and vcl transcriptional activation by actin binding drugs. Serum-deprived NIH3T3 cells were stimulated with 15% Fetal Calf Serum (SER), 0.5μM Jasplakinolide, 2μM Cytochalasin D and 0.2μM Swinholide A for the times indicated on the figure 5.13. Transcript levels were quantified by RNase protection. Relative pre-vc (C) and vcl (D) transcript levels expressed as a fold induction are shown.
Figure 5.15A. Kinetics of vcl transcriptional activation in Swiss3T3 cells. Serum-deprived Swiss3T3 cells were stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for the times indicated on the figure. Nuclease resistant fragments derived from precursor vcl (pre-vcl), vcl and GAPDH are indicated.
Figure 5.15B. Kinetics of vcl transcriptional activation in Swiss3T3 cells. Serum-deprived Swiss3T3 cells were stimulated with 15% Fetal Calf Serum (SER), 10μM LPA, 25 ng/ml PDGF or 50 ng/ml TPA for the times indicated on the figure. Relative pre-vcl and vcl transcript levels expressed as a fold induction are shown.
6. Discussion

6.1. Chapter Summary

The research presented in this thesis concerns SRF activation and the role of SRF in immediate early gene transcription. The role of actin dynamics in the transcriptional activation of SRF will be discussed and potential mechanisms will be presented. The question of how different signalling pathways lead to the activation of SRF, and therefore transcription of a subset of SRF target genes, will be presented, leading to a model for differential activation of endogenous SRF target genes. In addition, the complexity of endogenous gene promoters will be addressed. Finally, speculative models based on recent literature will be discussed with respect to the role of downstream effectors of MAP kinases, other than TCFs, in SRF target gene activation.

6.2. The Actin Cycle and Gene Activation

6.2.1. Actin polymerisation is necessary for SRF activation

The identification of LIM kinase as an SRF activator strongly suggested the importance of actin dynamics in signalling to SRF (Sotiropoulos et al., 1999). The specific actin polymerisation inhibitor Latrunculin B, which binds actin monomers and dissolves the actin cytoskeleton, was used to examine the role of actin dynamics in SRF function more directly. Induction of SRF reporter activity following serum, LPA, PDGF, or TPA treatment was critically dependent on actin polymerisation. Moreover, pretreatment of the cells with Latrunculin B inhibited SRF reporter gene induction to background levels. Interestingly however, the same concentration of Latrunculin B, at most, only partially affected Ras-ERK or PI-3 K pathways, as assessed by western blotting with p-ERK and p-Akt antibodies. In addition, the activity of a TCF-dependent reporter gene is almost unaffected by Latrunculin B,
suggesting that TCF-dependent transcription does not require actin dynamics and is primarily responsive to Ras-ERK pathways. Consistent with this, it has been shown that latrunculin does not substantially affect MAPK activation in Swiss3T3 cells following EGF treatment (Aplin and Juliano, 1999). These data demonstrate that, although cells undergo drastic cytoskeletal changes, multiple non-actin dependent signalling pathways remain largely intact.

Although latrunculin does not block certain signalling pathways, it is still possible that there are other non-specific effects in addition to the specific effect on actin polymerisation. To confirm the data obtained with Latrunculin B, similar experiments were performed with another inhibitor of actin polymerisation, C2 toxin. It has been previously demonstrated that toxin C2 toxin from Clostridium botulinum prevents polymerisation of actin by ADP-ribosylating it at residue R177 (Vandekerckhove, 1988). C2 pretreatment of cells completely blocked serum induction of the SRF-dependent reporter. These data confirm that actin polymerisation is absolutely necessary for extracellular signal-induced SRF activation.

### 6.2.2. Actin dynamics are sufficient for SRF activation

Experiments with actin binding drugs suggested that the actin treadmilling cycle is a convergence point for signal-induced SRF activation. Latrunculin is able to block not only extracellular stimuli-induced SRF reporter gene activity, but also substantially inhibits SRF activation by overexpression of signalling factors known to impinge on the actin treadmilling cycle, such as RhoAV12, LIM kinase and mDia, (Sotiropoulos et al., 1999); (John Copeland personal communication). This suggests that the actin cycle lies downstream of many components in the signalling pathway leading to activation of SRF-dependent transcription. Moreover, direct intervention to the actin treadmilling cycle is sufficient to activate SRF-dependent reporter genes in the absence of extracellular stimuli (see Figure 6.1). The actin binding drug Jasplakinolide, which stabilises F-actin, strongly induces the SRF reporter gene with kinetics similar to that of serum stimulation. However, some agents that bind G actin monomers (Cytochalasin D) or dimers (Swinholide A and Cytochalasin D) also are sufficient to induce the SRF reporter gene, as is overexpression of the actin binding protein profilin (Sotiropoulos et al., 1999). These agents do not induce actin polymerisation suggesting that the sensing mechanism for regulation of SRF activity must involve G-actin, rather than F-actin.
6.2.3. Potential mechanisms of SRF activation

Recent findings in the Treisman laboratory support the idea that depletion of the G-actin pool induces SRF activation. Overexpression of wild type actin itself inhibits signal-induced SRF activation (Sotiropoulos et al., 1999). But what is the mechanistic connection between G-actin and SRF? In a general sense, activation of SRF could occur in two ways: positive regulation by a coactivator that senses a drop in G-actin level by some means, or relief of negative regulation by a repressive protein complex, perhaps involving G-actin itself (see Figure 6.2). According to this model, the overexpression of actin could sequester cofactors that are necessary for SRF-induced activation or render them inactive. Conversely, treatment with actin binding drugs would result in a release of such a coactivator, either by inducing actin polymerisation (e.g. Jasplakinolide) or by directly preventing actin from interacting with the putative coactivator (e.g. Cytochalasin D).

However, another possibility exists that SRF activation occurs as a consequence of a shift in equilibrium of the actin treadmilling cycle. For example, some kinases which are necessary to potentiate SRF activity are perhaps activated upon treatment with actin binding drugs. It has been demonstrated that JNK activity is required for Cdc42- and Rho-induced SRF reporter gene activation (Alberts et al., 1998). Some reports have suggested that stress family kinases could be activated in certain cell types upon Cytochalasin D addition at late time points after treatment (Yujiri et al., 1999). Therefore, it is possible that JNK could contribute to SRF activation. However, this mechanism seems unlikely to contribute much to SRF activation given that the kinetics of the SRF reporter gene activation by serum, Jasplakinolide and Cytochalasin D are very similar and transient (peaking at 30 minutes after stimulation) and no JNK activation was seen at the 30 minute time point following Jasplakinolide treatment (Sotiropoulos et al., 1999). Interestingly, the strong JNK activator anisomycin did not activate the SRF reporter gene (DG, unpublished). This evidence suggests that JNK activation is neither sufficient nor necessary for SRF induction. However, it would be of interest to test whether any of the actin binding drugs that activate SRF also activate p38, JNK or other family kinases and to determine their contribution in SRF activation.
6.3. Signalling Pathways Leading to SRF Activation

6.3.1. Extracellular stimuli activate an SRF reporter gene

The 3D.AFos reporter gene has been used as a readout for SRF activation, since it comprises the \textit{c-fos} transcription unit controlled by a chimaeric promoter containing cytoskeletal actin TATA region (minimal promoter) and three adjacent SRF binding sites. An integrated version of 3D.AFos in NIH3T3 cells is activated by serum, LPA, growth factors and the phorbol ester TPA. In contrast, when the same reporter gene is transiently transfected into NIH3T3 cells, it is not substantially activated by PDGF or phorbol ester TPA. However, serum, LPA and Cytochalasin D activate both the transiently transfected and the integrated reporter gene with a similar efficiency. These effects were not dependent on the integration sites of the reporter, as similar results were obtained with two other independent NIH3T3 clones. In addition, another stably integrated SRF-dependent reporter gene, 3DA.CD2/CD8, was activated by PDGF, EGF and TPA. With the exception of one clone (clone 2-9), in which very low levels of SRF reporter gene inducibility were observed by TPA compared to other stimuli, all clones were inducible to a similar extent. In all cases serum- and LPA-induced reporter gene activity was greater than that of PDGF or TPA. It is possible that the discrepancies found are dependent on the sensitivity of the assay used. For example, activation of the SRF reporter by PDGF measured by CAT assay was only approximately one tenth of that registered following stimulation by serum (Hill et al., 1995). However, it is unlikely that the differences observed were due to the sensitivity of the assay used, as the results described in Chapter 2 using luciferase reporter gene assay which is known to be more sensitive than the CAT assay gave very similar results compared to those obtained previously in CAT assays (Hill et al., 1995). Direct comparison of stably integrated and transiently transfected SRF reporter genes suggests that the sensitivity of promoters towards certain signalling pathways may be dependent on the chromatin context of the reporter gene (for further discussion see section 6.5).

6.3.2. The role of PI-3 kinase in SRF activation
Specific inhibitors of known signalling cascades implicated in immediate early gene activation (Treisman, 1994) were used to determine the downstream signalling requirements for different stimuli. PI-3 kinase has been characterised as one of the downstream targets of serum and receptor tyrosine kinase signalling cascades, however, the potential involvement of PI-3 kinase in SRF activation is not well understood. SRF reporter activity was assayed using two specific PI-3 kinase inhibitors: wortmannin and LY294002. With pretreatment with these inhibitors, serum induction of the SRF reporter gene was unaffected, and LPA induction was reduced by only 20%. In contrast, PDGF-induced reporter gene activity was reduced by up to 80%, and TPA induction was substantially blocked. It has been demonstrated that PDGF-induced MAPK activation is dependent on PI-3 kinase activity in some cell lines (Grammer and Blenis, 1997). However, the results in Chapter 3 clearly demonstrate that in NIH3T3 cells PI-3 K inhibition had no effect on ERK activation. The requirement of PI-3 K for PDGF-induced SRF reporter gene activity is not totally surprising, since PDGF strongly activates PI-3 K, which in turn participates in a number of physiological processes, such as chemotaxis, membrane ruffling and cell proliferation (Valius and Kazlauskas, 1993; Wennstrom et al., 1994). It has been demonstrated that PDGF-induced Rac activation is dependent on PI-3 kinase binding sites on the PDGF receptor, resulting in Rac-induced cytoskeletal rearrangements (Hawkins et al., 1995). More recently, it has been suggested that the activity of the guanine nucleotide binding protein Vav, an exchange factor for Rac, is directly controlled by PI-3 K products (Han et al., 1998). As Rac itself is sufficient to activate SRF, it is tempting to speculate that PDGF signalling to SRF is mediated through Rac (see Figure 6.3). However, it has not been possible to examine this hypothesis due to the very low activation of the transfected SRF reporter gene achieved by PDGF.

Alternatively, PI-3 K activity might be required for activation of novel or atypical PKC isoforms. It has been demonstrated that PDGF activation of PKCe (Moriya et al., 1996) and PKCζ (Akimoto et al., 1996) requires PI-3 kinase activity. A more recent study suggests that PKCe is involved in the activation of SRE reporter genes (Soh et al., 1999). The finding that PKC inhibitors partially affect SRF reporter gene activation, as described in Chapter 3, suggests that PKCs can also contribute to PDGF-induced signalling to SRF (see Figure 6.4).

These data establish that signalling to SRF by PDGF and TPA differs significantly from that of serum and LPA. Interestingly, in contrast to PDGF, TPA requires only basal PI-3 kinase activity to activate SRF. Several lines of evidence support this view: first, constitutively active PI-3 K subunit p110 does not activate the
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SRF reporter gene in NIH3T3 cells, second, PI-3 K activity is not required for other stimuli-induced reporter activation and, third, TPA itself does not induce PI-3 kinase activity. It is not clear how TPA contributes to the signalling pathways that lead to SRF activation. Some lines of evidence suggest that TPA could impinge on the actin pathway via PKC. Experiments described in Chapter 3 show that TPA-induced SRF activation is absolutely dependent on PI-3 kinase, MEK and PKC activity (see Figure 6.4). Due to a lack of specific inhibitors for the PKC isoforms it is difficult to distinguish whether there are different PKC isoform requirements for SRF activation. However, the sensitivity to PI-3 K inhibitors observed suggests that a putative SRF-specific PKC could belong to the nPKC class, as this class is known to depend on PI3 K activity. Consistent with this two members of a novel PKC subfamily, PKCe and PKCS, have been implicated in activation of SRE reporter gene (Soh et al., 1999). Interestingly, a study by Prekeris et al. reported that PKCe, but not PKC β, -δ, -η or -ζ, binds filamentous actin in a TPA-dependent manner (Prekeris et al., 1998). However, if and how PKC contributes to changes in actin dynamics and SRF activation remains unclear.

It is worth noting that the dependence on PI-3 K for growth factor- and TPA-induced SRF activation is consistent with previously reported data demonstrating that PI-3 K activity is required for efficient activation of a fos promoter reporter lacking the TCF binding site (pm18Luc) in HeLa cells (Wang et al., 1998). However, Wang et al. also suggested that PI-3 K activity is required not only for EGF-, but also for serum-induced reporter induction. The data presented here, in contrast, has demonstrated that SRF activation by serum or LPA is not dependent on PI-3 K activity. This discrepancy could be due to the different cell lines used (NIH3T3 versus HeLa) or to the different SRF reporters used. The pm18Luc reporter (used by Wang et al.) contains CRE and SIE binding sites in addition to the SRF binding site (see section 6.6.1). Therefore, the pm18Luc reporter is not directly comparable with the 3DA.Fos reporter used in this study, which comprises only SRF binding sites and a minimal promoter.

6.3.3. SRF activation and MAPK signalling

It is well established that Ras-MEK–ERK signalling is crucial for the transcriptional activity of immediate early genes, such as c-fos and egr-1 (Price et al., 1996), for review see (Treisman, 1996). Since polypeptide growth factors and TPA...
activate the integrated SRF reporter gene, and both PDGF and TPA are strong activators of MAPK, the relative contribution of MEK-ERK signalling to SRF activation was examined. Using two specific MEK inhibitors, U0126 and PD 98059, it was demonstrated that the ability of serum, LPA, and PDGF to activate SRF is substantially independent of MAPK signalling. However, in contrast with other stimuli used, TPA activation of SRF was completely dependent on MEK activity. Control experiments demonstrated that U0126 and PD98059 strongly inhibited both MAPK phosphorylation and activation of a TCF-dependent reporter gene. In addition, TPA-induced activation of SRF was also dependent on PKC activity, since long pretreatment with TPA or PKC inhibitors blocked SRF activation following TPA, but not serum, LPA or PDGF stimulation. These data imply that TPA activates SRF through a different signalling pathway, which requires both MEK and PKC activities. However, TPA-induced SRF activation was also blocked by treatment with C3 or Latrunculin B. Thus, TPA signalling, similarly to other stimuli-induced signalling to SRF, also works via a Rho-actin pathway (see section 6.3.5). However, it remains unclear by which signalling route TPA impinges on the actin cycle. It is intriguing that TPA-induced SRF activation is dependent on MEK, PI-3 K, Rho and actin signalling components. Sensitivity to PI-3 K inhibition suggests that nPKC isoforms, which require PI-3 K products, might be involved in SRF signalling (discussed in previous section, see Figure 6.4). It is difficult to explain how MEK-ERK signalling contributes to SRF activation. The simplest explanation is that MEK inhibitors non-specifically inactivate another kinase, which is only involved in the TPA-PKC signalling route to SRF. However, this is unlikely as very similar results were obtained with two independent MEK inhibitors. Alternatively, perhaps a putative MEK/ERK substrate is required for TPA-, but not serum- or LPA-induced signalling to SRF.

6.3.4. Other kinases involved in signalling to SRF

p70 S6 kinase

The finding that PI-3 K inhibitors block PDGF-induced SRF activation, suggests that PI-3 K dependent kinase/kinases might be involved in signalling to SRF. One possible candidate is the p70 S6 kinase, since it has been shown to be activated by PDGF as well as other receptor tyrosine kinases. In addition, PI-3 K products have been shown to be necessary for p70 S6 kinase activation (Ming et al., 1994; Parekh et al., 1999; Pullen et al., 1998). The relatively specific inhibitor of mammalian TOR,
rapamycin, was used to test whether this pathway is involved in signalling to SRF, since mTOR activity is required for p70 S6 kinase activation. However, rapamycin was found to not affect SRF activation by serum, LPA, PDGF or TPA stimulation. This suggests that p70 S6 kinase is not required for PDGF-induced SRF activation, and thus, another PI-3 K dependent pathway to SRF must exist. Further experiments are required to resolve this issue.

**ROCK (RhoA kinase)**

A downstream target of Rho, the Rho kinase (ROCK) has been implicated in signalling to SRF. The specific inhibitor of ROCK, Y27632, was used to test whether ROCK is required for serum, LPA, PDGF or TPA induction of the integrated SRF reporter gene. All stimuli tested were insensitive to inhibition of ROCK, except LPA, where induction of the SRF reporter gene activity was reduced by approximately 50%. This result is consistent with previous findings reported by Sahai *et al.* where no inhibition by Y27632 on serum-induced SRF activation was observed in transfection assays (Sahai *et al.*, 1999). Although all stimuli were sensitive to Rho A inhibition by C3 (discussed in the following section), inhibition of the downstream target of RhoA, ROCK did not interfere with signalling to SRF. These data suggests that ROCK is not the downstream target of RhoA which leads to SRF activation in NIH3T3 cells.

### 6.3.5. The requirement of RhoA for SRF reporter gene activity

It was previously shown that Rho activity is absolutely necessary for transfected SRF reporter gene activation following serum and LPA stimulation (Hill *et al.*, 1995). Consistent with this, data presented in Chapter 3 demonstrates that RhoA activity is required for activation of a stably integrated SRF reporter gene following serum, LPA, PDGF, and TPA activation. However, in contrast to serum and LPA, PDGF and TPA do not activate RhoA. Recent studies have demonstrated that these stimuli rapidly decrease rather than increase GTP loading on RhoA in NIH3T3 cells (Sander *et al.*, 1999), Robert Grosse, personal communication), suggesting that activation of SRF by PDGF and TPA requires only basal RhoA activity. It is conceivable that PDGF acts to stabilise a pool of F-actin, whose assembly requires basal Rho activity. In addition, Rac has been implicated in activation of LIMK, which stabilises the F-actin pool by phosphorylating coflin (Arber *et al.*, 1998). PDGF is a strong activator of Rac, and could therefore activate SRF in a Rac- and LIMK-
dependent fashion (Edwards et al., 1999). This is consistent with recent findings in the Treisman laboratory where it was shown that, in PC12 cells, Rac and LIMK are stronger activators of SRF than RhoA (Geneste et al., 2001). Therefore, dependent on the signalling environment, signals to SRF could be mediated by inducing the activity of either RhoA or Rac. It is worth to note, however, that basal RhoA activity is required in both cases (see Figure 6.3).

TPA-induced signals to SRF are distinct in that TPA does not activate either Rho or Rac in NIH3T3 cells. Interestingly, in human neutrophils, TPA has been shown to activate Rac2, but in a PI-3 kinase-independent manner (Akasaki et al., 1999). The effect of TPA on cytoskeletal changes appear to be cell type, and possibly concentration, dependent. It has been reported that TPA-induced membrane ruffling is dependent on both Rac and Rho activities in MDCK cells (Fukata et al., 1999). In addition, TPA-induced MBS phosphorylation requires Rho kinase/ROCK and RhoA (Kawano et al., 1999). In Swiss3T3 and NIH3T3 cells, however, membrane ruffling is dependent on Rac, but not RhoA activity (reviewed by Hall, 1998). It is therefore tempting to speculate that actin-dependent SRF activation by TPA would require Rac or RhoA activity, but depending on the cell type. This model would be consistent with the idea that LIM kinase activity (and therefore the actin pathway) could be modulated in two ways to activate SRF: via Rho–ROCK (Maekawa et al., 1999; Ohashi et al., 2000) or Rac–PAK (Edwards et al., 1999; Watanabe et al., 1999) (see Figure 6.3 and Figure 6.4).

6.4. Immediate Early Genes and SRF Activation

In this study the signalling requirements for the activation of immediate early genes which contain an SRE in their promoters have been examined. SREs have been shown to play a regulatory role in activation of many immediate early genes, such as c-fos (Treisman, 1986). Previous studies suggest that the SRE in the c-fos promoter can be activated in both a TCF-dependent and a TCF-independent manner (Hill et al., 1995). Potentially, these two types of activation are related to the fact that the SRE of the c-fos promoter includes a binding site for TCFs adjacent to the SRF binding site. Although some other immediate early genes appear to have a similar SRE arrangement in their promoters to that of c-fos, in others the SRE appears to contain a binding site only for SRF. Perhaps SRF-only SREs respond just to TCF-independent mechanisms. This study has investigated the induction responses of several
endogenous immediate early genes and found that *srf* and *vcl* genes, which do not have a consensus TCF binding sites in close proximity to the SRE, behave similarly to the integrated SRF-dependent reporter gene, 3DA.Fos. In contrast, other genes, such as *c-fos*, *erg-1* and *junB*, which have TCF bindings sites in their promoters, are sensitive to the same pathways as a TCF-dependent reporter gene.

### 6.4.1. *srf* and *vinculin* genes require actin polymerisation, but not ERK signalling

The data presented in this study demonstrates that the SRF target genes *srf* and *vcl* behave like the SRF-dependent reporter. Both *srf* and *vcl* are inducible by stimuli such as serum, LPA, PDGF, and TPA, and, require Rho-actin, but not MEK-ERK signalling. The development of intronic probes for RNase protection studies (probing for pre-mRNA) helped to reveal that the kinetics of *srf* and *vcl* gene transcriptional activation is similar to that of the SRF reporter gene. Both the *srf* and *vcl* precursor RNA is rapidly induced after treatment with serum, growth factors or actin binding drugs. Increased *srf* precursor RNA level was observed 15 minutes following stimulation, reached a maximum level at 30 minutes and declined over a two hour period. Interestingly, *vcl* precursor RNA was not detectable until 30 minutes following induction and declined thereafter. In contrast, accumulation of the *srf* and *vcl* mRNAs proceeded gradually over a two hour period, although that of *vcl* was delayed, consistent with a delayed appearance of its precursor.

It has been demonstrated that actin dynamics plays a critical role in the regulation of a subset of SRF target genes. Indeed, induction of *srf* and *vcl* was found to be absolutely blocked by inhibitors of actin polymerisation, such as Latrunculin B and C2 toxin. Moreover actin binding drugs, such as Jasplakinolide, Cytochalasin D and Swinholide A, all induce *srf* and *vcl* genes with kinetics similar to that of serum stimulation. It therefore appears that changes in actin dynamics are not only necessary, but are also sufficient to induce transcription of these two endogenous SRF target genes. These findings confirm and extend previous reports that changes in actin levels can regulate cytoskeletal actin and vinculin synthesis (Bershadsky *et al.*, 1995; Lyubimova *et al.*, 1997; Reuner *et al.*, 1996). Interestingly both *srf* and *vcl* precursors and mRNA induction, by all stimuli except TPA, is relatively insensitive to the MEK-ERK signalling pathway inhibitors U0126 and PD98059, similarly to the SRF reporter gene. This data suggests that the MEK-ERK pathway contributes little to the induction
of \textit{srf} and \textit{vcl} genes. Interestingly, SRE sites in muscle-specific promoters, which similarly to \textit{srf} and \textit{vcl} genes generally lack associated TCF binding sites, have also been shown to be sensitive to actin dynamics (Mack et al., 2001; Wei et al., 2001).

The \textit{srf} gene responds almost identically to the SRF reporter gene in respect to activation and sensitivity to inhibitors, although the \textit{srf} promoter is more complex, containing SP1 sites and a consensus Ets binding motive in addition to the SRF binding site. Previous studies suggest that LPA-induced activation of the \textit{srf} promoter involves a Ras-dependent signalling input through an SP1 site, while FGF-induced activation involves both the SP1 and Ets motifs (Spencer et al., 1999; Spencer and Misra, 1999). Similarly to FGF, sensitivity of TPA-induced \textit{srf} gene activation to the MEK inhibitor could be explained by the involvement of the Ets binding motif. However, the 3D.AFos reporter, which does not contain a consensus Ets binding motif, is also sensitive to the inhibition of the ERK signalling pathway. Moreover, the \textit{vcl} promoter, which also does not contain a consensus Ets binding site (Moiseyeva et al., 1993), is also sensitive to MEK inhibition following TPA stimulation. This suggests that in TPA-induced signalling to SRF via the ERK pathway may contribute through an element other than the Ets motif and is most likely reliant on SRF binding itself. Further experiments are required to investigate this issue.

\subsection*{6.4.2. Activation of \textit{c-fos} and \textit{egr-1} is insensitive to actin dynamics, but dependent on ERK}

Having established that the actin pathway is necessary for SRF activation, it was expected that induction of \textit{c-fos} and \textit{egr-1}, both SRF target genes, would be dependent upon changes in actin dynamics. Surprisingly, alterations in actin dynamics contributed little to the transcriptional activation of \textit{c-fos} and \textit{egr-1} by serum, LPA, PDGF or TPA. Moreover, experiments with actin binding drugs demonstrated that the actin pathway is neither necessary nor sufficient for activation of \textit{c-fos} and \textit{egr-1} genes. Instead, it is apparent that these genes are absolutely dependent on ERK activation. Both the \textit{c-fos} and the \textit{egr-1} promoter SREs, unlike those of \textit{srf} and \textit{vcl} genes, contain a consensus binding site for the SRF cofactor TCF, which is regulated by the ERK signalling pathway. Activated ERK phosphorylates TCF at multiple residues, resulting in an activation of TCF-dependent transcription. However, the TCF-dependent (ERK) transcription of these promoters does not explain the concomitant absence of TCF-independent (i.e. Rho-actin-SRF) transcription. It is
conceivable that association of TCF with SRF may simultaneously render a promoter sensitive to MAPK signalling and refractory to actin dynamics. The overexpression of truncated, and therefore transcriptionally inactive, TCF inhibits induction of a SRE-dependent reporter by activated GTPases, which is consistent with this hypothesis (Hill et al., 1994; Hill et al., 1995).

6.4.3. *srf* and *vcl* induction is Rho dependent

Two specific recombinant toxins of Rho family GTPases, C3 toxin and toxin B (Just et al., 1994; Just et al., 1995a) were used to investigate the role of Rho activity in *srf* and *vcl* induction. C3 toxin specifically inhibits RhoA, while Toxin B inactivates Rho, Rac and Cdc42. Ras and downstream targets of Ras, such as ERK, are insensitive to these toxins (Clerk et al., 2001). Srf and vinculin gene activation was blocked by C3 toxin and Toxin B, both at precursor and mRNA level, following serum, LPA, PDGF and TPA stimulation. However, Cytochalasin D- and Jasplakinolide-induced activation of *srf* and *vcl* was insensitive to these toxins. This is consistent with the model in which changes in actin dynamics, that induce SRF reporter gene activation, are downstream of Rho family GTPases. Rho family GTPases themselves are activated by appropriate exchange factors following external stimulation. Therefore extracellular stimuli-induced signals to SRF can be blocked at two levels: either at (i) the GTPase level or (ii) the actin cycle level (see Figure 6.3).

6.4.4. *c-fos* activation is not dependent on Rho activity

Results in Chapter 4 suggest that, in NIH3T3 cells, signal-induced activation of the endogenous *c-fos* gene occurs largely independently of RhoA, consistent with a similar finding in Rat-1 cells (Beltman et al., 1999). These findings are in contrast to previous observation in transient transfection experiments in which *fos* activation exhibits a strong dependence on functional RhoA (Hill et al., 1995). One potential explanation for this discrepancy is that transient transfected promoters are somehow more sensitive to the Rho–actin pathway than their chromosomal counterparts. However, this would appear unlikely, since latrunculin does not block either activation of either the endogenous or the transfected *fos*. An alternative explanation can be based on the observation that, in fibroblasts, ERK activation is partially dependent on
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Rho: perhaps the presence of large number of transfected fos gene templates saturates the ability of ERK to signal to all, with the result that the dependence of ERK signalling upon RhoA would then become significant (Hill et al., 1995; Kumagai et al., 1993). Consistent with the latter, this study has shown that LPA is more sensitive to either latrunculin or C3 pretreatment than is serum or PDGF, which are known to activate ERK through different signalling pathways than LPA. However, most of these experiments are based on overexpressing C3 exoenzyme, which, although specific to RhoA, is toxic to cells. Therefore, it is important to express an optimal amount of C3 to inhibit RhoA with minimum toxicity. Data presented in Chapter 4 show that a low concentration of C3 is capable of inhibiting the SRF-dependent reporter gene to a background level, with only a partial effect on the \( c-fos \) gene. Similar patterns of inhibition were observed using Latrunculin B: the SRF reporter gene is inhibited to background level, while \( c-fos \) remains mostly unaffected.

Taken together these data clearly establish a different sensitivity of SRF-dependent reporter and \( c-fos \); signalling to SRF is absolutely dependent on RhoA activity and the actin pathway, while \( c-fos \) does not require either RhoA or the actin pathway.

6.4.5. The role of PI-3 kinase in SRF target gene activation

This study has shown that PI-3 K activity is required for PDGF- and TPA-induced 3D.AFos reporter gene activation. In contrast, serum- and LPA-induced SRF reporter gene activation occurred in a PI-3 K independent manner. Interestingly, the SRF target genes tested respond in a distinct way to inhibition of PI-3 K activity: (i) the \( srf \) and \( vcl \) genes respond in a similar way to the SRF reporter; serum and LPA induction was essentially unimpaired, while PDGF and TPA activation was substantially inhibited, by PI-3 K inhibitors; (ii) activation of \( c-fos \) and \( egr-1 \) by all stimuli was not diminished by PI-3 K inhibitors, displaying even a slight enhancement of induction.

The exact involvement of PI-3 kinase in SRF target gene activity remains unclear. Consistent with a report by Reif et al., the constitutively active p110 subunit of PI-3 kinase was found not to activate the SRF reporter gene (Reif et al., 1996). However, a recent report by Poser et al. suggested that an SRF-dependent reporter can in fact be activated by PI-3 kinase, and that this activation is not dependent on the actin pathway (Poser et al., 2000). The inhibition of \( c-fos \) and \( egr-1 \) genes by
dominant negative forms of PI-3 kinase can be attributed to the inhibition of the Ras-MEK-ERK signalling pathway, rather than directly to SRF itself (Yamauchi et al., 1993).

6.4.6. Two classes of SRF target genes

This study has used inhibitors specific for Rho GTPases, actin dynamics, MEK and PI-3 K to investigate signalling to the SRF transcription factor and four of its cellular target genes in response to different stimuli. The results potentially define two classes of SRF target gene (see Figure 6.5). One class, which includes srf and vcl, behaves in a similar fashion to that of an SRF-dependent reporter gene: regulation of these genes requires Rho activity and actin polymerisation, but is not dependent on MEK activity. Regulation of the second class, which includes c-fos and egr-1, occurs largely independently of actin dynamics and functional Rho, but instead is critically dependent on MEK-ERK signalling. This classification is strengthened by the observation that the srf-vcl class of genes are also sensitive to the PI-3 K inhibition following PDGF or TPA stimulation. It appears that, at least in the case of PDGF and TPA, SRF target genes that are sensitive to actin dynamics, are also sensitive to PI-3 kinase inhibitors. A direct link between actin and the PI-3 kinase pathway requires further investigation.

Among other immediate early genes, JunB exhibits similar signalling requirements to c-fos and egr-1. As yet, there is insufficient data to classify other SRF target genes. It is intriguing to note that the cyr61-related immediate-early gene, CTGF, exhibits similar signalling requirement to srf and vcl (Hahn et al., 2000); however, as the SRE in the promoter of this gene is not conserved between mouse and human, it is yet to be confirmed as an SRF target gene (Ryseck et al., 1991). Several SRF-controlled muscle-specific promoters are Rho-dependent (Carnac et al., 1998; Wei et al., 1998), and at least α-actin and SM22 promoters are dependent on alterations in actin dynamics (Mack et al., 2001; Wei et al., 2001), suggesting that they might fall into the srf-vcl class. It is tempting to speculate that muscle specific promoters require PI-3 K activity for transcriptional activation, at least following growth factor stimulation, and if this proves true it is interesting to note that insulin-induced skeletal myoblast differentiation is dependent on PI-3 kinase activity (Sarbassov and Peterson, 1998). The role of MEK-ERK signalling in the expression of muscle specific SRF target genes has not been resolved; however, the failure of the MEK inhibitor PD98059 to
block differentiation of C2 skeletal myoblasts suggests that MEK-ERK signalling may
not be essential for the expression of the muscle specific SRF target genes in these
cells (Cuenda and Cohen, 1999; Sarbassov and Peterson, 1998).

6.4.7. A model for SRF target gene activation

Based on the work presented in this thesis I have identified two classes of SRF
target genes which can be distinguished on the basis of their relative sensitivity to
RhoA-actin and MEK-ERK signalling pathways. It appears that SRF target genes are
either sensitive to actin dynamics and independent of MEK-ERK signalling, or vice
versa. How might such mutually exclusive linkage of different signalling pathways to
SRF-dependent promoters be achieved? It has been suggested that promoter-specific
combinatorial interactions between SRF and other transcription factors might control
the sensitivity of SRF to signalling via actin dynamics (Sotiropoulos et al., 1999). It is
tempting to suggest a refinement of this model, in which the physical interactions
between SRF and different cofactors, responsible for actin-dependent signalling and
MEK-ERK signalling, respectively, are mutually exclusive. Several observations
suggest that the TCF proteins are good candidates for factors controlling signalling
specificity at SRF target gene promoters (see Figure 6.5). First, they are direct targets
of MEK-ERK signalling (Gille et al., 1992; Marais et al., 1993); second, the SRF
binding sites in actin-dependent promoters such as srf and vcl do not have consensus
TCF sites associated with them, while SRF binding sites in MEK-ERK dependent
promoters such as fos and egr -1 do (McMahon and Monroe, 1995; Moiseyeva et al.,
1993; Shaw et al., 1989; Spencer and Misra, 1996); and third, expression of inactive
forms of TCF can interfere with RhoA-dependent signalling to SRF reporter genes
(Hill et al., 1995). However, it should be noted that SRF also functionally cooperates
with several other transcription factors, such as SP1 (Spencer and Misra, 1999),
GATA4 (Durocher et al., 1997), Nkx2.5 (Chen et al., 1996), the myogenic factors
(Sartorelli et al., 1990) (Belaguli et al., 1997), and ATF6 (Thuerauf et al., 1998; Zhu
et al., 1997). Combinatorial interaction between SRF and such other factors might
also, therefore, constrain its sensitivity to Rho-actin signalling.

6.5. Transient Transfection versus Stable Integration
Several differences in induction responses were observed between transiently transfected versus stably integrated reporter genes. The stably integrated SRF-dependent reporter gene, 3D.AFos, was more responsive to PDGF- and TPA-induced signalling compared to a transiently transfected version. In addition, differences in sensitivity to C3 toxin induced transcription were observed between endogenous c-fos and a transiently transfected version. In this section the possible reasons for these differences will be briefly discussed.

6.5.1. The transfection approach

Variations in results using different gene transfection methods is not surprising. The simplest explanation for the apparent differences is a variability in the number of plasmid copies delivered per single cell, depending on transfection method. The activation of reporter genes that respond to multiple signalling pathways could be especially susceptible to copy number differences, since a given pathway may not be able to fully engage all reporter copies, allowing other minor pathways to contribute. The balance of responses may be modified by a high copy number of reporter. It is worth to note, that transfection efficiency (expressed as a percentage of transfected cells) does not necessarily correlate with the amount of plasmid transfected per single cell. For example, DEAE-Dextran and Lipofectamine transfection reagents, clearly differ in transfection efficiency, but may not deliver the same amount of plasmid per cell. The commercially available transfection reagents are not well characterised in this respect. Cells transfected with a GFP expressing plasmid display a wide range of fluorescence intensity, indicating that the distribution of transfected plasmid per cell can vary even within the same transfection. All of these characteristics are considerations in the analysis of transient transfection data, but do not necessarily preclude their value.

6.5.2. Chromatin context

It is well established that acetylation, both of histone tails as well as some transcription factors, is actively involved in the regulation of transcription (reviewed by Roth et al., 2001). It has previously been reported that the activation of the SRF-dependent reporter gene requires an additional signal, which has been linked to histone
acetylation (Alberts et al., 1998). Accumulating evidence suggests that not only acetylation, but also phosphorylation, of histones plays a role in regulation of transcription (for review see Berger, 2001). Histone H3 phosphorylation has a role in the regulation of extracellular stimuli-induced transcription (Barratt et al., 1994; Mahadevan et al., 1991). Recent in vitro studies have suggested that EGF-induced H3 phosphorylation may act as a signal for histone acetyltransferase binding and acetylation of the particular locus during transcription initiation (Cheung et al., 2000; Lo et al., 2000). In addition, CBP/300, which has HAT activity, has been shown to bind TCF at the c-fos promoter (Janknecht and Nordheim, 1996). More recently, Yang and colleagues demonstrated that Elk can recruit Sin3A histone deacetylase, which is involved in shutting off transcription, suggesting that the balance of acetylation-deacetylation is used to tightly regulate transcription [Yang, 2001 #34]. Chromatin modifications, such as acetylation and phosphorylation, are therefore actively involved in both activation and repression of transcription.

The differences observed between transiently transfected and stably integrated reporter genes may reflect the differences in nucleosomal structure of the respective gene. For instance, transiently transfected reporters displayed a delay in induction compared to integrated reporters. This difference in kinetics was not relieved upon transfection of reduced amounts of reporter, suggesting that it is not influenced by reporter copy number.

6.6. The Different Contributions of Response Elements in the c-fos Promoter

Mutation of the SRF binding site significantly reduces the serum inducibility of the c-fos promoter. Interestingly, polypeptide growth factor induction is also reduced, but to a lesser extent. Conversely, mutations that eliminate the TCF binding site more dramatically effect growth factor and TPA induction than that of serum (Hill and Treisman, 1995). Consistent with these data, the specific MEK inhibitor U0126, used in this study, completely abolished PDGF and TPA induced c-fos expression. Serum-induced c-fos expression, although significantly inhibited was not completely blocked by the same concentration of inhibitor. Interestingly, a small residual activation of these genes observed in U0126 treated cells was also sensitive to Latrunculin B treatment. Thus, it appears that complete inhibition of c-fos activation by serum can be achieved when both MAPK and actin signalling pathway are inhibited. The simple model suggests that TCF responds to MAPK and SRF to actin,
but this idea is not in exact agreement with previous studies in which double SRF/TCF binding site mutants are more growth factor inducible than single SRF or TCF mutants (Hill and Treisman, 1995). Thus, it seems more likely that the inhibitors used could be affecting the induction of *c-fos* via other response elements in addition to the SRE (discussed in section 6.5.1).

### 6.6.1. The CRE (cAMP response element) contribution to *c-fos* activation

Results presented in Chapter 3 show that U0126 and PD980059 inhibit *c-fos* and *egr-1* activation almost to background levels, suggesting that the Ras-MEK-ERK pathway is essential for activation of these genes. Most of this inhibition is probably due to inhibition of MAPK-dependent phosphorylation of TCF at the SRE element (Marais *et al.*, 1993). However, it remains possible that inhibition of *c-fos* transcriptional activation is also partially mediated by other response elements, such as the CRE, either independently or in cooperation with the SRE. The downstream effector of MAPK, Rsk2 can phosphorylate CREB and therefore activate a CRE reporter gene (De Cesare *et al.*, 1998; Xing *et al.*, 1996). Additionally, microinjection of CBP (CREB binding protein) antibodies were able to block SRE-dependent transcription (Arias *et al.*, 1994). Another study has shown that the overexpression of the RBD (Rsk binding domain) of CBP can inhibit *c-fos* induction by NGF (Nakajima *et al.*, 1996). However, it has been reported that NGF induced CREB phosphorylation is not blocked by PD98059 inhibitor, and therefore not MEK-dependent (Xing *et al.*, 1998). It is therefore unlikely that the inhibition of growth factor-induced *c-fos* expression by MEK inhibitors can be attributed solely to the inhibition of CREB. Interestingly, p38 kinase inhibitor SB203580 blocks CREB phosphorylation following NGF stimulation, although this has no inhibitory effect on *c-fos* activation (Xing *et al.*, 1998). This suggests that inhibition of signals leading to CREB activation does not necessarily result in inhibition of *c-fos*. Previous reports using SB203580 inhibitor suggest that p38 kinase is not essential for serum- or growth factor-induced *c-fos* activation (Hazzalin *et al.*, 1997; Price *et al.*, 1996). The contribution of the CRE site to *c-fos* activation, in the context of whole promoter, is still not completely understood, but it seems doubtful that it plays a major role in serum- and growth factor-induced transcription. However, the CRE response element is involved in response induced by calcium signalling pathways (Johnson *et al.*, 1997).
6.6.2. The role of AP1 site in the \( c-fos \) promoter

Recent work from the Prywes laboratory suggests an important role for the FAP site (located on the 3' side of the SRE) in TPA, but not serum, induction of the \( c-fos \) gene. It appears that this site can bind ATF1 and possibly AP1 (Wang and Prywes, 2000). Although both CREB and ATF1 are phosphorylated following TPA treatment, the relative contribution for transcriptional activity of each transcription factor is not clear. It would be of interest to know how fos promoter mutants which can no longer bind both TCF and ATF/AP1 compare to the response of wild type to distinct signalling pathways.

6.7. Other Downstream Targets of MAPK

In addition to TCF, another substrate of ERK implicated in activation of \( c-fos \) transcription is Rsk. Recent studies with a fibroblast cell line carrying a non-functional Rsk-2 revealed that it is required for efficient activation of \( c-fos \) following EGF, PDGF, and IGF stimulation, but not serum (De Cesare \textit{et al.}, 1998). It is also required for CREB phosphorylation stimulated by EGF, but not serum. These findings strongly suggest that a Rsk2 substrate, possibly CREB, is necessary for EGF-induced \( c-fos \) activation.

Another study with the Rsk-2 knockout fibroblast cell line revealed that, although Elk becomes phosphorylated, TCF reporter activity is substantially reduced following growth factor stimulation (Bruning \textit{et al.}, 2000). Moreover, a \( c-fos \) promoter mutant that can no longer bind TCF did not display a reduced inducibility when compared with the wild type fos reporter gene, suggesting that Rsk-2 might be involved in transducing an addition signal other than TCF phosphorylation, and that this signal is required for transcriptional activity (see Figure 6.6). It is not clear how the specificity arising from the specific extracellular signal is achieved, since there are at least 3 isoforms of Rsk. It is unlikely that the difference observed in \( c-fos \) induction by serum versus EGF is due to the activation of distinct Rsk isoforms (Bruning \textit{et al.}, 2000; De Cesare \textit{et al.}, 1998).

Rsk2 has also been implicated in histone H3 phosphorylation. It is therefore conceivable that \( c-fos \) induction and TCF transcriptional activation in Rsk-2 knockout cells is compromised due to non-phosphorylated H3 or other targets (eg. HMG-14) (Sassone-Corsi \textit{et al.}, 1999). It has been reported that H3 is phosphorylated following
UV-induced activation of p38 kinase (Zhong et al., 2000). A recent study by Thomson et al. demonstrated that H3 phosphorylation is necessary for c-fos induction (Thomson et al., 1999). Since Rsk-2 is not a substrate of p38 kinase, the authors propose that another MAPKAPK family related kinase, MSK1, could be a downstream target of p38 kinase, and phosphorylate H3. Consistent with this, more recently it has been demonstrated that UV-induced H3 phosphorylation is mediated by MSK1 (Zhong et al., 2001). Drosophila studies demonstrate an in vivo role for MSK1 in H3 phosphorylation. Loss of JIL-1, an MSK homologue, results in a significant reduction in H3 phosphorylation at Ser10 (Wang et al., 2001). Further analysis is required to determine which kinases are responsible for signal-induced H3 phosphorylation. It is possible that serum partially activates MSK1 and therefore does not require Rsk-2 to achieve sufficient levels of H3 phosphorylation at c-fos promoter. It is still unclear what determines the specificity of histone phosphorylation at the specific gene promoters. Studies which address these question are very limited.

6.8. Conclusions

1. It has been shown that an integrated SRF reporter gene is activated by polypeptide growth factors and phorbol ester TPA, in contrast to transiently transfected templates. SRF activation by all stimuli is dependent on RhoA and actin polymerisation. It has been demonstrated that activation by growth factors and TPA is dependent on PI-3 kinase activity, while activation by serum and LPA is not.

2. It has been demonstrated that the actin treadmilling cycle is the point at which extracellular stimuli-induced signalling pathways converge to activate SRF. Moreover, changes in actin dynamics are not only necessary but also sufficient to activate SRF.

3. Activation of a subset of target genes, including c-fos and egr-1, are independent of actin and RhoA signals, but are dependent on ERK activity. Another subset of SRF target genes, including srf and vcl, are actin and RhoA dependent, but largely independent of MEK-ERK signalling.

4. Two classes of SRF target genes are defined. It is proposed that the two classes of SRF target genes are regulated in a mutually exclusive manner, and that the presence of TCF may control signalling specificity at SRF target gene promoters.
6.9 Future Directions

It remains possible that other type of SRF target gene exist. Therefore it is important to characterise a larger number of SRF target genes by either using the RNase protection technique or by a high throughput assay, such as DNA microarray technology. As more SRF target genes are characterised it will be of great importance to understand the mechanisms by which promoter specificity to different signalling pathways is determined. The *fos* promoter has been a useful tool in identifying SRF and its associated factors; mutants of this promoter will prove invaluable in determining what renders an SRF target gene sensitive to the actin pathway.

Rapid advances in Human and Mouse Genomic projects allow analysis of gene promoter structure using a bioinformatic approach. Due to the high conservation of promoter sequence in evolution it will be possible to use the consensus binding site of SRF in conjunction with the binding sites of other transcription factors to screen for putative SRF target genes. Combining this bioinformatic approach together with experimental data will deepen our understanding of how different signals activate genes and gene clusters. This will allow a broadening of knowledge in the field of gene regulation, and an understanding of how distinct gene expression patterns result in a different physiological outcomes, such as cell proliferation and differentiation.
Figure 6.1. Perturbation of the actin cytoskeleton by drugs. The actin treadmill cycle is regulated by LIM kinases and GTPases. Cofilin facilitates dissociation of monomers from pointed end of the F-actin, while polymerisation is dependent on Rho family GTPases. LIMK phosphorylates cofilin, preventing its interaction with actin and thereby stabilising F-actin. Jasplakinolide binds and stabilise F-actin, in contrast to Latrunculin, which sequesters G-actin. Clostridium botulinum C2 toxin prevents polymerisation of actin by ADP ribosylation. Swinhöide A sequesters G-actin as dimers. The effects of the Cytochalasin D binding to actin is not clear (binds G-actin either as monomers or dimers).
Figure 6.2. Positive or negative regulation of SRF via actin. It is proposed that depletion of the G-actin pool induces SRF activation. However it is not clear by what mechanism this would act. This theoretical model predicts in general how SRF activity may be controlled; either by released an unknown actin-interacting SRF coactivator "X" or relief of SRF from some repression (perhaps even involving actin directly).
Figure 6.3. Actin polymerisation is required for signalling to SRF. A minimal model for signal-induced changes in actin polymerisation, leading to SRF activation is presented. Differential signalling by serum/LPA and PDGF signalling cascades are color coded. Both signals are dependent on actin polymerisation. The link between GTPases (Rho/Rac) and kinase ROCK/PAK is less clear; Dia and ROCK are downstream effectors for the serum/LPA-induced pathway. It is proposed that PDGF signalling may possibly be linked to SRF via PAK and WAVE.
Figure 6.4. PI-3 kinase is involved in signalling to SRF. A proposed model for the PI-3 K requirement in PDGF- and TPA-induced SRF activation (the model is explained in detail in section 6.3.2). PDGF induces PI-3 K activity which in turn is required for Rac activity. TPA causes PKC activation, but does not however, activate PI-3 K. It is proposed that PDGF signalling to SRF is dependent on the induced PI-3 K activity, while TPA requires only basal PI-3 K activity. It is not clear if this occurs via PKC or possibly some other kinase. Both signalling pathways appears to be dependent on basal Rho activity and actin polymerisation (see also Figure 6.3).
Figure 6.5. Two classes of SRF target genes controlled through different signalling pathways. Signal transduction pathways controlled by the small GTPases Ras and Rho are shown with known SRF targets in red and blue, respectively. ERK indicates the Raf-MEK-ERK pathway, previously shown to control TCF activity; actin dynamics indicates the actin treadmilling cycle, which controls SRF activity and is regulated by the RhoA effector systems: ROCK-LIM kinase-cofilin and Diaphanous (for further discussion see text). Pathway specific inhibitors, U0126 and latrunculin, are indicated. TCF binding to the c-fos and egr-1 promoters is known to be important for signal transduction, however, it remains unclear whether TCF plays a role in regulation of srf and vcl.
Figure 6.6. The effects of ERK inhibition on transcription. A possible role for the MAPK pathway inhibition in transcriptional activation is discussed. Although it is well established that ERK activates TCF, other possible target exists, such as Rsk2. Rsk 2 inhibition could lead to H3 phosphorylation, which in turn could effect transcription (see section 6.7)
7. Materials and Methods

7.1. Reagents

Chemicals

Most chemicals and solvents were obtained from Sigma, BDH Laboratory Supplies or BioRad. All radioactive chemicals were obtained from Amersham International plc. Details of more specialised reagents are given along with the protocols in which they are used.

Other chemicals were obtained as follows:

- Agarose: GibcoBRL
- Ammonium persulphate: Sigma
- Ampicillin: Sigma
- Aprotinin: Boehringer Mannheim
- Benzamidine: Sigma
- Bromophenol Blue: Biorad
- Dithiothreitol (DTT): Calbiochem
- Ethidiumbromide: Boehringer Mannheim
- Glycogen: Boehringer Mannheim
- Guanidine hydrochloride: Fluka
- Leupeptin: Sigma
- dNTPs, rNTPs: Pharmacia
- Orange G: Sigma
- Pepstatin: Sigma
- Phenylmethyl-sulfonyl fluoride (PMSF): Sigma
- Sarcosyl: Fluka
- Spermidine: Sigma
- TEMED: BioRad
- Tris-base: Sigma
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Tris-HCl  Sigma
Xylene cyanol  Biorad

**Inhibitors, stimuli, actin binding drugs**

<table>
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<th>Supplier</th>
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</tr>
<tr>
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<tr>
<td>LY294002</td>
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**Enzymes**

All restriction enzymes were obtained from New England Biolabs (NEB). Other enzymes are as follows:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
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<tr>
<td>AMV reverse transcriptase</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>DNase I</td>
<td>Worthington</td>
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<tr>
<td>Proteinase K</td>
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<tr>
<td>RNase T1</td>
<td>Calbiochem</td>
</tr>
</tbody>
</table>
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RNase inhibitor: Boehringer Mannheim 799 025
SP6 RNA polymerase: NEB
T7 RNA polymerase: Promega
Taq DNA polymerase (AmpliTaq): Promega M 1865
Taq DNA polymerase: Takara RR002A

Buffers and solutions

Protein gel loading buffer: 62.5 mM Tris-HCl pH 8.0, 2% SDS, 2.5%
glycerol, 180 mM 8-mercaptoethanol, 0.1%
w/v Xylene Cyanol and 0.1% w/v Bromophenol Blue
1xTBS: 25 mM Tris-HCl pH 7.5, 150 mM NaCl
Agarose gel loading buffer: 10% glycerol, 10 mM EDTA pH 7.4, 0.1%
w/v Orange G
TBE: 89 mM Tris Base, 89 mM boric acid, 2 mM EDTA
TE: 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0
TE 0.1: 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0
PBS: 0.17 mM NaCl, 3 mM KCl, 1 mM
Na_2HPO_4: 1.8 mM KH_2PO_4 pH 7.4
Ridley Buffer: 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl_2
SDS running buffer: 192 mM glycine, 25 mM Tris Base, 0.1% SDS
Protein transfer buffer: 48 mM Tris Base, 40 mM glycine, 20% methanol, 0.01% SDS
7.2. General Procedures

All nucleic acid manipulations were done in Treff 1.5 ml tubes unless otherwise stated. All centrifugation steps were done in a bench top micro-centrifuge or Beckman GS6KR underbench centrifuge unless stated otherwise. Autoradiography was either with Kodak XAR-5 or BioMAX MS film, except for ECL which was done on Amersham Hyperfilm. Exposed films were scanned using a UMAX Power-look scanner and processed as TIF or Photoshop files using Adobe Photoshop 5.0. Alternatively radiolabelled samples were scanned and quantified using a Molecular Dynamics PhosphorImager with ImageQuant software, images were converted to TIF files and processed using Adobe Photoshop 5.0.

7.3. Bacterial Techniques

7.3.1. Bacterial media and plates

LB media 1% w/v Bacto-tryptone, 0.5% w/v Bacto-

yeast extract, 1% w/v NaCl

LB agar 1% w/v Bacto-tryptone, 0.5% w/v Bacto-

yeast extract, 1% w/v NaCl, 1.5% w/v

Bacto-agar

Ampicillin 100 µg/ml was added to media for selection.

7.3.2. Bacterial strains

DH5α: [F-, recA1, endA1, gyrA96, thi-1, hsdR17, relA1, deoR, supE44,l-] was used for all cloning manipulations.

TG1: [Δ(lac pro), supE, thi-1, hsdD5/F' traD36, proA+B+, LacIq, LacZΔM15] was used to amplify the low copy plasmids.
7.3.3. Transformation of E.coli

Preparation of electrocompetent E.coli: A single colony was inoculated in a sterile tube containing 5 ml of BHI broth and grown overnight at 37°C at 220 rpm. 1 ml of the saturated culture was then transferred to a fresh 500 ml flask containing 100 ml of BHI medium. Cells were then incubated at 37°C with shaking until they reached OD<sub>600</sub> = 0.5. The flask was then chilled on ice for 20 min and the cells were collected by centrifugation at 1200xg for 5 min at 4°C. The pellet was resuspended in 100 ml of ice-cold 10% glycerol made in highly purified water and incubated on ice for 20 min. Cells were then pelleted by centrifugation at 4000 rpm (1200xg) at 4°C for 10 min. After removing carefully as much supernatant as possible, cells were gently resuspended in 10 ml of ice-cold 10% glycerol and incubated for further 20 min. Cells were again repelleted by centrifugation for 10 min at 4°C and finally resuspended in 800 μl of ice-cold 10% glycerol. These cells were ready for electrotransformation and were stored at -70°C in 40 μl aliquots.

Electroporation: Electroporation of DNA into E.coli was performed with a BioRad Gene pulser with a pulse controller. 40 μl of bacterial cell suspension was mixed on ice with 1μl of DNA dissolved in TE or 1.5 μl of ligation mix. The suspension was placed in the bottom of an ice cold electroporation cuvette (BioRad 0.2 cm separation). The cuvette is then placed into the holder of the electroporator. The cells are subjected to an electric pulse of 2.5 kV (capacitance setting 25 μF and resistance setting 200 Ω). 1 ml of LB was then added immediately to the cell suspension which is then incubated at 37°C for 1 hr. Cells were then plated on LB plates containing 100 μg/ml of ampicillin.

7.4. Nucleic acid Manipulations

7.4.1. Preparation of DNA

Mini-prep: Cells from a 1.5 ml overnight culture were pelleted by centrifugation in a microcentrifuge (13000 rpm, 5 min). The media was decanted and
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the cell pellet resuspended in 75 μl solution 1 and lysed by addition of 150 μl of solution 2. After addition of 90 μl ice-cold solution 3, lysates were centrifuged for 3-5 min. the supernatant was transferred to a fresh tube, precipitated with 1ml cold ethanol on ice and centrifuged for 3-5 min. This yielded a pellet of plasmid DNA and cellular RNA, both of which were resuspended in 25 μl TE. RNA was precipitated by the addition of an equal volume of 5 M LiCl solution, followed by centrifugation for 3-5 min. The supernatant was then added to 200μl of TE, phenol extracted and plasmid DNA precipitated by centrifugation for 3-5 min following the addition of 0.7 ml of ice-cold ethanol. Plasmid DNA was then resuspended in 50 μl of TE containing 40 μg/ml RNase A.

Alternatively 4 ml overnight cultures grown in LB were pelleted by centrifugation at 3500 rpm for 10 min and plasmid DNA isolated using the Qiagen Biorobot 9600. On average this yielded 30 μg of plasmid DNA.

Maxi-prep: Cells were pelleted from a 100 ml overnight culture by centrifugation at 3500 rpm for 5 min in a Dupont Sorvall RC-5B centrifuge. The cell pellet was resuspended in 5 ml of ice-cold solution 1 and the cells were lysed by the addition of 10mls of solution 2. Cell debris and chromosomal DNA were then precipitated from the lysate by the addition of 5ml of ice cold solution 3 and pelleted by centrifugation at 3000 rpm for 5 min in Falcon 2098 tubes in an IEC Centra-8R centrifuge. The supernatant was decanted. Nucleic acids were isopropanol precipitated and resuspended in 1.5 ml TE buffer. RNA was precipitated by addition of equal volume of cold 5 M LiCl. Following centrifugation (3000 rpm, 5 min) the supernatant was poured in to a Falcon 2095 tube and plasmid DNA was precipitated by the addition of 2 volumes of ethanol followed by centrifugation (3000 rpm, 10 min). DNA was dissolved in 0.5 ml TE, transferred to a microcentrifuge tube and incubated at 37°C with 40 μg/ml RNase A for 15 min to remove remaining cellular RNA. Plasmid DNA was then precipitated by addition of 0.5 volumes 20% PEG8000, 2.5 M NaCl, leaving on ice for 5 min and centrifugation for 5 min. DNA was resuspended in 0.4ml TE and subjected to one chloroform extraction, two phenol extractions followed by two chloroform extraction. DNA was ethanol precipitated, resuspended in TE and quantified at absorbance 260 nm.

Solutions
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Solution 1: 50 mM glucose, 25 mM Tris-HCl pH 7.4, 10 mM EDTA
Solution 2: 200 mM NaOH, 1% SDS
Solution 3: 5 M K acetate pH 4.8

Alternatively maxi-preps were prepared using Qiagen midi size columns according to manufacturer's recommendations.

7.4.2. Nucleic acid Quantification

Double-stranded DNA, RNA and synthetic oligonucleotides were quantified by spectrophotometer using an LKB Biochrom Ultraspec II spectrophotometer. 1 absorbance unit at 260 nm corresponds to a 50 μg/ml double stranded DNA solution, a 40 μg/ml RNA solution and a 33 μg/ml single stranded DNA solution.

7.4.3. Subcloning procedures

Restriction enzyme digestion: Restriction enzyme digestions were performed for 1-2 hr in the appropriate NEB buffers at 37°C unless recommended otherwise in manufacturer's specification sheet. 10 μl of reaction mixture and 1 unit of enzyme were used per microgram of DNA. Products were analysed by agarose gel electrophoresis.

Filling in 5' overhangs with AMV reverse transcriptase: End-repair of 5' overhangs was performed at 37°C for 30 min using 5 units reverse transcriptase in restriction enzyme digestion buffer containing 0.1 mM dNTPs.

Phosphatase treatment: before phosphatase treatment, reactions were ethanol precipitated using sodium acetate for restriction enzyme digested samples or ammonium acetate for end-repair reactions. The pellet was resuspended in 50 μl 10 mM Tris-HCl pH 9.5, 1 mM spermidine, 0.1 mM EDTA, 1 mM MgCl₂, 0.1 mM ZnCl₂ and incubated at 37°C for 1 hr with 1 unit of alkaline phosphatase (Boehringer Mannheim 1097 075).
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Agarose gel electrophoresis: 0.8-2% agarose gels containing TBE buffer and 2 µg/ml ethidium Bromide (Boehringer Mannheim) were used to analyse DNA fragments. Gels were run at 10 v/cm and DNA visualised at 310/320nm.

DNA fragment purification: DNA fragments were purified on 1-2% Tris borate agarose gels. The DNA fragments were cut out of the gel by visualisation under the UV lamp. The cut agarose fragment was then centrifuged for 10 min at 8000 rpm in 0.5 ml tubes containing silanized glass wool with a small hole at the bottom of the tube held in a 1.5 ml tube. This separated the buffer containing the DNA fragment from the agarose that remained trapped within the glass wool. The supernatant was then phenol extracted and ethanol precipitated with 0.3 M Na acetate pH 5.2 (5 µg of glycogen carrier was routinely added (Boehringer Mannheim 901 393)) and finally resuspended in 10 µl of distilled water.

For sequencing, PCR products were purified using MicroSpin S-400 HR columns (Pharmacia) pre-equilibrated in TE 0.1. 50 µl of PCR product was diluted into 100 µl of dH2O and spun at 4000 rpm for 2 min. The concentration of DNA in the flow through was quantified roughly by agarose gel electrophoresis.

Ligations: 10 µl ligation reactions were carried out at room temperature using 200 units of T4 DNA ligase in 10 mM MgCl2, 60 mM Tris-HCl pH 7.4, 6 mM DTT, 1 mM ATP and left for a minimum of 3 hr or overnight.

7.4.4. Generation of DNA fragments by PCR

Standard 50 µl PCR reactions contained 5 ng plasmid template (unless template was a library, then 200-500ng of template was used), 50 mM KCl, 10 mM Tris-HCl pH8.3, 1.5 mM MgCl2, 100 µg/ml BSA, 0.2 mM dNTPs, approximately 25 ng of each oligonucleotide primer and 2.5 units of Taq polymerase. 28 amplification cycles were performed (unless stated otherwise). Amplification cycles were: 94°C, 1 min; 48-56°C (unless stated otherwise), 1 min; 72°C, 1 min. All constructs made using PCR-generated fragments were verified by DNA sequencing.
7.4.5. DNA sequencing

All sequencing reactions were carried out according to ABI PRISM Dye terminator cycle sequencing kit using ICRF in house sequencing facilities. 0.75 μg of plasmid DNA (either maxiprep or miniprep) or 50 ng of PCR product DNA was mixed with 3.2 pmol of primer and 8 μl of Perkin Elmer Dirhodamine big dye terminator cycle sequencer in a 20 μl reaction. Thermal cycling was as follows; 96°C, 30 sec; 50°C, 15 sec; 72°C, 4 min; 25 cycles. After ethanol precipitation the DNA sequence was analysed using an ABI PRISM 377 DNA sequencer. Sequence analysis was done with ABI Sequence Navigator software.

7.4.6. RNase protection assay

RNA probes were synthesised using the linearized reporter and reference templates (see section 7.8.3). For transcription, 10 μl reaction mixture containing 1 μl of 10x transcription buffer (400 mM Tris-HCl pH 7.5, 60 mM MgCl₂, 20 mM Spermidine), 0.5 μl of each 10 mM ATP, 10 mM GTP and 10 mM CTP, 0.5 μl of RNase inhibitor, 0.5 μl of 200 mM DTT, 0.5 μg of DNA template, 2.0 μl of ³²P-UTP(400 Ci/mm), 0.5 μl of 0.2 mM UTP and 10 units SP6 polymerase was incubated at 40°C for 1 hr. For the reference probes, different concentration of cold and hot UTP were added (0.5 μl of ³²P-UTP(400 Ci/m mol) and 0.5 μl 0.4 mM UTP, respectively). After RNA synthesis, DNA template was digested in 90 μl of 50TE, 0.5 μl of RNase inhibitor, 1.25 μl mixture (10 μl of 1M of MgCl₂, 5 μl of 1 M CaCl₂, 10 μl of 200 mM DTT), and 0.75 μl of DNase I at 37°C for 30 min. Following addition of 4 μl of 0.5 M EDTA pH 8.0 and 10 μg tRNA, the reactions were phenol extracted and precipitated with 20 μl 5 M Ammonium acetate and 0.9 ml ethanol on dry ice. Precipitates were spun down at 13000 rpm for 10 min, washed with 80% ethanol and redissolved in 50 μl of hybridisation buffer (400 mM NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA, 80% v/v formamide (deionised).

10 μg of total RNA slurry was spun out for each sample and dissolved in 20 μl of hybridisation buffer (allowed pellet to rehydrate on ice for 30 min). Approximately 200 000 cpm of each probe was added per sample. Samples were denatured at 85°C for 5 min and hybridized at 45°C overnight. 350 μl of RNase digestion mixture (10
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mM Tris-HCl pH 7.5, 5 mM EDTA, 300 mM NaCl, 50 μg/ml RNase A and 2.5 μg/ml RNase T1) was added, to each hybridized RNA sample and incubated at 37°C for 30 min. Reactions were stopped by adding 5 μl of 20% SDS and 5 μl of 10 mg/ml proteinase K, and incubated at 37°C for 10 min. Following phenol extraction, RNA fragments were precipitated with 15μg of tRNA and 900 μl of ethanol. After centrifugation at 13 000 rpm for 10 min, the pellets were washed with 80% ethanol and resuspended in 5 μl of formamide loading buffer containing bromophenol blue and xylene cyanol. All samples were analysed on thin 6% acrylamide sequencing gels (19:1 acrylamide-bis acrylamide crosslinking (Anachem), 48% w/v urea, in TBE buffer), running at 24 W for approximately 1 hr and 40 min, dried on Whatman 3MM paper and exposed to film.

7.5. Protein Manipulations

7.5.1. Protein quantification

Protein samples were quantified by mixing with 200 μl of BioRad Protein Assay reagent (diluted 1:5 in dH₂O) and measuring absorbance at OD₅₉₅ that was calibrated against readings with known concentrations of Bovine Serum Albumin (Sigma A 7906)

7.5.2. SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE (Laemmli, 1970) using a minigel apparatus (ATTA). 10 ml of gel (10-15% polyacrylamide (30:0.8 crosslinking; Anachem), 0.375 M Tris acetate pH 8.8, 0.1% SDS) was poured between two glass plates and the surface overlaid with isobutanol. After polymerisation, isobutanol was washed off and 2 ml of stacking gel (5% polyacrylamide (30:0.8 crosslinking), 0.125 M Tris-HCl pH 6.8, 0.1% SDS) was poured on top. All acrylamide gels were polymerised by the addition of 1/1000 volume TEMED and 1/200 volume 20% w/v ammonium persulphate. Samples were boiled for 5 min in SDS loading buffer to denature protein before loading. Gels were run at 140 V in SDS running buffer. After SDS-PAGE, gels were either fixed in 10% methanol-10% acetic acid, stained with
0.1% Comassie R250, destained with 10% methanol-10% acetic acid, and dried on Whatmann 3MM paper or transferred on to nitro-cellulose filters for western blotting (see below).

Protein Gel Size Markers: Prestained (Rainbow) markers used for western Blot analysis were purchased from Amersham(200kD, 92.5kD, 69kD, 46kD, 30kD, 14.3kD).

7.5.3. Western blots

Routinely, western blots were carried out as follows: after electrophoresis, gels were transferred on to methanol pre-soaked PVDF membranes (Millipore IPVH00010) sandwiched between Whatmann 3MM paper for 1 hr at 100 V using a Mini Trans-Blot Cell (Biorad) or overnight at 50 V using a Trans-Blot Cell (Biorad) with transfer (48 mM Tris Base, 40 mM glycine, 20% methanol, 0.01% SDS): an ice pack was used to keep the buffer cool. After transfer, the filter was pre blocked with 3% Marvel milk, 1xPBS for 60 min at room temperature with shaking. The filter was incubated with the primary antibody (diluted 1:1000, except pan ERK 1:5000) in a buffer containing 1.5% Marvel milk, 0.1% Tween 20, 1xPBS either overnight at 4°C or for 3 hr at room temperature with rotation. The filter was then washed with in 1xPBS buffer containing, 0.1% Tween 20 for 10 min three times at room temperature. Incubation with the secondary antibody (diluted 1:2000) was same as for the primary antibody except 2 hr incubation at room temperature. Following this, the filter was washed same as before and then incubated for 1 min with the 3 ml ECL reagents (A and B mixed 1:1 from Amersham RPN 2106). The excess liquid was removed and the filter was wrapped in Saran wrap and exposed to Amersham ECL Hyperfilm.

For phospho-tyrosine blots 3% w/v BSA was used instead of 3% w/v Marvel milk when blocking the filter and 1.5% w/v BSA was used instead of Marvel milk during the antibody incubations and washes. TBS was used instead of PBS for all procedures when using phospho-tyrosine antibody.
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7.5.4. Antibodies for western blotting

Antibodies used were as follows: anti HA, 12CA5, mouse monoclonal (Boehringer Mannheim); anti panERK, mouse monoclonal (Transduction Laboratories, E17120); anti-phospho ERK1 (T202,Y204) mouse monoclonal E10 (NEB, 9106S); Akt anti-Ser(P)473 (NEB, 9271S); Akt (NEB, 9272S); 4G10, mouse monoclonal (Upstate Biotechnology Incorporated); anti-mouse HRP conjugated, goat polyclonal (DAKO), anti-rabbit HRP conjugated, swine polyclonal (DAKO).

7.6. Mammalian Cell Culture and Other Techniques

7.6.1. Cell culture media

- E4 (equivalent to DMEM)
- Foetal Calf Serum (FCS)
- OPTIMEM 1
- LIPOFECTAMINE
- Trypsin 0.25% w/v trypsin (Flow Labs) in PBS
- Versene 0.5mM EDTA in PBS

7.6.2. Cell lines

- NIH3T3: A mouse fibroblast cell line (Richard Treisman Laboratory)
- Swiss3T3: A mouse fibroblast cell line (ICRF)
- SRE.FosHA (clone13): A gift from A. Alberts
- SRE.FosHA (clone1-6)
- SRE.FosHA (clone2-9)
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3D.ACD2/CD8

A gift from A. Alberts

SRE.FosHA clones 1-6 and 2-9 was generated by cotransfection of the 3D.AFosHA reporter and TKneo plasmid with a molar ratio 10:1. Cells were transfected using lipofection and maintained for two weeks in medium containing 10% FBS and 1 mg/ml G418 (Gibco-BRL). Medium was changed every 3 days. After 2 weeks cells were picked from individual colonies, expanded and tested for the 3D.AFosHA reporter expression following serum stimulation by western blotting. Following expansion SRE.FosHA clones were maintained in medium containing 10% FBS and 0.4 mg/ml G418.

7.6.3. Transfection

7.6.3.1. Transfection for CAT assay

3x10^5 NIH3T3 cells were seeded in 60mm wells 16hrs prior to transfection in E4+10% FBS. 2μg of DNA (0.3μg 3D.ACAT reporter, 0.5μg MLV LacZ and 1.4 μg MLVβ128 carrier plasmid) was diluted in 200μl of optimem (GibcoBRL), to this 200μl of lipofectamine mixture was added (200:12 optimem:lipofectamine (GibcoBRL)). The DNA-lipofectamine mixture was left at room temperature for 30 min before adding to the cells, of which media had been replaced with 2ml of OPTIMEM1 after washing once in same media. The cells were then incubated at 37°C and 10% CO₂ for 4-5 hr before replacing the DNA mixture with 4 ml of E4+0.5% FBS. After 16-20 hr following starvation, cells were stimulated for 8 hr with an appropriate stimuli.

7.6.3.2. Transfection for TCF reporter assay

NIH3T3 cells in a 6-well plate were transiently transfected using Lipofectamine (GibcoBRL) according to manufacturer's recommendations 1x10⁵ NIH3T3 cells were seeded 16 hr prior to transfection in E4+10% FBS. 0.8 μg of DNA (0.05 μg LexOP2tkLuc reporter, 0.02 μg NLexElkC, 0.1 μg MLV LacZ and 0.65 μg MLVβ128 carrier plasmid) was diluted in 100 μl of optimem (GibcoBRL) and mixed.
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with 100 μl of lipofectamine mixture (100:6 optimem:lipofectamine (GibcoBRL)).
The DNA-lipofectoamine mixture was left at room temperature for 30 min before
adding to the cells, of which media had been replaced with 0.8 ml of OPTIMEM1
after washing once in same media. The cells were then incubated at 37°C and 10%
CO_2 for 4-5 hr before replacing the DNA mix with 2 ml of E4+0.5% FBS. After 16-20
hr following starvation, cells were stimulated for 6 hr with an appropriate stimuli.

7.6.3.3. Transfection for RNase protection assay

3x10^5 NIH3T3 cells were seeded in 60 mm wells 16 hr prior to transfection in
E4+10% FBS. 2 μg of DNA (0.5 μg 3D.AFos (F711) reporter, 0.3 μg MLVα118 and
1.2 μg MLVβ128 carrier plasmid. When additional plasmids were cotransfected, such
as 0.1 μg EFC3, the amount of DNA transfected in each experiment were kept
constant by adding control plasmid) was diluted in 200 μl of optimem (GibcoBRL)
and mixed with 200 μl of lipofectamine mixture (200:12 optimem:lipofectamine
(GibcoBRL)). The DNA-lipofectoamine mixture was left at room temperature for 30
min before adding to the cells, of which media had been replaced with 2 ml of
optimem after washing once in same media. The cells were then incubated at 37°C
and 10%CO_2 for 4-5 hr before replacing the DNA mixture with 4 ml of E4+0.5% FBS.
After 20-24 hr following starvation, cells were stimulated with an appropriate stimuli.

7.6.4. Preparation of whole cell extract for western blotting

For western blot analysis, 4 x 10^6 cells were seeded per 60-mm dish, serum-
starved for 24 hr, pretreated with inhibitors if required, and then stimulated with
appropriate stimuli. Cells were washed twice using ice-cold PBS, 150 μl of 2x Protein
gel loading buffer was added to 60 mm dishes and the cells were scraped off the dish
using a 'rubber policeman'. The extracts were then transferred to microcentrifuge
tubes, sonicated twice for 10 sec, and stored at -20°C. For western blotting. 20 μl of
extract was loaded per lane.
7.6.5. Preparation of lysates for phospho-Akt and phospho-ERK assays

4 x 10^9 cells were seeded per 60-mm dish, serum-starved for 24 hr, pretreated with inhibitors if required, and then stimulated with appropriate stimuli. Cells were washed twice using ice-cold PBS, and lysed into ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 40 mM β-glycerophosphate, 50 mM NaF, 0.1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/m of leupeptin, pepstatin and aprotinin). After clarification by centrifuging, equal amounts of lysate were resolved by 10% SDS-PAGE, transferred onto polyvinylidenedifluoride membrane (Immobilon-P, Milipore) and probed with an appropriate antibodies.

7.6.6. Preparation of extract for CAT and β-galactosidase assays

Cells were washed once with ice-cold PBS. 1 ml PBS was then added to the plates cells were scraped into an micro-centrifuge tube. After spinning at 8000 rpm for 3 min, supernatant was aspirated off and pellet resuspended in 200 μl of 0.25 M Tris-HCl pH7.5. Samples were then frozen on dry ice for 5 min. The samples were then thawed at 37°C for 5 min, vortexed, frozen, and this procedure was repeated three times. After spinning at 13000 rpm for 2 min the supernatant was transferred to fresh tubes. Protein concentration was then measured on 2.5 μl extract using 200 μl BioRad protein assay reagent (see section 7.5.1). Extracts were then equalised for protein concentration, and up to a maximum of 80 μl were used for either CAT assays or β-galactosidase assays.

7.6.7. β-galactosidase assay

80 μl extract was incubated with 400 μl LacZ buffer (60 mM Na₂HPO₄.7H₂O, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 2.7 ml/l β-mercaptoethanol) and 100 μl of 4 mg/ml O-Nitro-Phenyl-β-D-galactoside (ONPG) at 37°C. Reactions were stopped after 2-6 hr (or until reactions turned lemon yellow), by the addition of 250 μl 1M Na₂CO₃. β-galactosidase activity was quantified at absorbance 595 nm.
7.6.9. CAT assay

80 μl of cell extract (equalised for amount of protein between samples) was incubated with 2.5 μl of 40 mM Acetyl CoA (Sigma A 2056), 1.5 μl of 14C-Chloramphenicol (0.2 μCi/μl) and 6 μl of dH₂O for 2 hr at 37°C. 500 μl ethyl acetate was then added to the reaction, vortexed for 15 sec and spun for 2 min. The top organic layer was transferred to a fresh tube and re-extracted with 200 μl ethyl acetate. The ethyl acetate extract was combined and dried down in a Speed Vac for 20-30 min. 30 μl of ethyl acetate was then added to each tube and vortexed to mix. Samples were spotted on to the TLC plates (20 cm x 20 cm Aldrich Z12, 277-7) and were run in tank containing 250 ml buffer (5% methanol, 95% chloroform) for 75 min. Plates were then dried and autoradiographed.

7.6.10. Luciferase assay

Firefly luciferase was measured using luciferase reporter assay system according manufacturer’s recommendations (Promega). Cells were washed twice with ice-cold PBS, and lysed in 200 μl reporter lysis buffer (Promega, Cat. No E4030). 20 μl of lysate was used to determine firefly luciferase activity. The activity was normalised to protein content in the lysate. Shown values are means of at least three independent experiments. Arrow bars represent standard error of the mean (SEM). The data are normalised to the serum response (15% FBS), which is taken as 100.

7.6.11. RNA preparation

RNA from transfected NIH3T3 cells in 60 mm tissue culture dishes was prepared as follows. The cells were washed twice with ice-cold PBS. 500 μl of guanidinium solution was added to each plate. The cell lysate was scraped with a rubber policeman and transferred to a 1.5 ml eppendorf tube. 50 μl of 2 M Na acetate pH 4 was added to each sample and mixed well. Subsequently 500 μl of acid phenol was added and mixed well. 100 μl of chloroform was added, the solution was vortexed for 30 seconds and then spun for 3-5 min at room temperature at 6000 rpm. The top phase was transferred to new tube and precipitated with an equal volume (~750 μl) cold isopropanol at -20°C or overnight. After spinning for 15 min at 13000
rpm the pellet was resuspended in 200 µl of TES, debris was removed by spinning for a further 3 min at 13000 rpm. The supernatant was transferred to a fresh tube and precipitated with 20 µl of 3 M Na Acetate pH 5.6 and 700 µl cold ethanol on dry ice. For RNA from transfected cells, following centrifugation for 10 min at 13000 rpm, the pellet was resuspended in 100 µl of 50TE. 0.5 µl of 1 M CaCl₂, 1.25 µl of 1 M MgCl₂, 0.5 µl of RNase inhibitor, 0.5 µl of 200 mM DTT, 0.5 µl of DNase 1 (RNase-free) was added and incubated at 37°C for 30 min. After extraction with 100 µl of 2FC, the aqueous phase was transferred to a fresh tube and precipitated with 10 µl 3 M Na acetate pH 5.6 and 300 µl of ethanol. For RNA from non-transfected cells, DNase 1 treatment was omitted. After centrifugation at 13000 rpm for 10 min the pellet was resuspended in 50 µl of TE and quantified at absorbance 260 nm. The total cell mRNA was then analysed as described above (see 7.4 Nucleic Acid manipulations).

**Solutions**

Guanidinium solution: 236.25 g guanidinium isothyocyanate (Fluka 50990), 3.675 g sodium citrate, 2.5 g N-lauryl sarcosine, 3.575 ml β-mercaptoethanol in 500 ml with water.

TES: 10 mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, 0.1% SDS

50TE: 50 mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0

Acid phenol: Phenol saturated with water and 8-hydroxyquinoline to 0.2% w/v or alternatively, premade acid phenol (Sigma P-4682)

DNase 1: Worthington enzyme: RNase free. 2.7 units/µl dissolved in 10 mM Tris-HCl pH 7.5, 50% glycerol.

2FC: 50% Phenol, 50% Chloroform.

**7.6.12. Microinjection**

NIH3T3 cells were seeded on glass coverslips 48 hr before injection and serum starved in EM + 0.5% FCS for 24 hr prior to injection. Injections were done on a Zeiss 5171 semi-automated machine using glass pulled capillaries (Intrafil 01-020-06 1.2mm outer diameter 0.82mm inner diameter, vertically pulled on a KOPF 720 puller, heater set at 12.3). All DNAs were injected into the cell nucleus in Ridley buffer, when necessary pEF-GFP plasmid was co-injected as marker. Cells were
routinely harvested 5 hr after injection; starved cells were stimulated with 15% FCS or PDGF-BB 1 hr prior to harvesting.

7.6.13. Immunofluorescence

Cells were fixed in fresh 4% formaldehyde in PBS for 10 min followed by extraction in 0.3% Triton X100. Antibodies were diluted 1:100 in PBS + 5% calf serum and the cells were washed four times in PBS after antibody incubations and prior to mounting. Images were obtained using a Zeiss Axiovert microscope and Smart Capture system (Vysis UK) and processed as PICT files using Adobe photoshop 5.0. Antibodies used were as follows: 12CA5 (mouse monoclonal - Boehringer Mannheim), Y-11 (rabbit polyclonal - Santa Cruz anti-rabbit-Texas Red or -FITC (FlouresceinIsoThioCyanate), anti-mouse-FITC or -AMCA (AminoMethylCoumarin), and also TRITC (Tetramethyl Rhodamine IsoThioCyanate) phalloidin (Sigma P 1951).

7.6.14. Immunofluorescence analysis by flow cytometry

Following stimulation, cells were washed twice in PBS and detached from the dish incubating for 10 min in 1ml of PBS containing 2 mM EDTA at 37°C. Antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerethrin (PE) were used at final concentrations of 5 µg/ml. All incubations for FACS analysis were performed at 4°C. For staining cells were transferred into FACS tubes (Falcon) and pelleted by centrifugation 1000 rpm for 4 min. The pellet was resuspended in 0.5 ml E4 containing 1% FBS and appropriate antibody and incubated for 30 min on ice in the dark. Cells were then washed free of unbound antibody by the addition of 2 ml of E4 containing 1% FBS, centrifuged at 1000 rpm for 4 min and finally resuspended in 0.5 ml E4 + 1% FBS. Acquisition and analysis of stained cells was performed on FACScan (Becton Dickinson), using Cellquest software. Cells were gated on forward and side scatter on a linear scale, and data for other parameters collected in logarithmic scale. Unstained cells and cells stained with single fluorochrome were used to set up the electronic gating collection parameters. Usually, at least 10⁶ events were collected per sample. All events were saved and gates were introduced to allow analysis of specific cell populations.
7.6.15. Recombinant toxins

C3 fusion toxin (C2IN-C3 and C2II), C2 toxin (C2I and C2II) and toxin B were kindly provided by H.Barth and K. Aktories (Freiburg University, Freiburg). C2IN-C3 and C2II proteins were added directly to the tissue culture medium at 0.4 μg/ml concentration 5 hr prior to stimulation. C2I and C2II proteins were added directly to the tissue culture medium at 0.2 μg/ml 0.4 μg/ml concentration, respectively. Toxin B was added at 5 ng/ml concentration 60 min before stimulation. At these times more than 90% of the cells were rounded up.

7.7. Plasmids and Oligonucleotides

7.7.1. Mammalian reporter plasmids

3D.ACAT
Described (Mohun et al., 1987)

3D.ALUC
Made by O. Geneste
3D.ALUC is reporter plasmid is a derivative of 3D.ACAT with firefly luciferase in place of the CAT sequence

LexOP2tkLUC
Made by R. Thomas
LexOP2tkLUC comprise the LexA operator and HSVtk promoter sequence from LexOP2tkCAT (Marais et al., 1993) inserted into the XhoI site of pGL3Basic (Promega)

3D.AFosHA
Made by E. Sahai
Based on 3D.AFos described (Hill et al., 1995)
3xHA epitope (includes BamHI site) cloned in NcoI site of exon 4 of c-fos gene
CC ATG TAC CCA TAC GAT GTT CCT GAC TAT GCG GGC TAT CCC TAT GAC
GTC CCG GAC TAT GCA GGA TCC TAT CCA TAT GAC GTT CCA GAT TAC
GCT GCT CAG TGC GCC ATG G

pF711 (Fos wt)
Described (Treisman, 1985)

MLVα118 (α-globin)
Described (Hill and Treisman, 1995)

7.7.2 Mammalian expression plasmids

NLexELKC
Described (Marais et al., 1993)

MLVLacZ
Described (Marais et al., 1993)

MLV128β
Described (Marais et al., 1993)

EFC3
Described (Hill et al., 1995)

EFGFP
Described (Alberts and Treisman, 1998)

7.7.3 RNase protection probes

SP6AF5'AH2
Chapter 7: Materials and Methods

Based on SP6AF5' described (Hill et al., 1995). RNase protection probe used for 3D.AFosHA detection. Contains HincII-HincII deletion resulting in an intron 1 excision. Generates 242-nucleotide protected fragment. Linearised with EcoRI.

GEM1GAPDHAX (SP6)

Mouse GAPDH probe, a 165-nucleotide probe spanning cDNA nucleotides 92-241 (GenBank accession number M32599) and generating 150-nucleotide protected fragment. Linearised with BamH1.

SP132


SP65M-c-fos5'

Mouse c-fos probe, a 199-nucleotide probe spanning 5'- flanking region and part of exon 1, nucleotides 540-573 (GenBank accession number V00727), generating 185-nucleotide protected fragment. Linearised with HindIII.

SP6egr1

Made by J.Copeland.

Mouse egr-1 RNase protection probe, a 348-nucleotide probe spanning 5'- flanking region and part of exon 1, nucleotides 1348-1661 (GenBank accession number V22326), generating 267-nucleotide protected fragment. Linearised with EcoRI.

GEMsrf(SP6)

Mouse srf RNase protection probe, a 252-nucleotide probe spanning exon 3 nucleotides 1128 -1334 (R. Nicolas, personal communication) and generating a 175-nucleotide protected fragment. Linearised with EcoRI.

GEMvinc(SP6)
Mouse vcl RNase protection probe, a 316-nucleotide probe spanning cDNA nucleotides 1204-1444 (GenBank accession number L18880) and generating a 239-nucleotide protected fragment. Linearised with BamHI.

GEMsrf-IN4(SP6)

Mouse pre-srf probe, a 343-nucleotide probe spanning the exon 5 - intron 5 boundary, nucleotides 8181-8450 (GenBank accession number AB03837), generating a 276-nucleotide protected fragment (exon 5 - intron 5 precursor) and a 192-nucleotide protected fragment (exon 5 mRNA). Linearised with EcoRI.

TOPOvcl-IN2(T7)

Mouse pre-vcl probe, a 343-nucleotide probe spanning the exon 3 - intron 3 boundary, nucleotides 413-769 (GenBank accession number L13299), generating a 357-nucleotide protected fragment (exon 3 - intron 3 precursor) and a 108-nucleotide protected fragment (exon 3 mRNA). Linearised with BamHI.

7.7.4 Oligonucleotides used for RNase protection probes

GEMsrf(SP6)

CCAGAATTCACAGTCACCAACCTGC - TOP
TGTAAGCTTGAGCACAGTCCCGTTG - BOTTOM

GEMvinc(SP6)

TGGCGGATCCAAATGGTGACCTGAGGGA - TOP
TTGGTCTGCCAGGTCTGTAGTGCCGTCGC - BOTTOM

GEMsrf-IN4(SP6)
GTGAATTCCAGTGACGTTGCCCGCCACCATC - TOP
GGTGATCCAGGGGTGCATGTTGGCGCTGGAG - BOTTOM

TOPOvel-IN2(T7)

GTCCGGATCCCTTCAGTCAGACCCATACTCG - TOP
GGAGAATTCACTCCAGGTCAAGAACTCATG - BOTTOM
REFERENCES


Alessi, D.R. (1997) The protein kinase C inhibitors Ro 318220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1beta (Rsk-2) and p70 S6 kinase. *FEBS Lett*, 402, 121-123.


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Signal-Regulated Activation of Serum Response Factor Is Mediated by Changes in Actin Dynamics

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Summary

Serum response factor (SRF) regulates transcription of many serum-inducible and muscle-specific genes. Using a functional screen, we identified LIM kinase-1 as a potent activator of SRF. We show that SRF activation by LIM kinase-1 is dependent on its ability to regulate actin treadmilling. LIM kinase activity is not essential for SRF activation by serum, but signals depend on alterations in actin dynamics. Studies with actin-binding drugs, the actin-specific C2 toxin, and actin overexpression demonstrate that G-actin level controls SRF. Regulation of actin dynamics is necessary for serum induction of a subset of SRF target genes, including vinculin, cytoskeletal actin, and srf itself, and also suffices for their activation. Actin treadmilling provides a convergence point for both serum- and LIM kinase-1-induced signaling to SRF.

Introduction

The MADS box transcription factor serum response factor (SRF) regulates both cellular immediate-early genes and genes expressed in skeletal, smooth, and cardiac muscle (for references, see Arsenian et al., 1998). Like many transcription factors, SRF exhibits combinatorial interactions with other transcription factors. At the growth factor-regulated c-fos serum response element, it forms a ternary complex with members of the ternary complex factor (TCF) family of Ets domain proteins, whose activity is regulated by MAP kinase signaling pathways (for review, see Treisman, 1994). In contrast, at muscle-specific promoters, SRF acts cooperatively with other transcription factors, including GATA-4, Nkx2.5, and the myogenic group of E box proteins (Sartorelli et al., 1990; Chen et al., 1996; Durocher et al., 1997; Sepulveda et al., 1998).

Extracellular signals can also control SRF activity in the absence of serum binding by a pathway that involves Rho family GTPases. Serum- or lysophosphatidic acid (LPA)-induced activation of SRF is dependent on functional RhoA, as is the apparently constitutive activity of SRF at muscle-specific promoters (Hill et al., 1995). Although constitutively active RhoA can transform fibroblasts, and Ras-induced transformation and cell cycle reentry is Rho dependent (reviewed by Van Aelst and D'Souza-Schorey, 1997), Studies with RhoA and Rac effector loop mutants show that SRF activation does not correlate with transformation or cytoskeletal rearrangements but have failed to identify the effector proteins involved (Westwick et al., 1997; Sahai et al., 1998; Zohar et al., 1998). Although the RhoA effector kinase ROCK can activate SRF, this process is itself Rho dependent, and ROCK activity does not appear necessary for serum-induced SRF activation (Chihara et al., 1997; Sahai et al., 1998). The mechanism of SRF activation and its relationship to other RhoA-controlled processes has therefore remained unclear.

We developed an in vivo functional screen for SRF activators and describe here its use to identify the actin regulator LIM kinase-1 (LIMK-1) as a powerful SRF activator. We show that although LIMK-1 is not essential for serum-regulated activation of SRF, it is the ability of both LIMK-1 and extracellular signals to regulate the actin treadmilling cycle that regulates SRF activity. Depletion of G-actin is both necessary and sufficient for activation both of SRF reporters and a subset of SRF target genes. Our results show that the actin treadmilling cycle represents a convergence point in signaling pathways to SRF and reveal a direct link between cytoskeletal reorganization and gene transcription.

Results

Expression Screening for SRF Activators

To establish an in vivo screening assay for SRF activators, we first constructed an indicator COS cell line, COS/3DA.CD8, containing an SRF-controlled CD8 reporter gene (see Experimental Procedures). These cells were then used to screen a VP16-tagged NIH3T3 cDNA library by FACS sorting for cell-surface CD8 expression. We expected to recover two types of activator: cDNAs that stimulate transcription directly, encoding proteins that interact with SRF or proteins bound to it; and cDNAs that act indirectly, encoding proteins that activate SRF-linked signal transduction pathways (Figure 1A). Following sorting, DNA was recovered from the top 0.7% of CD8-expressing cells and divided into pools of 50 plasmids. Of 150 pools screened, 5 positive pools

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SRF in the absence of extracellular signals; Cdc42 and Rac1 function independently of RhoA, but their relevance to regulation of SRF by extracellular signals remains unclear (Hill et al., 1995). Since serum- and LPA-induced activation of SRF does not require known posttranslational modifications of the protein, we have previously proposed that it is mediated by an as yet uncharacterized SRF accessory factor (Hill et al., 1994; Johansen and Prywes, 1994).

RhoA controls diverse cellular processes concerned with the cytoskeleton, including PIP2 synthesis, actin polymerization, F-actin bundling, myosin-based contractility, focal adhesion formation, and cytokinesis. Constitutively active RhoA can transform fibroblasts, and Ras-induced transformation and cell cycle reentry is Rho dependent (reviewed by Van Aelst and D'Souza-Schorey, 1997). Studies with RhoA and Rac effector loop mutants show that SRF activation does not correlate with transformation or cytoskeletal rearrangements but have failed to identify the effector proteins involved (Westwick et al., 1997; Sahai et al., 1998; Zohar et al., 1998). Although the RhoA effector kinase ROCK can activate SRF, this process is itself Rho dependent, and ROCK activity does not appear necessary for serum-induced SRF activation (Chihara et al., 1997; Sahai et al., 1998). The mechanism of SRF activation and its relationship to other RhoA-controlled processes has therefore remained unclear.

We developed an in vivo functional screen for SRF activators and describe here its use to identify the actin regulator LIM kinase-1 (LIMK-1) as a powerful SRF activator. We show that although LIMK-1 is not essential for serum-regulated activation of SRF, it is the ability of both LIMK-1 and extracellular signals to regulate the actin treadmilling cycle that regulates SRF activity. Depletion of G-actin is both necessary and sufficient for activation both of SRF reporters and a subset of SRF target genes. Our results show that the actin treadmilling cycle represents a convergence point in signaling pathways to SRF and reveal a direct link between cytoskeletal reorganization and gene transcription.

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Activation of SRF by LIM Kinases

LIMK-1 and its close relative LIMK-2 define a novel family of serine/threonine kinases containing two N-terminal LIM motifs and a PDZ domain (Bernard et al., 1994; Mizuno et al., 1994). To investigate the specificity of transcriptional activation by LIMK-1, we first performed transfection assays using reporters controlled by various derivatives of the c-fos SRE (Hill et al., 1994; Figure 2A). Clone Q1 activated the wild-type c-fos SRE at levels comparable to SRF-VP16 or serum stimulation; mutation of the TCF-binding site actually increased activation (Figure 2A). The VP16 tag was not required for activation, suggesting that activation occurs via the action of the kinase (Figure 2A). Neither intact LIMK-1 nor its kinase domain alone could activate the MAP kinases ERK2 and SAPK/JNK (data not shown), and, consistent with this, they did not activate a reporter system controlled by the MAPK-controlled TCF Elk-1 (Figure 2A, right panel). We next compared the ability of several LIMK-1 and LIMK-2 derivatives, shown in Figure 2B, to activate the SRF-controlled reporter gene 3D.ACAT (Mohun et al., 1987; Hill et al., 1995). LIMK derivatives activated SRF at levels comparable to that by activated Rho family GTPases. The isolated LIMK-1 kinase domain was identified and the active plasmids isolated by sibling selection (Figure 1B). Two different partial cDNAs were recovered (Figure 1C): clone G5, encoding mouse SRF residues 59–276, which encompass the DNA-binding domain, and clone Q1, which encodes mouse LIM K-1 residues 293–646, including the kinase domain. The repeated isolation of these cDNAs (G5, twice; Q1, three times) shows that under the screening conditions used, the library was screened at saturation.

Figure 1. COS Cell Expression Screen for SRF Activators

(A) Experimental strategy. Left panel, the SRF-controlled reporter in 3D.ACD8 cells is shown with the SRF dimer as shaded circles, and below are two expected scenarios for activation of the reporter gene by VP16-tagged cDNAs. Right panel, the screening protocol. (B) Sibling selection of a positive pool. FACs analysis of positive pool 20 (thick lines) compared to cells transfected with vector alone (thin lines). (C) Schematic representation of the clones isolated in the screen compared with the intact proteins.

Figure 2. LIMK-1 Is a Strong Activator of SRF

(A) LIMK-1 activates the SRF-linked signaling pathway. Left panel, behavior of different c-fos SRE derivatives: SRE.WT binds both SRF and TCF; SRE.L binds SRF only; SRE.LM binds neither SRF nor TCF. LIMK-1 derivatives used are shown in (B). SRF.M2-VP16 is an altered-specificity SRF that can activate the SRE.LM reporter (Hill et al., 1994). NIH3T3 cells were transfected using DEAE-dextran; data shown are from a representative experiment. Right panel, LIMK-1 does not activate the ternary complex factor Elk-1. Cells were transfected with the reporter LexOP.CAT together with expression plasmids encoding the LexA/Elk-1 fusion protein NLex.ElkC and LIMK-1 derivatives. Data shown represent the mean ± SEM from three independent transfections. (B) LIMK-1 and LIMK-2 derivatives used in this paper. Point mutations are represented by black lines. (C) LIMK-1 kinase activity is required for SRF activation. NIH3T3 cells were transfected with the SRF-controlled 3D.ACAT reporter together with the indicated activators. Data shown represent the mean ± SEM from at least three independent transfections.
was more effective than that of LIMK-2, and both were more effective than the intact proteins, even though all were expressed at similar levels (Figure 2C; data not shown); it is likely that this reflects the increased activity of the isolated kinase domain (Edwards and Gill, 1999). SRF activation by LIMK-1 was abolished by kinase-inactive mutations (Yang et al., 1998; Edwards and Gill, 1999); these mutations also inactivated VP16-tagged clone Q1, indicating that activation cannot occur via two hybrid interactions between the kinase and the reporter gene. Taken together, these results show that LIM kinases specifically activate the SRF-linked signal pathway.

LIMK-1-Induced SRF Activation Requires F-Actin Stabilization

Currently, the only known LIMK substrates are the cofilins, small actin-binding proteins that facilitate the dissociation of actin monomers from F-actin pointed ends (see Figure 3A; Carlier et al., 1997; Lappalainen and Drubin, 1997). Phosphorylation of cofilin at serine 3 by LIMKs prevents its interaction with actin and thereby stabilizes F-actin. Overexpression of LIMK-1 therefore promotes formation of F-actin aggregates, an effect that can be blocked by coexpression of the nonphosphorylatable cofilin mutant Cof-S3A (Arber et al., 1998; Yang et al., 1998). We exploited this observation to test whether SRF activation by LIMK-1 is mediated via its effect on F-actin.

NIH3T3 cells were microinjected with LIMK and cofilin expression plasmids, together with the SRF-controlled reporter 3D.FosHA. The LIMK-1 kinase domain induced formation of large F-actin aggregates, as did intact LIMK-1, although less effectively, and this required kinase activity (Figure 3B; data not shown). Both intact LIMK-1 and its kinase domain also activated the coexpressed SRF reporter gene (Figure 3C). Expression of Cof-S3A but not wild-type cofilin blocked formation of actin aggregates by LIMK-1 or its kinase domain (Figure 3B; data not shown) and substantially inhibited activation of the SRF-controlled reporter gene (Figure 3C). These effects were dependent on interaction of the mutant cofilin with actin, since neither phenotype was affected by coexpression of Cof-S3A/K112,114A, a mutant that can neither be phosphorylated nor bind actin (Figures 3B and 3C; see Lappalainen et al., 1997). These results strongly suggest that the ability of LIMK-1 derivatives to activate SRF is dependent on its ability to regulate actin treadmilling.

We used overexpression of Cof-S3A or kinase-inactive LIMK-1 to test whether SRF regulation by extracellular signals also requires cofilin phosphorylation by LIMK. Under conditions sufficient to block activation by coexpressed LIMK-1, Cof-S3A expression had no effect on SRF activation by serum or only a marginal effect on that by LPA (Figure 3C). Similarly, under conditions where the kinase-inactive LIMK-1 mutant D460A/T580V (Edwards and Gill, 1999; see Figure 2B) could partially inhibit SRF activation by LIMK-1, it had no effect on SRF activation by serum or LPA (Figure 3D). Together, these data show that although LIMK-1 regulates SRF activity via its effects on actin, its activity is not essential for transmission of serum- or LPA-induced signals to SRF.
A. Microinjection, 3D.AFosHA reporter

![Image of microinjection experiment showing phalloidin and HA staining]

(A) Both cytoskeletal rearrangements and SRF activation by LIMK-1 are RhoA dependent. NIH3T3 cells were microinjected with plasmids encoding LIMK-1 derivatives (100 μg/ml) and C3 transferase (25 μg/ml) together with the SRF-controlled reporter 3D.AFosHA (50 μg/ml). Five hours later, FosHA protein was detected by indirect immunofluorescence and F-actin visualized using Texas red-phalloidin. Microinjected cells, identified by staining for LIMK-1, are arrowed. Similar results were observed when endogenous RhoA was sequestered by coexpression of the RhoA-binding domain of its effector kinase PKN (data not shown).

(B) Sensitivity of SRF activation to C3 transferase in the microinjection assay. Data represent mean ± SEM from three independent microinjection experiments performed as in (A).

(C) SRF activation by LIMK-1 is largely RhoA dependent in the transfection assay. NIH3T3 cells were transfected with the SRF-controlled 3D.ACAT reporter together with the indicated activators. Data shown represent the mean ± SEM from three independent transfections.

Aelst and D’Souza-Schorey, 1997; Wasserman, 1998; Bl and Zigmond, 1999). Since even serum-starved cells possess basal levels of RhoA activity (C. Hill, A. S. Alberts, and R. T., unpublished data), we used C3 transferase, which inactivates RhoA and its close relatives, to test whether LIMK-induced SRF activation and actin reorganization were dependent on RhoA.

In microinjection assays, expression of C3 transferase antagonized the formation of F-actin aggregates in cells expressing the LIMK-1 kinase domain expression plasmid and had a similar effect on the less dramatic actin rearrangements induced by intact LIMK-1, LIMK-2, or the LIMK-2 kinase domain (Figure 4A, upper panels, and data not shown). LIMK-1 expression partially alleviated the cytoskeletal disruption induced by C3 transferase, demonstrating that functional RhoA is not essential for LIMK activity (data not shown; Figure 4A, upper panels; see Discussion). Activation of SRF by intact LIMK-1 or LIMK-2 was strongly inhibited by C3 transferase. However, SRF activation by the LIMK-1 kinase domain was only partially inhibited at low plasmid input and apparently unaffected at high plasmid concentration (Figures 4A and 4B; see Discussion). Similar results were obtained in the more quantitative transfection assay: SRF activation by the LIMK-1 kinase domain was sensitive to C3 transferase at low plasmid concentrations but substantially RhoA independent at high plasmid inputs, while LIMK-2 was RhoA dependent at all concentrations of input plasmid (Figure 4C).

These results show that in serum-starved NIH3T3 cells, LIMK-induced actin rearrangements and SRF activation are substantially dependent on basal levels of RhoA signaling. Actin treadmilling therefore represents a potential convergence point for LIMK- and Rho-dependent signaling to SRF.

Actin Polymerization Inhibitors Prevent SRF Activation by LIMK-1, Rho Family GTPases, and Extracellular Signals

To examine the role of actin dynamics in SRF activation more directly, we tested the effects of actin polymerization inhibitors. Cells were treated either with the toxin latrunculin B, which sequesters G-actin monomers (Coue et al., 1987), or with Clostridium botulinum C2 toxin, which prevents polymerization of actin by ADP-ribosylating it at residue R177 (Vandekerckhove et al., 1988).

We first tested the effect of latrunculin B treatment on activation of SRF induced by cotransfection of signaling components. Latrunculin B significantly impaired activation of the SRF reporter 3D.CAT by LIMK-1 derivatives, constitutively active Rho family GTPases, and the activated RhoA exchange factor mNet1A; it did not affect activation by the constitutively active SRF derivative
role of actin polymerization in serum- or LPA-induced SRF activation, which are dependent on endogenous GDPDh transcripts. Latrunculin B did not affect endogenous SRF activity via Actin Dynamics

Figure 5. Inhibition of Actin Polymerization by Latrunculin or C. botulinum 2B Toxin Inhibits SRF Activation

(A) Latrunculin B inhibits SRF but not TCF. Cells were transfected with reporters and activator plasmids as indicated. Eight hours after the start of the transfection, latrunculin B was added; cells were harvested 12 hr later. Left panel, SRF-controlled 3D.ACAT reporter system. Data are mean ± SEM from three independent transfections, with activity in the absence of inhibitor set to 100 (see Figure 2C). Right panel, Elk-1-controlled Nlex.ElkC/LexOP.CAT reporter system. (B) NIH3T3 cells maintained in 0.5% FCS for 24 hr were stimulated with 10% FCS for 2 hr were stimulated with 10% FCS for the indicated times or for 30 min with 50 ng/ml anisomycin (aniso), UV irradiation (UV), or 0.5 μM jasplakinolide; latrunculin B pretreatment (60 min) was as indicated. Cell lysates were analyzed by immunoblotting with ERK2 or phospho-JNK antibodies.

(C) Latrunculin B blocks SRF activation by serum and LPA. SRE.FosHA cells, which contain the SRF-controlled reporter 3D.AFOsHA, were serum-starved for 36 hr. Following a 60 min latrunculin B pretreatment as indicated, cells were stimulated with 15% FCS or 10 μM LPA for 30 min before analysis by RNase protection for 3D.AFOsHA and GAPDH transcripts. Latrunculin B did not affect endogenous RhoA level (data not shown).

(D) C2 toxin treatment blocks SRF activation by serum. SRE.FosHA cells were serum-starved for 36 hr and then treated with C2 toxin (C2I, 200 ng/ml; C2II, 400 ng/ml) for 4 hr before serum stimulation and analysis as in (C).

SRF-VP16 and had no effect alone (Figure 5A; see also Figure 6B). Similar results were obtained with latrunculin A (data not shown). Latrunculin B had little effect on serum-induced ERK2 phosphorylation or stress-induced activation of SAPK/JNK, showing that it specifically blocks SRF-linked signaling pathways (Figure 5B). Consistent with this, latrunculin treatment had no effect on the ability of constitutively active Ras to activate a reporter gene system controlled by the MAP-responsive SRF cofactor TCF Elk-1 (Figure 5A, right panel).

Latrunculin B treatment led to a reduction in protein accumulation, as assessed by immunoblotting, but this was observed with all activators (data not shown).

We next used latrunculin B and C2 toxin to test the role of actin polymerization in serum- or LPA-induced SRF activation, which are dependent on endogenous RhoA (Hill et al., 1995). For these experiments, we performed RNase protection assays using the NIH3T3-derived cell line SRE.FosHA, which carries an integrated SRF-controlled reporter gene (Alberts et al., 1998). Latrunculin B treatment completely blocked serum- and LPA-induced transcription of the reporter (Figure 5C). Similar results were obtained upon treatment of the cells with C2 toxin (Figure 5D). Taken together, these data show that although LIMK is not required for serum-induced SRF activation, both LIMK- and serum-induced signals acting through RhoA converge at the level of actin treadmilling.

Induction of SRF by Actin-Polymerizing Agents

The experiments described in the preceding sections suggest that stimuli that activate SRF do so by inducing F-actin accumulation. We therefore tested the effect on SRF activity of other drugs and proteins known to increase F-actin levels. These agents include jasplakinolide, which binds to and stabilizes F-actin (Bubb et al., 1994), and proteins belonging to the WASP and Diaphanous families that promote actin polymerization (Beckerle, 1996; Wasserman, 1996; Blanks and Zigmond, 1999).

Jasplakinolide strongly activated transcription of the SRF reporter gene in SRE.FosHA cells, although with somewhat slower and more prolonged kinetics than serum (Figure 6A). Latrunculin B pretreatment retarded jasplakinolide-induced SRF activation but did not block it, consistent with the fact that the two drugs bind reversibly to different types of actin target (Figure 6B). Jasplakinolide also activated the transfected SRF-controlled reporter 3D.ACAT (Figure 6B) but did not activate the MAP kinase–controlled Elk-1 TCF reporter gene system or MAP kinases (data not shown, see Figure 5B). Overexpression of the WASP family proteins VASP, WASP, and N-WASP activated the SRF-controlled reporter, and the C-terminal 110 residues of N-WASP (VCA), which are sufficient to promote actin polymerization in vitro (Machesky and Gould, 1999; Rohatgi et al., 1999), were also sufficient for SRF activation (Figure 5C). Removal of the N-terminal Rho-binding domains of mDia1 and mDia2 generates constitutively active forms that promote F-actin accumulation (Watanebe et al., 1999; Tominaga et al., submitted), and these mutants also strongly activated SRF (Figure 5C). Thus, in addition to LIMK, both drugs and proteins that promote F-actin accumulation activate SRF.

SRF Responds to G-Actin Levels

Although the experiments presented showed that increased F-actin levels are associated with increased SRF activity, they do not address whether SRF senses increased F-actin level itself, an increased F-/G-actin ratio, or a decreased G-actin level. To address this issue, we examined the effects of two other drugs, swinholide A and cytochalasin D. Swinholide A sequesters G-actin as dimers (Bubb et al., 1995; Lyubimova et al., 1997; Saito et al., 1998), while cytochalasin D caps actin filaments and stimulates ATP hydrolysis on G-actin (discussed by Sampaio and Pollard, 1991). Both of these compounds strongly activated SRF reporter genes with prolonged activation kinetics similar to those of jasplakinolide (Figure 6 and data not shown). Although a recent
Actin Overexpression Inhibits Activation of SRF by Serum

The observation that certain drugs that bind G-actin are sufficient to activate SRF provides strong support for the idea that actin itself directly participates in SRF regulation. We reasoned that overexpression of actin would titrate cofactors involved in SRF regulation and thereby inhibit its activation. To test this idea, we increased actin levels in SRE-FosHA cells by microinjection of a plasmid expressing Flag-tagged actin (Figure 7A, panels 1–3). Similar results were obtained using a microinjected reporter (Figure 7B). The effect of actin overexpression was specific, since in parallel experiments, serum induction of the immediate-early gene Egr-1 was unimpaired (Figure 7A, panel 4; see below). We used a similar approach to test whether overexpression of profilin, an actin-binding protein thought to be involved in the polymerization process (Schlöter et al., 1997), could also affect SRF activity. Overexpression of wild-type profilin activated expression of the reporter, but profilin H119E, which cannot bind actin, did not (Figure 7C; Suetsugu et al., 1998). Taken together, these results are consistent with participation of actin itself in the regulatory process.

A Subset of SRF Target Genes Is Regulated by Actin Dynamics

Finally, we investigated to what extent alterations in actin dynamics contribute to the regulation of endogenous SRF target genes. Previous studies have suggested that expression of two SRF target genes, cytoskeletal actin and vinculin (Mohun et al., 1987; Ben-Ze’ev et al., 1990; Moiseyeva et al., 1993), responds to actin expression levels (Reuner et al., 1995, 1996; Schevzov et al., 1995; Lyubimova et al., 1997). We therefore examined these genes, as well as the well-characterized SRF targets c-fos and egr-1, consistent with the actin overexpression experiments (data not shown). Both the srf and vinculin genes were induced by serum, although less dramatically and with slower kinetics than c-fos, reflecting the greater stability of these mRNAs. In contrast to c-fos induction, however, serum induction of srf and vinculin was completely blocked by latrunculin B or C2 toxin (Figure 8A, compare lanes 3–5 with 6–8); similar results were obtained with egr-1, consistent with the actin overexpression experiments (data not shown). The srf and vinculin genes were induced by serum, although less dramatically and with slower kinetics than c-fos, reflecting the greater stability of these mRNAs. In contrast to c-fos induction, however, serum induction of srf and vinculin was completely blocked by latrunculin B or C2 toxin (Figure 8A, compare lanes 3–5 with 6–8 and 10 and 11 with 12 and 13). Induction of the c-fos gene by jasplakinolide, swinholide A, or cytochalasin D was barely detectable (<2-fold, compared with 100-fold induction by serum); in contrast, these drugs induced the srf and vinculin mRNAs, although somewhat less efficiently than serum stimulation (Figure 8B; compare Figure 8A, lanes 1–5). Since cytoskeletal actin synthesis is controlled at both the transcriptional and posttranscriptional levels (Bershadsky et al., 1995), we used a reporter system to study actin regulation. The Xenopus laevis type-5 cytoskeletal actin promoter, which like the mammalian genes contains multiple SRF-binding sites (Mohun et al., 1987), behaved in an identical manner to the SRF-controlled reporter genes: induction by serum or LPA stimulation was blocked by latrunculin B, and the gene was activated by the other actin-binding drugs, LIMK-1 expression, and activated RhoA (Figure 8C). Taken together, these data demonstrate that potentiation of SRF activity by depletion of the cellular G-actin pool is necessary and sufficient for induction of some but not all SRF target genes.

Discussion

Actin Dynamics: A Convergence Point for SRF-Linked Signal Pathways

We used an in vivo functional screen based on activation of an SRF-controlled reporter gene to identify activators

Figure 6. Activation Responds to Depletion of the G-Actin Pool

(A) Activation of SRF following F-actin stabilization by jasplakinolide. SRE-FosHA cells maintained in 0.5% FCS were stimulated for 24 hr, and then stimulated with 10% FCS, 0.03–0.1 μM swinholide A (Sw), and 0.8–2 μM cytochalasin D (C D ) as indicated. Data shown represent the mean ± SEM from three independent transfections.

(B) Activation of SRF by WASP and Diaphanous family proteins. NIH3T3 cells were transfected with the SRF-controlled 3D.ACAT reporter, maintained in 0.5% FCS for 24 hr, and then stimulated with 10% FCS, 0.03–0.1 μM swinholide A (Swm), and 0.8–2 μM cytochalasin D (CD) as indicated. Data shown represent the mean ± SEM from three independent transfections.

(C) Activation of SRF by WASP and Diaphanous family proteins. NIH3T3 cells were transfected with the SRF-controlled 3D.ACAT reporter together with expression plasmids encoding activated mDia1 or mDia2 (codons 263–1255 and 255–1153, respectively), VASP, WASP, N-WASP, or VCA (N-WASP codons 394–505) as indicated and maintained in 0.5% FCS for 24 hr before harvest. Data shown represent the mean ± SEM from three independent transfections.

Figure 7A. 3D.ACAT reporter

Figure 7B. Microinjected reporter

Figure 7C. Activated SRF reporter together with expression plasmids encoding activated mDia1 or mDia2 (codons 263–1255 and 255–1153, respectively), VASP, WASP, N-WASP, or VCA (N-WASP codons 394–505) as indicated and maintained in 0.5% FCS for 24 hr before harvest. Data shown represent the mean ± SEM from three independent transfections.
of the SRF-linked signal pathway. In addition to SRF itself, we recovered a cDNA encoding the kinase domain of the actin regulator LIMK-1 (Bernard et al., 1994; Mizuno et al., 1994; Arber et al., 1998; Yang et al., 1998). Expression screens of this type may facilitate identification of further regulators of both SRF and other transcription factors. SRF activity can be increased by overexpression of intact LIMK-1, its close relative LIMK-2, or either kinase domain. The only currently known function of LIMK is to phosphorylate cofilin, which results in the stabilization of F-actin (Carlier et al., 1997; Lappalainen and Drubin, 1997; Arber et al., 1998; Yang et al., 1998). Both F-actin stabilization and SRF activation by LIMK are inhibited by overexpression of nonphosphorylatable cofilin, and this inhibition is dependent on the interaction of the mutant cofilin with actin. LIMK-induced SRF activation is also inhibited by latrunculins, drugs that prevent actin polymerization by destabilizing F-actin (Bubb et al., 1994), or are associated with de novo actin polymerization (reviewed by Beckerle, 1998; Wasserman, 1998; Bi and Zigmond, 1999). The 110 residue N-WASP VCA region sufficient to nucleate actin polymerization in vitro (Rohatgi et al., 1999) activates SRF, and, consistent with our model, a recent study indicates that Diaphanous family proteins are essential components of the serum-induced RhoA-dependent signaling pathway (Tominaga et al., submitted). Third, overexpression of cofilin itself specifically inhibits serum-induced signaling to SRF. Finally, the actin-binding drugs swinholide A and cytochalasin D are sufficient to activate SRF, as is overexpression of the actin-binding protein profilin. These agents do not induce polymerization, however, demonstrating that the sensing mechanism for regulation of SRF activity must involve G-actin, rather than the level of F-actin or the F-/G-actin ratio.

We propose that it is depletion of the G-actin pool that induces SRF activation. This could occur in either of two ways: positive regulation by a coactivator that perhaps senses G-actin level by direct physical interaction, or negative regulation by a repressive complex perhaps involving G-actin itself (see Figure 9). Increased actin overexpression would inhibit SRF function by titrating such cofactors. Further experiments are required to distinguish between these possibilities. Although cofilin itself could in principle act as an SRF cofactor, this would appear unlikely, because nonphosphorylatable cofilin mutants do not impair SRF activation by extracellular signals and phosphomimetic cofilin mutants do not activate SRF (A.S., unpublished data). We are currently
cells were stimulated or not for 8 hr with 10% FCS, 10 μM LPA, the indicated activators. Following maintenance in 0.5% FCS for 16 hr, the NIH3T3 cells were cotransfected with a CAT reporter controlled by (C) Control of a cytoskeletal actin promoter via actin dynamics.

swinholide A, or 2 μM cytochalasin D for the indicated times before each panel. Transcripts were analyzed by RNase protection. Relative transcript or C2 toxin (lanes 9-13) as in Figure S. After serum stimulation for 0.5% FCS for 36 hr and then treated with latrunculin B (lanes 1-8) vinculin, but not c-fbs, genes. SRE.FosHA cells were maintained in (A) Latrunculin B and C2 toxin block serum induction of the srf and maintained as in (A) were treated with 0.5 μM jasplakinolide, 0.1 μM swinholide A, or 2 μM cytochalasin D, which also bind actin, are themselves sufficient to activate SRF. We propose that this contrasting behavior occurs because their association with G-actin, unlike latrunculins and C2 toxin, prevents interaction with the partner molecule involved in SRF regulation. Although we think it most likely that regulation occurs at the level of transcriptional initiation, this need not necessarily be the case, and it is interesting to note that cytoskeletal actin has recently been found to be associated with the BAF chromatin remodeling complex (Zhao et al., 1998). Latrunculin B and C2 toxin inhibit signal transduction to SRF by binding to or modifying G-actin and preventing its polymerization. In contrast, profilin, swinholide A, and cytochalasin D, which also bind actin, are themselves poorly defined. Swinholide A sequesters G-actin as dimers and can sever F-actin (Bubb et al., 1995; Lyubimova et al., 1997; Saito et al., 1998); cytochalasin D induces dimerization of G-actin, promotes ATP hydrolysis, and caps filament barbed ends (see Sampath and Pollard, 1991); and binding of profilin to actin promotes nucleotide exchange (Schluter et al., 1997). Further insights into the mechanism of SRF regulation will result from complete characterization of the interactions between these agents and actin.

The failure of our RhoA effector loop mutant studies to implicate Diaphanous family proteins in SRF activation (Sahai et al., 1998) may reflect either inadequacies of using inhibition of SRF regulation by actin overexpression as an assay with which to map regions of the molecule that participate in SRF regulation. Although we think it is likely that regulation occurs at the level of transcriptional initiation, this need not necessarily be the case, and it is interesting to note that cytoskeletal actin has recently been found to be associated with the BAF chromatin remodeling complex (Zhao et al., 1998).

Latrunculin B and C2 toxin block serum induction of the srf and vinculin, but not c-fos, genes. SRE.FosHA cells were maintained in 0.5% FCS for 36 hr and then treated with latrunculin B (lanes 1-8) or C2 toxin (lanes 9-13) as in Figure 5. After serum stimulation for the indicated times, c-fos (Fos), srf (SRF), vinculin, and GAPDH transcripts were analyzed by RNase protection. Relative transcript levels, as determined by Phosphorimager analysis, are shown below each panel.

(B) Activation of SRF target genes by actin-binding drugs. Cells maintained as in (A) were treated with 0.5 μM jasplakinolide, 0.1 μM swinholide A, or 2 μM cytochalasin D for the indicated times before RNAs analysis.

(C) Control of a cytoskeletal actin promoter via actin dynamics. NIH3T3 cells were cotransfected with a CAT reporter controlled by the Xenopus type 5 cytoskeletal actin promoter (A431.CAT) and the indicated activators. Following maintenance in 0.5% FCS for 16 hr, cells were stimulated or not for 8 hr with 10% FCS, 10 μM LPA, 0.3 μM jasplakinolide, 0.03 μM swinholide A, or 2 μM cytochalasin D, with latrunculin B pretreatment as indicated, and harvested. Data shown are from a representative experiment.
the approach or requirements for additional effectors. One candidate might be phosphatidilinositol 5-kinase, which controls synthesis of PIP2, a regulator of actin dynamics (reviewed by Van Aelst and D'Souza-Schorey, 1997).

Although our data show that serum or LPA induction of SRF is not dependent on LIMK, the localization of LIMKs at the plasma membrane suggests that they may well be true signaling intermediates on as yet unidentified signaling pathways to SRF. Recent studies of LIMK have placed it downstream of both Rac and Rho GTPases (Arber et al., 1998; Yang et al., 1998; S. Narumiya, personal communication). However, although we found that LIMK-induced SRF activation and actin aggregate formation are largely Rho dependent, LIMK-1 expression alleviated cytoskeletal disruption induced by inactivation of Rho, indicating that LIMK activity is not dependent on Rho in our cells. We propose that LIMK-induced SRF activation is Rho dependent because the F-actin that is sensitive to LIMK is absolutely dependent on basal levels of RhoA activity. According to this view, RhoA-independent SRF activation by high-level LIMK expression would occur because higher LIMK activity is required to affect RhoA-independent F-actin. We are currently investigating the involvement of LIMK-1 in SRF regulation in other signaling contexts, particularly those that, unlike the serum pathway, involve Rac.

**Actin Dynamics as a Regulator of SRF**

**Target Genes**

Our data show that actin dynamics plays a critical role in regulation of a subset of SRF target genes. Induction of the expression of actin-related proteins, vinculin, and SRF itself by serum is blocked by latrunculin or C2 toxin, and each responds to actin-binding drugs in a similar way to our SRF-controlled reporter gene. Thus, alterations in actin dynamics appear both necessary and sufficient for regulation of these genes. These findings confirm and extend previous reports that G-actin levels regulate cytoskeletal actin and vinculin synthesis (Bershadsky et al., 1995; Reuner et al., 1995; Schevzov et al., 1995; Lyubimova et al., 1997). Vinculin overexpression correlates inversely with transformation (see Rodriguez Fernandez et al., 1992), so our results might explain the observation that LIMK-1 overexpression can suppress transformation (Hughes et al., 1996). By contrast, and to our surprise, we found that alterations in actin dynamics both contribute little to serum-induced transcription of the c-fos and egr-1 genes and also appear insufficient for their activation. The basis for the differential sensitivity of SRF target genes remains to be determined. It is intriguing to note that the SRF sites in the c-fos and egr-1 promoters, unlike those of the others, contain well-defined binding sites for the SRF cofactor TCF and that regulation of TCF activity via the ERK pathway plays a major role in their activation. We speculate that the association of TCF with SRF may simultaneously render a promoter sensitive to MAPK signaling pathways and relatively refractory to signaling via actin dynamics. Indeed, in reporter gene experiments, inactive TCF can inhibit activation of SRF by activated GTPases (Hill et al., 1994, 1995). SRF sites in muscle-specific promoters generally lack associated TCF-binding sites, and we expect that these promoters will also be sensitive to G-actin levels.

**Experimental Procedures**

**Plasmids**

Reporter plasmids and systems have been described previously: LexOP.CAT-NLS.ErbB, LS.RE.CAT, and LMS.RE.CAT (Hill et al., 1994); A413, CAT and 3D.ACAT (Mohun et al., 1989). 3D.ACAD is a derivative of 3D.ACAT in which the CAT coding sequences are replaced by a 0.8 kb CD8 cDNA fragment encoding human CD8 (GenBank number M38712). 3D.ACAD-neo (a gift from Alberts) contains the TK.Neo gene from pTREPS (Baltimore) inserted at the 3D.ACAD Pst site.

The library vector EF.NLS-VP16 is an EF.BOS derivative encoding [5SV40.NLS]MAPPKKKKKVVG-S-(VP16 codons 410-490) upstream of a BstXI-flanked cDNA. Random primed cDNA from growing mouse NIH3T3 cells was synthesized by standard techniques, ligated to BstXI adaptors, and inserted into EF.NLS-VP16 backbone. The library contained 4.5 x 10^7 independent clones with a median cDNA insert size of ~5 kb.

The LIMK-1 kinase domain in clone Q1 deviated from the published sequence (GenBank number M501416) at G1770-1771—CC. The 5' end of the mouse LIMK-1 cDNA was amplified from the EF.NLS-VP16 library by PCR, sequenced, and used to reconstruct the full-length ORF: human LIMK-2 cDNA (Invitrogen) was used following correction of a presumed PCR error at Y336. LIMKs were expressed with N-terminal KDEL epitope tag using the EF.BOS derivative EF.Tplink. EF.Flink (N-terminal Flag epitope) was used to express the following ORF's (GenBank numbers in parentheses): human collagen XI (X00662), β-actin (NM001010), profilin (NM000502), and bovine N-WASP (NM003070). Expression plasmids for mouse mDax1 (AF094519) and mDia2 (U69693), human VASP (NM003070) and WASP (NM003077), and bovine N-WASP (NM003070) were gifts from Shuh Narumiya, Art Alberts, Nick Brindle, and Alan Hall, respectively.
SRF Activator Screen

COS/3:ACD8 cells were established by transfection with 3D:ACD8-neo and G418 selection and screening for robust and uniform response to cytochalasin D using FACS. For screening, 5 × 10^4 COS/3:ACD8 cells were transfected with 30 μg of the cDNA library using DEAE-Dextran, replated the following day, and a day later stained with FITC-conjugated anti-CD8 monoclonal antibody (DAKO clone DX2 at 1:200). The top 0.7% CD8-positive cells were recovered by FACS and Hirt supernatants transformed into DH5α (190 pools of 50 plasmids). For screening, 5 × 10^4 COS/3:ACD8 cells were transfected with plasmid pool DNA (0.75 μg) together with 0.4 μg 3D:CD8 and 0.25 μg GFP expression plasmids using lipofectamine; GFP-positive cells were analyzed for CD8 expression by FACS using RPE-conjugated anti-CD8 (DAKO) as above. Positive pools were subdivided and rescreened.

Microinjection and Immunofluorescence

Expression plasmid DNAs were microinjected into the nuclei of cells grown on glass coverslips using a Zeiss S171 semiautomated machine (Alberts et al., 1996). For each experiment, 50-100 cells were injected. Plasmid concentrations are given in the figure legends. Cells were fixed with 4% formaldehyde in PBS for 15 min and then permeabilized for 10 min in 0.3% Triton X-100 in PBS. Antibodies were diluted 1:100 in PBS, 1% gelatin, 0.5% NP-40, and 10% FCS; cells were washed four times in PBS after the antibody incubation prior to mounting. Microscopy was as described (Sahai et al., 1996).

Cell Transfection and Gene Expression Assays

SRE:FoxA cells are a NIH3T3 line with an integrated 3D:ACD8 reporter (Hill et al., 1995; Alberts et al., 1998). SRE:FoxA or NIH3T3 cells were transfected using lipofectamine unless otherwise stated (per 60 mm dish: reporter plasmids, 0.3 or 0.5 μg; activators, 0.3 to 1 μg; RSV-lacZ standard, 0.6 μg). Transfected plasmid DNA was made up to 2 μg by addition of MLV128p or EFplink vector DNA. DEAE-dextran transfections were as described (Hill et al., 1995). Stimulation with either serum, LPA, C2 toxin, or actin-binding drugs 38 hr following transfection was as specified in the legends (Hill et al., 1995; Reuner et al., 1995). Actin-binding drugs were cytochalasin D and latrunculin B (Caldexchem); jaspilnolide and latrunculin A (Molecular Probes); and swinholide A (Alexis). For latrunculin treatment during expression of intracellular activators, the drug was added 2–3 hr following transfection treatment. RNAse protection assays with c-fos and 3D:AFos probes were as described (Hill et al., 1995). Other probes were as follows: vinculin, 316 nt probe spanning cDNA nucleotides 1201–1444 (Genbank number L12151); generating 270 nt protected fragment; arf, 252 nt probe spanning exon 3 nucleotides 1128–1134 (H. Nichols, personal communication) and generating 175 nt protected fragment; GAPDH, 165 nt probe spanning cDNA nucleotides 92–241 (Genbank number M33259) and generating 150 nt protected fragment.

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References


Control of SRF Activity via Actin Dynamics


Differential Usage of Signal Transduction Pathways Defines Two Types of Serum Response Factor Target Gene*

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Activation of the transcription factor serum response factor (SRF) is dependent on Rho-controlled changes in actin dynamics. We used pathway-specific inhibitors to compare the roles of actin dynamics, extracellular signal-regulated kinase (ERK) signaling, and phosphatidylinositol 3-kinase pathway activation on the transcriptional activity of a stably integrated SRF reporter gene dependent on functional RhoA GTPase. Inhibition of mitogen-activated protein kinase-ERK kinase (MEK) signaling reduced activation of the SRF reporter by all stimuli by about 50%, except for PMA, which was effectively blocked. Inhibition of phosphatidylinositol 3-kinase (PI-3K) slightly reduced reporter activation by serum and lysophosphatidic acid, but substantially inhibited activation by platelet-derived growth factor and PMA. Reporter induction by all stimuli was absolutely dependent on actin dynamics. Regulation of the SRF (srfrl) and vinculin (vcl) genes was similar to that of the SRF reporter gene; activation by all stimuli was Rho-dependent and required actin dynamics but was largely independent of MEK activity. In contrast, activation of fos and egrl occurred independently of RhoA and actin polymerization but was almost completely dependent on MEK activation. These results show that at least two classes of SRF target genes can be distinguished on the basis of their relative sensitivity to RhoA-actin and MEK-ERK signaling pathways.

Serum response factor (SRF) is a transcription factor that controls many "immediate-early" genes whose transcription is induced by extracellular signals and many genes constitutively expressed in muscle (for references see Ref. 1). The activity of SRF is regulated both by cellular signal transduction pathways and by its interaction with other transcription factors. At the immediate-early fos and egrl promoters, for example, SRF forms a ternary complex with members of the ternary complex factor (TCF) family of mitogen-activated protein kinase-regulated Ets domain proteins (Ref. 2; for review see Ref. 3). It remains unclear whether all SRF-controlled immediate-early gene promoters bind TCF, however, because the TCF recognition site is simple and can be located at variable distances from that of SRF (4). SRF also exhibits functional cooperation with a number of other, constitutively active, transcription factors including Sp1, ATF6, GATA4, Nkx2.5, and the myogenic regulatory factors (5–10).

The signaling pathways impinging on SRF and its TCF partners at immediate-early promoters have been extensively studied. Transcriptional activation by the TCF proteins is potentiated by signal-induced phosphorylation of a conserved C-terminal activation domain (3, 11). Promoter mutant and TCF expression studies suggest that TCF binding is required to link the fos and egrl promoters to the Ras-Raf-MEK-ERK signaling pathway (12–16). Consistent with this, the specific MEK inhibitor PD98059 (17, 18) inhibits fos induction by a number of stimuli (19, 20). By contrast, serum stimulation potentiates SRF activity via a signaling pathway involving the Rho GTPase (21). In transfection assays, both the serum-induced activity of SRF and fos reporter genes and the constitutive activity of certain muscle-specific promoters are strongly dependent on functional Rho (21–23). Phosphatidylinositol 3-kinase (PI-3K) has also been implicated in signaling to SRF via both Rho-dependent and -independent mechanisms, although this is not detectable in all cell types (24–27).

Recent studies have shown that Rho GTPases activate SRF via their ability to induce depletion of the G-actin pool (28–30). Although this pathway is required for serum-induced transcription of the cellular SRF, vinculin, and cytoskeletal actin genes, it contributes little to activation of fos or egrl (28). This suggests that the efficiency of Rho-actin signaling to SRF is dependent on promoter context and that other signal pathways must control the activity of SRF target genes not responsive to Rho-actin signaling. Here we use pathway-specific inhibitors to investigate the roles of Rho-actin signaling, MEK-ERK, and PI-3K in activation of an SRF reporter gene by different stimuli. We compare the results to those obtained with a panel of endogenous SRF target genes. Our results define two classes of SRF target gene controlled by Rho-mediated actin dynamics and MEK-ERK signaling, respectively.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfections, and General Methods—SRE FoxHA cells are NIH3T3 cell-derivative cells carrying an integrated 3D.AFos HA reporter (21, 31). NIH3T3 cells in a 6-well plate were transiently transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's recommendations; 6 μl of LipofectAMINE, 0.05 μg of

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§ The abbreviations used are: SRF, serum response factor; TCF, ternary complex factor; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; PI-3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase-ERK kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis.
The cells were rinsed twice with ice-cold phosphate-buffered saline and inhibitors as required, and then stimulated with different agents. The cells were plated per GO-m dish, serum-starved for 24 h, pretreated with 5'-flanking region and part of exon 1, nucleotides 540-738 (GenBank accession number M22356), generating a 267-nucleotide protected fragment; pre-srf, a 343-nucleotide probe spanning the exon 5-intron 3 boundary, nucleotides 8181-8450 (GenBank accession number AB03837), generating a 357-nucleotide protected fragment (exon 3-intron 3 precursor) and a 108-nucleotide protected fragment (exon 3 mRNA). For quantitation of RNase protection assays, images were obtained using a Phosphorlmager (Molecular Dynamics). Protected fragments were quantified after background subtraction with ImageQuaNT software and normalized to the GAPDH signal.

Immunoblotting and Antibodies—For Western blot analysis 4 × 106 cells were plated per 60-mm dish, serum-starved for 24 h, pretreated with inhibitors as required, and then stimulated with different agents. The cells were rinsed twice with ice-cold phosphate-buffered saline and lysed into ice-cold lysis buffer (20 mm Tris-HCl, pH 7.5, 1 mm EGTA, 40 mm (β-glycerophosphate, 50 mm NaF, 0.1 mm vanadate, 1 mm phenylmethylsulfonlfyl fluoride, and 1 μg/ml of leupeptin, pepstatin, and apro tinin). Following clarification, equal amounts of lysate were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membrane (Millipore), and probed with the following antibodies: phospho-ERK, anti-phospho ERK1(T202,Y204) monoclonal E10(New England Biolabs, 9272S); and Akt (New England Biolabs, 910GS); pan-ERK (Transduction Laboratories, E17120); Akt anti-Ser(P)8(P)(New England Biolabs, 9271S), and Akt (New England Biolabs, 9272S). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse goat antibodies were from DAKO; ECL detection reagents were from Amersham Pharmacia Biotech. Western blots were stripped in 0.1 M glycine, pH 2.5, 0.1% SDS for 30 min and reprobed with the appropriate antibodies.

Stimuli, Inhibitors, and Toxins—Stimuli were used at the following concentrations: fetal bovine serum (Life Technologies, Inc.), 15% for serum, 200 ng/ml transfected onto polyvinylidene difluoride membrane (Immobilon-P, Millipore), and probed with the following antibodies: phospho-ERK, anti-phospho ERK1(T202,Y204) monoclonal E10 (New England Biolabs, 9106S); pan-ERK (Transduction Laboratories, E17120); Akt anti-Ser(P)8(P) (New England Biolabs, 9271S); and Akt (New England Biolabs, 9272S). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse goat antibodies were from DAKO; ECL detection reagents were from Amersham Pharmacia Biotech. Western blots were stripped in 0.1 M glycine, pH 2.5, 0.1% SDS for 30 min and reprobed with the appropriate antibodies.

Results

RhoA-dependent Activation of an Integrated SRF Reporter by Different Stimuli—Although responsive to serum, Fos reporter genes controlled by the minimal fos SRE are unresponsive to receptor-tyrosine kinase activation and PMA stimulation upon transient transfection into NIH3T3 cells (21, 33). To test the possibility that SRF reporter genes maintained in stable transfectants might respond to a greater range of stimuli, we examined the NIH3T3 cell line SRE.FosHA (31). These cells contain the SRF reporter gene 3D.AFos, which comprises the human c-fos transcription unit controlled by a chimeric promoter comprising a cytoskeletal actin TATA region and three SRF binding sites. Reporter activity was evaluated by RNase protection at various times following stimulation of serum-deprived cells with serum, LPA, PDGF, or PMA. The reporter gene showed a robust response to stimulation with each agent (Fig. 1A). Activation by PMA, but not the other stimuli, required protein kinase C activation because it was blocked by a prolonged PMA pretreatment and by the protein kinase C inhibitor GF109203X (data not shown). Maximal reporter RNA accumulation following serum, LPA, and PMA stimulation occurred 60 min after stimulation, followed by a slow decline; in contrast, PDGF-induced reporter RNA was maximal at 30 min and declined rapidly thereafter (Fig. 1A). Similar results were obtained with two further NIH3T3 cell clones carrying the 3D.AFos reporter (data not shown).

Activation of a transfected SRF reporter gene by serum and LPA requires functional RhoA (21); therefore we next investi-
Differential Signaling to SRF Target Genes

Fig. 2. Dependence of SRF-linked signaling pathways on PI-3K and MEK. A, effect of the MEK inhibitor U0126 on SRF reporter gene activity. Serum-deprived SRE.FosHA cells were pretreated for 30 min with 10 μM U0126 and then stimulated as indicated on the figure. SRF 3D.AFos reporter and GAPDH transcripts were quantitated by RNase protection. The transcript levels at 30 min, expressed as percentages of those in untreated cells, were as follows (means ± S.E., three independent experiments): fetal calf serum (SER), 54.2 ± 6.0; LPA, 53.6 ± 3.2; PDGF, 36.4 ± 7.5; PMA (TPA), 10.2 ± 0.5. B, effect of the PI-3K inhibitor LY294002 on SRF reporter gene activity. Serum-deprived SRE.FosHA cells were pretreated for 30 min with 20 μM LY294002 and then stimulated as indicated on the figure. SRF 3D.AFos reporter and GAPDH transcripts were quantitated by RNase protection. The transcript levels at 30 min, expressed as percentages of those in untreated cells, were as follows (means ± S.E., three independent experiments): fetal calf serum (SER), 108 ± 8.3; LPA, 83.2 ± 3.2; PDGF, 35.3 ± 3.4; PMA (TPA), 16.0 ± 1.0. C, U0126 does not affect PI-3K activation by the stimuli tested. Serum-deprived cells were pretreated for 30 min with 10 μM U0126 and stimulated for the times indicated. Whole cell lysates were prepared, fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane before analysis by immunoblotting for diphospho-ERK and total ERK (top panels) and Akt Ser(P)' and total Akt (bottom panels). D, LY294002 does not affect ERK activation by the stimuli tested. Serum-deprived cells were pretreated for 30 min with 20 μM LY294002 and stimulated for the times indicated. Whole cell lysates were prepared, fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane before analysis by immunoblotting for Akt Ser(P)' and total Akt (top panels) and diphospho-ERK and total ERK (bottom panels).

 gated the involvement of RhoA in activation of the integrated SRF reporter gene. We used two different toxins to inactivate Rho family GTPases: the chimeric toxin C2-C3, which ADP-ribosylates and inactivates RhoA (32), and Clostridium difficile toxin B, which glucosylates and inactivates the Rac1, Cdc42, and Rho GTPases (34, 35). The cells were treated with toxin for a period sufficient to induce rounding up of the entire cell population (data not shown) and then stimulated as before. Activation of the integrated SRF reporter gene by serum, LPA, PDGF, and PMA was completely inhibited in cells pretreated either with C2-C3 toxin (Fig. 1B) or toxin B (Fig. 1C). In contrast, activation of the reporter by cytochalasin D, which alters actin dynamics directly by interacting with actin, was not affected (data not shown).

Differential Dependence of SRF Activation on MEK and PI-3K Signaling—All the stimuli are strong activators of the ERK pathway; therefore we next investigated the contribution of this pathway to activation of the SRF reporter gene. The cells were pretreated for 30 min with the specific MEK inhibitor U0126 (36) before stimulation and analysis of reporter activity as before. Serum- and LPA-induced transcription was reduced by almost 50%, whereas activation by PDGF was reduced by 60%; only induction by PMA was reduced to background levels (Fig. 2A). To examine the efficacy of the inhibitor, we measured ERK activation by immunoblotting using an antiserum specific for the activated form of ERK1/2. U0126 treatment completely blocked ERK activation by all the stimuli except serum, where a low level of activation persisted at late times; the inhibitor did not affect PI-3K activation (Fig. 2C). Similar results were obtained using another MEK inhibitor, PD98059 (18). The ability of serum, LPA, and PDGF to activate the SRF reporter gene is thus substantially independent of ERK signaling (see "Discussion").

We next investigated whether reporter activity was dependent on PI-3K, which has previously been implicated in SRF activation in transfected HeLa cells (24). Cells were pretreated with the PI-3K inhibitor LY294002, then stimulated, and analyzed as before. The inhibitor had variable effects according to the stimulus. Serum induction was unaffected, and LPA induction was reduced by some 20%; in contrast, PDGF-induced reporter activity was reduced by 70%, and PMA induction was substantially blocked (Fig. 2B). Similar results were obtained.
using another PI3K inhibitor, wortmannin (data not shown). To evaluate the activation of PI3K in response to the various stimuli, we examined phosphorylation of Akt, a downstream target of PI3K pathway, using an antisera specific for Akt Ser(\*P)\(^{273}\). Serum and PDGF strongly induced Akt phosphorylation, whereas induction by PMA was not detectable (Fig. 1A, upper panels). Moreover, LY294002 treatment did not affect ERK activation by any of the stimuli (Fig. 2D, lower panels). The apparent failure of PMA stimulation to activate Akt phosphorylation suggests that, at least in this case, inhibition of SRF activation by LY294002 reflects a requirement for basal PI3K activity.

**Activation of SRF by All Stimuli Requires Akt Polymerization**—We previously showed that serum and LPA induction of Akt, a downstream target of PI3K pathway, using an antisera specific for Akt Ser(\*P)\(^{273}\). Serum and PDGF strongly induced Akt phosphorylation, whereas induction by PMA was not detectable (Fig. 1A, upper panels). The apparent failure of PMA stimulation to activate Akt phosphorylation suggests that, at least in this case, inhibition of SRF activation by LY294002 reflects a requirement for basal PI3K activity.

**Kinetics of Vinculin and srf Activation by Extracellular Stimuli**—We previously demonstrated that serum-induced transcription of various SRF target genes exhibits a differential sensitivity to actin dynamics; induction of the genes encoding vinculin (vcl), cytoskeletal actin (actb), and SRF (srf) was sensitive to latrunculin B, like the SRF reporter gene, whereas induction of fos transcription was not (28). Having established the contributions of different signaling pathways to activation of the SRF reporter gene, we set out to determine their contribution to SRF target gene activation. The kinetics of transcriptional activation of fos and egfr are well established (38, 39); however, although it is clear that growth factors activate ccl and srf gene expression at the transcriptional level (40, 41), the kinetics of this have not been investigated in detail. The high basal levels and stabilities of the srf and ccl mRNAs allow only strong and prolonged transcriptional changes to be reliably measured by quantitation of mRNA and preclude the use of the mRNA level as a measure of transcription rate. To circumvent these problems, we developed an RNA protection assay that allows simultaneous measurement of the levels of both mRNA and unspliced precursor transcripts of each gene, by use of RNA probes spanning the srf exon 5-intron 5 and ccl exon 3-intron 3 borders.

Serum stimulation of NIH3T3 cells led to rapid and transient appearance of srf and ccl mRNA precursors. Increased srf precursor RNA level was observed 15 min following stimula-
Differential Signaling to SRF Target Genes

Serum-induced transcriptional activation of the different SRF target genes was not significantly different in NIH3T3 cells treated with at least 100-fold less VEGF receptor kinase inhibitor U0126 than in mock-treated conditions. We therefore evaluated the contribution of MEK-ERK signaling to transcriptional activation of the different genes using U0126 pretreatment (Fig. 5B). Similar results were obtained with cycloheximide (see below) and serum (Fig. 5C). Taken together, these data establish that the kinetics of sr-/vcl mRNA accumulation, apart from the delayed appearance of the vcl precursor, resemble those of the SRF reporter gene (compare Figs. 1A and 5).

SRF Target Genes Differ in Their Sensitivity to Actin Dynamics, MEK, and PI-3K Signaling—We used latrunculin B pretreatment to examine sensitivity of the different SRF target genes to alterations in actin dynamics. Induction of srf and vcl mRNA by serum was strongly delayed, consistent with the delayed appearance of its precursor (Fig. 5A). We previously showed that srf and vcl mRNAs accumulate in response to treatment with the actin binding drugs jasplakinolide, cytochalasin D, and swinholide (28). Use of the precursor-specific probes demonstrated that Jasplakinolide treatment induced transient accumulation of srf and vcl precursor RNA with kinetics similar to those of serum stimulation (Fig. 5B). Similar results were obtained with cytochalasin D and swinholide (data not shown). LPA and PMA activated srf and vcl mRNA accumulation with similar kinetics to serum stimulation, whereas PDGF-induced activation was much more transient, reverting to prestimulation levels after 1 h (Fig. 5). Taken together, these data establish that the srf and vcl genes are transiently and transiently induced at the transcriptional level by the stimuli under investigation. Moreover, the kinetics of srf and vcl precursor accumulation, apart from the delayed appearance of the vcl precursor, resemble those of the SRF reporter gene (compare Figs. 1A and 5).

Fig. 4. TCF Elk-1 activation is insensitive to latrunculin B but inhibited by U0126. NIH3T3 cells were transfected with an expression plasmid encoding the chimeric transactivator NLS-ErkC together with Lex operator-controlled luciferase reporter gene, maintained in 0.5% fetal calf serum for 24 h and then stimulated following a 30-min pretreatment with 20 μM LY294002, 10 μM U0126, or 0.5 μM latrunculin B as indicated. Data are analyzed as in Fig. 5A. No inhibitor; 0.5 μM LB; 20 μM LY294002; 10 μM U0126.

Fig. 5. Transient kinetics of srf and vcl gene activation in NIH3T3 cells. A, serum activation and independence of new protein synthesis. Serum-deprived cells were stimulated with 15% serum for the indicated times after pretreatments with protein synthesis inhibitors or before analysis with probes specific for srf exon 5-intron 5 (top panels) or vcl exon 3-intron 3 (bottom panels) hybridized to GAPDH reference probe. Chx, 10 μg/ml cycloheximide; An, 10 μg/ml anisomycin. Protected fragments from srf precursor (pre-srf), srf mRNA (srf), vcl precursor (pre-vcl), vcl mRNA (vcl), and GAPDH are indicated. B, activation by jasplakinolide. Serum-deprived cells were stimulated for the indicated times with serum or the F-actin stabilizing drug jasplakinolide (0.5 μM). RNA was prepared and analyzed as in A. C, activation by LPA, PDGF, and PMA (TPA). Serum-deprived cells were stimulated for the indicated times with LPA, PDGF, and PMA. RNA was prepared and analyzed as in A.
although a residual fos induction by serum was detectable (Fig. 6A, lower panels). Thus with respect to these treatments, the behavior of the srf and cvel genes is similar to that of the SRF reporter, whereas that of fos and egr1 resembles that of the TCP reporter.

We also examined the effect of the PI-3K inhibitors LY294022 and wortmannin on activation of the various target genes, because this treatment also affects differentially activation of the SRF and TCP reporters. Transcriptional induction of the srf and cvel genes was sensitive to LY294022; induction by PDGF and PMA was effectively blocked, and induction by LPA was reduced some 50%, whereas serum induction was either slightly impaired (srfl) or not affected (cvel) (Fig. 6B). In contrast, activation of fos and egr1 transcription by all stimuli was insensitive to LY294022, with fos transcription actually showing a slight enhancement (Fig. 6B). As with MEK-ERK and RhoA-actin signaling, PI-3K thus makes qualitatively distinct contributions to srf and cvel compared with fos and egr1.

**SRF Target Genes Exhibit Differential Dependence on Rho GT Pases**—Finally, we used C2-C3 toxin and toxin B treatment to investigate the dependence of each of the target genes upon functional RhoA. Activation of srf and cvel transcription by all stimuli was blocked in cells treated with C2-C3 toxin; in contrast, egr1 and fos induction was substantially less sensitive to C2-C3 toxin, with egr1 unaffected and fos reduced by up to 50% (Fig. 7, left panel). A similar result was obtained when cells were treated with toxin B; induction of srf and cvel transcription by all stimuli was completely sensitive to toxin treatment, whereas activation of egr1 and fos was at most only partially affected (Fig. 7, right panel). In the latter case toxin B treatment caused ~50% reduction in serum- and PDGF-induced fos and egr1 transcription but substantially reduced activation by LPA; PMA induction was not affected. Thus only those SRF target genes whose activation is critically dependent on actin dynamics exhibit a requirement for Rho GTPase activity.

**DISCUSSION**

Recent studies have led to the identification of a number of inhibitors and toxins specific for signaling molecules that regulate the expression of cellular immediate-early genes, including kinases, small GTPases, and cytoskeletal components (for references see Refs. 28, 42, and 43). Here we have used inhibitors specific for Rho GTPases, actin dynamics, MEK, and PI-3K to investigate signaling to the SRF transcription factor and four of its cellular target genes in response to different stimuli. Our results define two types of SRF target gene, illustrated in Fig. 8. One class, which includes srf and cvel, behaves in a fashion similar to that of an SRF reporter gene: regulation of these genes requires functional Rho and actin polymerization but is only partially dependent on MEK activity. Regulation of the second class, which includes fos and egr1, occurs largely independently of functional Rho and actin dynamics but is instead critically dependent on MEK-ERK signaling.

SRF reporter activity is critically dependent on Rho GTPase and actin polymerization, whether induced via activation of serpentine receptors (serum and LPA), receptor tyrosine kinases (PDGF), or intracellular activation of protein kinase C (PMA). Signaling to SRF by PDGF and PMA differs in several ways from the other stimuli. First, only PDGF- and PMA-induced reporter activity requires PI-3K activity. PMA treatment does not induce activation of PI-3K itself, at least as assessed by Akt phosphorylation; therefore it would appear...
**Fig. 7.** Activation of the srf and vcl genes but not fos or egr1 is RhoA-dependent. Serum-deprived cells were pre-treated for 5 h with C2-C3 toxin (left panels) or for 1 h with toxin B (right panels) as described under "Experimental Procedures" before stimulation and analysis as in Fig. 6. The protected fragments are indicated on the figure. Pre-srf and pre-vcl transcript levels were reduced to background in both cases; for egr1 and fos transcript levels (C2-C3/toxin B expressed as percentages of untreated signal at 30 min) were as follows: fetal calf serum (SER): egr1 88.4/66.9, fos 62.5/47.3; LPA: egr1 102.3/33.0, fos 46.8/19.6; PDGF: egr1 97.7/67.2, fos 50.8/50.1; PMA (TPA): egr1 106.4/122.8, fos 77.7/93.6. Un., untreated.

**Fig. 8.** Extracellular signals control at least two distinct classes of SRF target gene. Outline signal transduction pathways controlled by the small GTPases Ras and Rho are shown with known SRF targets in italics. Downstream pathway components are boxed. ERK indicates the Raf-MEK-ERK pathway previously shown to control TCF activity in response to mitogenic stimuli; Actin dynamics indicates the actin treadmill cycle, which controls SRF activity and is regulated by the Rhoa effector systems ROCK-LIM kinase-cofilin and Diaphanous (Refs. 28 and 29; O. Geneste, J. Copeland, and R. Treisman, manuscript in preparation). Pathway specific inhibitors U0126 and latrunculin are indicated. Dotted arrows indicate that signal transmission through each pathway may also respond to (i) changes in Ras and Rho GTP loading induced by cell cycle/growth cues and cytoskeletal events respectively and (ii) cross-talk between the pathways. TCF binding to the fos and egr1 promoters is known to be important for signal transduction (2, 12, 15); however, it remains unclear whether TCF plays a role in regulation of srf or vcl. For discussion see text.

likely that SRF activation by PMA, and probably PDGF, requires only basal PI-3K activity. Consistent with this idea, expression of activated PI-3K p110 does not activate the SRF reporter gene in our cells. Second, PMA-induced reporter activation, which unlike the other stimuli results from the activation of protein kinase C, is substantially blocked by inhibition of MEK. This observation is consistent with a model in which SRF can be activated by both MEK-dependent and -independent routes, with only the former being activated by PMA, although further studies are necessary to confirm this. Finally, although PMA and PDGF induction of the SRF reporter gene (and cellular srf and vcl) is absolutely dependent on functional RhoA, previous studies indicate that these stimuli rapidly decrease rather than increase GTP loading of RhoA in NIH3T3 cells (44), suggesting that activation of SRF by these agents requires only basal RhoA activity. We propose that PDGF and PMA might act to stabilize a pool of F-actin whose assembly requires basal RhoA activity; PDGF is a strong activator of Rac in NIH3T3 cells, and one way this could be achieved is by Rac-dependent activation of the actin stabilizer LIM kinase (45). Further work will be necessary to clarify the connection between PDGF and PMA-induced signaling and actin dynamics.

Our data show that the SRF target genes srf and vcl behave like the SRF reporter, requiring Rho-actin but not MEK-ERK signaling, whereas activation of fos and egr1 requires MEK-ERK but not Rho-actin signaling. At present it remains unclear whether all SRF target genes fall into these two classes. In principle further classes of SRF target gene might exist, perhaps dependent on both RhoA-actin and MEK-ERK signaling or regulated by other signaling pathways with or without input from RhoA-actin signals. Among immediate-early genes, the junB gene exhibits similar signaling requirements to fos and egr1. However, there is as yet insufficient data to classify other SRF target genes. Several SRF-controlled muscle-specific promoters are Rho-dependent (22, 23), and at least the smooth muscle α-actin and SM22 promoters are dependent on alterations in actin polymerization (30), suggesting that they might fall into the srf-vcl class. The role of MEK-ERK signaling in the expression of such muscle-specific SRF target genes has not been resolved; however, the failure of the MEK inhibitor PD98059 to block differentiation of C2 skeletal myoblasts suggests that MEK-ERK signaling may not be essential for expression of SRF target genes in these cells (46, 47). It is also intriguing to note that the cyr61-related immediate-early gene CTGF exhibits similar signaling requirements to srf and vcl (48, 49), however, it remains to be confirmed whether CTGF actually is an SRF target. We are currently comparing signal-

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8. R. Grosse, unpublished observations.
Differential Signaling to SRF Target Genes

Our results extend previous characterization of signaling to SRF target promoters. Previous transfection studies have clearly established the functional significance of the SRF sites present in the srf and ocl promoters (50–52). In addition to signal inputs through the SRF site, LPA-induced activation of the srf promoter involves a Ras-dependent signaling input through an adjacent Sp1 site, whereas FGF-induced activation involves both the Sp1 and a Rho family-dependent input via a nearby Ets motif (53, 54). However, signal-regulated srf and ocl transcription is absolutely dependent on functional RhoA and actin polymerization; therefore, cooperating signals that act through other elements in these promoters must be insufficient for activation of transcription. Moreover, because neither the ocl nor srf promoters require active MEK, it is unlikely that they contain sequences directly regulated by ERK. Vcl transcription exhibits a delayed onset compared with that of srf and the SRF reporter gene; this does not reflect a requirement for new protein synthesis, and its basis is currently under investigation. The role of PI-3K in SRF target gene activation is less clear. Although experiments involving activated and inhibitory PI-3K mutants have implicated PI-3K in activation of SRF target genes in some signaling systems, at least some of these effects can be attributed to inhibition of Ras-Raf-MEK-ERK signaling rather than SRF itself (24–27, 55–57). In our cells, the SRF signal generated in response to inhibition of PI-3K; the srf and ocl genes respond in a similar way to the SRF reporter, although the effects of the inhibitor on these genes are less marked, whereas activation of the fos or egfr genes by all stimuli is unimpaired.

We found that in NIH3T3 cells signal-induced activation of the endogenous fos and egfr genes occurs largely independently of RhoA; a similar finding has been reported using Rat-1 cells (58). These findings contrast with our own previous transient transfection experiments and those of others, in which fos activation exhibits a strong dependence on functional RhoA (21). One potential explanation for this discrepancy is that transiently transfected promoters are somehow more sensitive to the Rho-actin pathway than their chromosomal counterparts; however, this would appear unlikely because a transfected fos gene is insensitive to latrunculin. An alternative explanation can be based on the observation that in fibroblasts ERK activation is partially dependent on functional RhoA (21, 59); perhaps the presence of a large number of transfected fos gene templates is sufficient to render ERK signaling limiting, with the result that the dependence of ERK signaling upon RhoA would then become significant. We also found that the stably transfected SRF reporter gene was more responsive to PDGF- and PMA-induced signaling than in transient transfection assays. The reason for this is unclear but again might reflect reporter copy number; transfected reporters may have a relatively high basal level of activity, and if signal strength by PDGF and PMA, but not serum and LPA, is limiting, the transfected reporter might appear less sensitive to PDGF and PMA. Further experiments will be required to resolve these issues, which caution against the use of transfected, high copy, reporter systems.

In this work we have identified four SRF target genes that are either sensitive to actin dynamics and independent of MEK-ERK signaling or vice versa (Fig. 8). How might such mutually exclusive linkage of different signaling pathways to SRF-dependent promoters be achieved? We previously suggested that promoter-specific combinatorial interactions between SRF and other transcription factors might control the sensitivity of SRF to signaling via actin dynamics (28). The results described here suggest a refinement of this model, in which the physical interactions between SRF and different cofactors responsible for actin-dependent signaling and MEK-ERK signaling respectively are mutually exclusive. Several observations suggest that the TCF proteins are good candidates for factors controlling signaling specificity at SRF target promoters (Fig. 8). First, they are direct targets for MEK-ERK signaling (3). Second, the SRF binding sites in actin-dependent promoters such as ocl and srf do not have obvious TCF sites associated with them, whereas SRF sites in MEK-ERK-dependent promoters such as fos and egfr do (2, 15). Third, expression of inactive forms of TCF can interfere with RhoA-dependent signaling to SRF reporter genes (21). It should be borne in mind, however, that SRF also functionally cooperates with several other transcription factors including Sp1, GATA4, Nkx2.5, the myogenic factors, and ATF6 (5–10); moreover, SRF sites are frequently associated with API/ATF sites, which are also targets for signaling pathways (60). Combinatorial interactions between SRF and such other factors might also therefore constrain its sensitivity to Rho-actin signaling. We are currently studying the role of a number of different SRF-controlled promoters to elucidate the role of TCF and other transcriptional regulators in the control of signaling to SRF.

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