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

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Dziugas Gineitis‡ and Richard Treisman§
From the Transcription Laboratory, Imperial Cancer Research Fund Laboratories, 44 Lincoln’s Inn Fields, London WC2A 3PX, United Kingdom

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 (PI-3K) has also been implicated in signaling to SRF. Inhibition of phosphatidylinositol 3-kinase 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 (sr) and vinculin (rc) genes was similar to that of the SRF reporter gene; activation by all stimuli was Rho-dependent and required 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 egr1 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 egr1 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 egr1 (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—SRF.FosHA 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 SRF.FosHA, and 2 µg of pSG5 (Invitrogen, Carlsbad, CA). The PCR primers used for the SRF targeted were: sense, 5′-ACCTGGCTGAGCTGCTGACG-3′; antisense, 5′-GTCTTTGCCCTGTTGGTGAG-3′.

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§ To whom correspondence should be addressed: Transcription Laboratory, Imperial Cancer Research Fund Laboratories, 44 Lincoln’s Inn Fields, London WC2A 3PX, United Kingdom.

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; GADD, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis.

2 O. Geneste, J. Copeland, and R. Treisman, manuscript in preparation.

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LexOP2tkLuc, 0.02 μg of NLexElkC, 0.1 μg of MLVlacZ, and 0.65 μg of MLVΔ8β (11) were used per well. The cells were lysed in 200 μl of reporter lysis buffer, and luciferase activity was determined by standard methods. DNA manipulations and plasmid DNA preparation were by standard methods. RNase protection probes were synthesized from appropriate derivatives of pCR2.1TOPO (TT; pre-ccl), pGEM-T (Sp6; pre-srf), pSP65 (Sp6; egr1), and pSP64 (Sp6; fos). LexOP2tkLCUC comprises the LexA operator and HSVtk promoter sequences from LexOP2tkCAT (3, 11) inserted into the XhoI site of pGLOBasic (Promega).

**RNase Protection Assays**—RNA preparation and RNase protection assays were as described (13, 21). GAPDH and 3D.Afos probes were as described (21, 28). Other probes were: fos, a 199-nucleotide probe spanning 5'-flanking region and part of exon 1, nucleotides 540–738 (GenBank accession number V00727), generating a 185-nucleotide protected fragment; egr1, a 348-nucleotide probe spanning 5'-flanking region and part of exon1, nucleotides 1348–1661 (GenBank accession number M22326), generating a 267-nucleotide protected fragment; pre-ccl, a 443-nucleotide fragment 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 192-nucleotide protected fragment (exon 5 mRNA); pre-egr, a 443-nucleotide fragment 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 192-nucleotide protected fragment (exon 5 mRNA). For quantitation of RNase protection assays, images were obtained using a PhosphorImager (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 × 10^5 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 EDTA, 40 mM β-glycerophosphate, 50 mM NaF, 0.1% SDS, and 1 μg/ml each of aprotinin, leupeptin, pepstatin, and aprotonin). Following clarification, equal amounts of lysate were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore), and probed with the following antibodies: phospho-ERK, anti-phospho ERK1(Thr202/Tyr204) monoclonal E12 (New England Biolabs, 9271S); Akt anti-Ser(P)473 (New England Biolabs, 9271S); and Akt (New England Biolabs, 9106S); pan-ERK (Transduction Laboratories, E17120); phospho-ERK, anti-phospho ERK1(T202,Y204) monoclonal E10 (New England Biolabs, 9272S). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies were from Amersham Pharmacia Biotech. Western blots were stripped and reprobed with the appropriate antibodies.

**Stimuli, Inhibitors, and Toxins**—Stimuli were used at the following concentrations: fetal bovine serum (Life Technologies, Inc.), 15%; LPA (Sigma), 10 μg/ml; PDGF-BB (Calbiochem), 25 ng/ml; PMA (Sigma), 50 ng/ml; cytochalasin D (Calbiochem), 2 μM; and leupeptin, pepstatin, and aprotonin (Sigma), 1 μg/ml final concentration. GAPDH and 3D.Afos probes were analyzed by standard methods. DNA manipulations and plasmid DNA preparation were by standard methods. RNase protection probes were synthesized from appropriate derivatives of pCR2.1TOPO (TT; pre-ccl), pGEM-T (Sp6; pre-srf), pSP65 (Sp6; egr1), and pSP64 (Sp6; fos). LexOP2tkLCUC comprises the LexA operator and HSVtk promoter sequences from LexOP2tkCAT (3, 11) inserted into the XhoI site of pGLOBasic (Promega).

**RESULTS**

**RhoA-dependent Activation of an Integrated SRF Reporter by Different Stimuli**—Although responsive to serum, Fos reporter genes controlled by the minimal fos promoter 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).

![Image](image-url)

**FIG. 1. Activation of SRF reporter genes by different stimuli.** A, integrated 3D.Afos reporter gene. SRE.FosHA cells were seeded at 4 × 10^5/cm^2 and after 24 h in medium containing 10% serum transferred to medium containing 0.5% serum for 36 h before stimulation with 15% fetal calf serum (SER), 10 μg/ml LPA, 25 ng/ml PDGF, or 50 ng/ml phorbol 12-myristate 13-acetate (TPA) for the times indicated on the figure. Transcripts of the reporter and a control gene, GAPDH, were analyzed by RNase protection assays; nuclelease-resistant fragments derived from the two RNAs are indicated on the figure. At peak accumulation, relative transcript levels were expressed as a percentage of those induced by serum at 60 min (means ± S.E., three independent experiments) were as follows: LPA, 55.7 ± 5.3; PDGF, 29.4 ± 4.5; and PMA, 42.9 ± 3.4. Similar results were obtained with two further cell lines containing the 3D.Afos reporter (data not shown). B, inhibition of SRF reporter activity by C2-C3 toxin. Serum-deprived cells were pretreated for 5 h with C2-C3 toxin as described in Experimental Procedures before stimulation and analysis for 3D.Afos reporter and GAPDH reference transcripts. Transcript levels were reduced to background in each case. Protected fragments are indicated. C, inhibition of SRF reporter activity by Toxin B. Serum-deprived cells were pretreated for 1 h with Toxin B before stimulation and analysis as in part (B).
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 by the stimuli tested. 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\(^{(P)}\)473 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 \(\mu\)M 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\(^{(P)}\)473 and total Akt (top panels) and diphospho-ERK and total ERK (bottom panels).

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 \(\mu\)M 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 \(\mu\)M 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 \(\mu\)M 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 Akt Ser\(^{(P)}\)473 and total Akt (top panels) and diphospho-ERK and total ERK (bottom panels).
Differential Signaling to SRF Target Genes

Activation of SRF by All Stimuli Requires Actin Polymerization—We previously showed that serum and LPA induction of an SRF reporter gene is absolutely dependent on signal-induced changes in actin polymerization (28). Because activation of this reporter by PDGF and PMA exhibits a different requirement for MEK and PI-3K signaling compared with serum and LPA, we next investigated whether activation by these stimuli is also dependent on signal-induced actin polymerization. Serum-starved reporter cells were pretreated for 60 min with 0.5 μM latrunculin B, which inhibits actin polymerization by sequestering G-actin monomer (37), prior to stimulation and analysis of reporter RNA activity. Latrunculin B treatment completely blocked transcriptional induction by all stimuli including PDGF and PMA (Fig. 3A; U0126 + latrunculin B allows comparison with Fig. 6). To confirm that latrunculin B treatment did not affect the activation of the ERK and PI-3K pathways, we performed immunoblotting experiments with the activated ERK and Akt Ser(P)473 antibodies. Latrunculin B treatment did not inhibit activation of either ERK or PI-3K by any stimuli (Fig. 3B).

Activation of TCF Elk-1 Requires MEK but Not Actin Polymerization—All the stimuli tested potentiate transcriptional activation by members of the ternary complex factor family of Ets domain proteins. The SRF reporter gene does not contain consensus TCF binding sites, but we nevertheless considered the possibility that its enhanced ability to respond to stimuli when integrated into cellular DNA might reflect TCF binding. To test this idea, we evaluated the effects of the inhibitors on TCF activation using a TCF Elk-1 reporter system. The cells were transfected with a LexA operator-controlled reporter plasmid and an expression vector encoding a LexA/Elk-1 fusion protein (11). Following treatment with the various inhibitors, cells were stimulated, and reporter gene activity was measured. Activation of the Elk-1 reporter by all stimuli was reduced to background level by U0126 pretreatment but was not affected by pretreatment with latrunculin B (Fig. 4). Although Elk-1 reporter activation by PDGF and LPA was reduced some 50% by LY294002 pretreatment, LY294002 had no substantial effect on ERK activation by these stimuli (compare Fig. 4 with Fig. 2D).

Taken together, these results provide strong support for the notion that TCF is not involved in activation of the integrated SRF reporter gene. The availability of inhibitors specific for the signal transduction pathways that regulate SRF and TCF activation provides a simple way to evaluate the potential contributions of these transcription factors to immediate-early gene transcription.

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 egr1 are well established (38, 39); however, although it is clear that growth factors activate vcl 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 vcl 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 vcl exon 3-intron 3 borders.

Serum stimulation of NIH3T3 cells led to rapid and transient appearance of srf and vcl mRNA precursors. Increased srf precursor RNA level was observed 15 min following stimula-
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 NLexElkC 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. The data are normalized to the serum response, which is taken as 100; the error bars indicate S.E. from three independent transfections; where not shown, the S.E. was always less than 20% of the experimental value. Un., untreated; SER, fetal calf serum; TPA, phorbol 12-myristate 13-acetate.

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) each together with 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-vinc), vcl mRNA (vinc), and GAPDH are indicated. B, activation by jasplakinolide. Serum-deprived cells were stimulated for the indicated times with serum or the 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 vcl genes is similar to that of the SRF reporter, whereas that of fos and egr1 resembles that of the TCF reporter.

We also examined the effect of the PI-3K inhibitors LY294002 and wortmannin on activation of the various target genes, because this treatment also differentially affects activation of the SRF and TCF reporters. Transcriptional induction of the srf and vcl genes was sensitive to LY294002; induction by PDGF and PMA was effectively blocked, and induction by LPA was reduced some 50%, whereas serum induction was either slightly impaired (srf) or not affected (vcl) (Fig. 6B). In contrast, activation of fos and egr1 transcription by all stimuli was insensitive to LY294002, 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 vcl compared with fos and egr1.

**SRF Target Genes Exhibit Differential Dependence on Rho GTPases**—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 vcl 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 vcl 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 GTase 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 GTases, and cytoskeletal components (for references see Refs. 28, 42, and 43). Here we have used inhibitors specific for Rho GTases, 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 vcl, 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 GTase 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 activation 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...
Differential Signaling to SRF Target Genes

**Fig. 7.** Activation of the srf and vcl genes but not fos or egr1 is RhoA-dependent. Serum-deprived cells were pretreated 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 expression of untreated signal at 30 min) were as follows: fetal calf serum (Un.): egr1 102.3/33.0, fos 62.5/47.3; LPA: egr1 97.7/67.2, fos 50.8/50.1; PDGF: egr1 108.4/122.8, 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 treadmilling 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.\(^3\) 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.\(^3\) However, there is as yet insufficient data to classify other SRF targets. Several SRF-controlled muscle-specific promoters are Rho-dependent (22, 23), and at least the smooth muscle a-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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\(^3\) D. Gineitis, unpublished data.

\(^4\) R. Grosse, unpublished observations.
ing requirements for activation of different 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 vcl 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 vcl 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 vcl 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 target genes tested respond in distinct ways to inhibition of PI-3K; the srf and vcl 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 egr1 genes by all stimuli is unimpaired.

We found that NIH3T3 cells signal-induced activation of the endogenous fos and egr1 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.3 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 vcl and srf do not have obvious TCF sites associated with them, whereas SRF sites in MEK-ERK-dependent promoters such as fos and egr1 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 SPI, 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 signaling to 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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REFERENCES
1. Arsenian, S., Weindhoel, B., Oelgeschlager, M., Ruther, U., and Nordheim, A. (1998) EMBO J. 17, 6269–6299
2. Shaw, P. E., Schroter, H., and Nordheim, A. (1989) Cell 56, 563–572
3. Treisman, R. (1994) Curr. Opin. Gen. Dev. 4, 96–101
4. Shaw, P. E. and Nordheim, A. (1992) EMBO J. 11, 4631–4640
5. Zhu, C., Johannessen, F. E., and Prywes, R. (1997) Mol. Cell. Biol. 17, 4957–4966
6. Durocher, D., Charron, F., Warren, R., Schwartz, R. J., and Nemer, M. (1997) EMBO J. 16, 5687–5698
7. Chen, C. Y., Croissant, J., Majesky, M., Topouzis, S., McQuinn, T., Frankovsky, M. J., and Schwartz, R. J. (1996) Dev. Genet. 19, 119–130
8. Santorelli, V., Webster, K. A., and Kedes, L. (1990) Genes Dev. 4, 1811–1822
9. Belaglu, N. S., Sepulveda, J. L., Nigam, V., Charron, F., Nemer, M., and Schwartz, R. J. (2000) Mol. Cell. Biol. 20, 7550–7558
10. Moore, M. L., Wang, G. L., Belaglu, N. S., Schwartz, R. J., and McMillin, J. B. (2001) J. Biol. Chem. 276, 1632–1633
11. Marias, R., Wynne, J., and Treisman, R. (1993) Cell 75, 381–393
12. Graham, R., and Gilman, M. (1991) Science 251, 189–192
13. Hill, C. S., Wynne, J., and Treisman, R. (1994) EMBO J. 13, 5421–5432
14. Kortenjann, M., Thomae, O., and Shaw, P. E. (1994) Mol. Cell. Biol. 14, 4845–4842
15. McMahon, S. B., and Monroe, J. G. (1995) Mol. Cell. Biol. 15, 1086–1093
16. Hill, C. S., and Treisman, R. (1995) EMBO J. 14, 5037–5047
17. Alesi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 27489–27494
18. Dudley, D. T., Pang, L., Decke, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7686–7689
19. Price, M. A., Cruzalegui, F. H., and Treisman, R. H. (1996) EMBO J. 15, 6552–6560
20. Lazar, D. F., Wiese, R. J., Brady, M. J., Mastick, C. C., Waters, S. B., Yamauchi, K., Pessin, J. E., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 20801–20807
21. Hill, C. S., Wynne, J., and Treisman, R. (1995) Cell 81, 1159–1170
22. Carnac, G., Primig, M., Rostaing, M., Chafey, P., Tulli, D., Lamb, N., and Fernandez, A. (1998) Mol. Biol. Cell 9, 1891–1902
23. Wei, L., Zhou, W., Croissant, J. D., Johannsen, F. E., Prywes, R., Balasubramanyam, A., and Schwartz, R. J. (1998) J. Biol. Chem. 273, 30297–30304
24. Wang, Y., Falasca, M., Schlessinger, J., Malstrom, S., Tsichlis, P., Settleman, J., Wu, L., Lim, B., and Prywes, R. (1998) Cell Growth Differ. 9, 513–522
25. Wei, L., Zhou, W., Wang, L., and Schwartz, R. J. (2000) Am. J. Physiol. 278, H1736–H1743
26. Poser, S., Impey, S., Trinh, K., Xia, Z., and Storm, D. R. (2000) EMBO J. 19, 4955–4966
27. Reif, K., Nohes, C. D., Thomas, G., Hall, A., and Cantrell, D. A. (1996) Curr. Biol. 6, 1445–1455
28. Sotiriou, C., Gineitis, D., Copeland, J., and Treisman, R. (2000) Cell 99, 159–169
29. Tominga, T., Sahai, E., Chardin, P., McCormick, F., Courtneidge, S. A., and Alberts, A. S. (2000) Mol. Cell 5, 13–25
30. Mack, C. P., Sumlyo, A. V., Hautmann, M., Sumlyo, A. P., and Owens, G. K. (2001) J. Biol. Chem. 276, 341–347
31. Alberts, A. S., Geneste, O., and Treisman, R. (1998) Cell 92, 475–487
32. Barth, H., Hofmann, F., Olenik, C., Just, I., and Aktories, K. (1996) Infect.
Differential Signaling to SRF Target Genes

33. Wagner, B. J., Hayes, T. E., Hoban, C. J., and Cochran, B. H. (1990) *EMBO J.* 9, 4477–4484
34. Just, I., Selzer, J., Wilm, M., von Eichel-Streib, C., Mann, M., and Aktories, K. (1995) *Nature* 375, 500–503
35. Just, I., Wilm, M., Selzer, J., Rex, G., von Eichel-Streib, C., Mann, M., and Aktories, K. (1995) *J. Biol. Chem.* 270, 13932–13936
36. Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feerer, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., and Trzaskos, J. M. (1998) *J. Biol. Chem.* 273, 18623–18632
37. Coue, M., Brenner, S. L., Spector, I., and Korn, E. D. (1987) *FEBS Lett.* 213, 316–318
38. Greenberg, M. E., and Ziff, E. B. (1984) *Nature* 311, 433–438
39. Lau, L. F., and Nathans, D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 1182–1186
40. Misra, R. P., Rivera, V. M., Wang, J. M., Fan, P. D., and Greenberg, M. E. (1991) *Mol. Cell. Biol.* 11, 4545–4554
41. Ben-Ze’ev, A., Reiss, R., Bendori, R., and Gorodecki, B. (1990) *Cell Regul.* 1, 621–636
42. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) *Biochem. J.* 351, 95–105
43. Aktories, K. (1997) *Trends Microbiol.* 5, 282–288
44. Sander, E. E., ten Klooster, J. P., van Delft, S., van der Kammen, R. A., and Collard, J. G. (1999) *J. Cell Biol.* 147, 1009–1022
45. Edwards, D. C., Sanders, L. C., Bokoch, G. M., and Gill, G. N. (1999) *Nat. Cell Biol.* 1, 253–259
46. Sarbassov, D. D., and Peterson, C. A. (1998) *Mol. Endocrinol.* 12, 1870–1878
47. Cuenda, A., and Cohen, P. (1999) *J. Biol. Chem.* 274, 4341–4346
48. Reiser, C. O., Lanza, T., Hofmann, F., Hofer, G., Rupprecht, H. D., and Goppelt-Struebe, M. (1998) *Biochem. J.* 330, 1107–1114
49. Hahn, A., Heusinger-Ribeiro, J., Lanza, T., Zenkel, S., and Goppelt-Struebe, M. (2000) *J. Biol. Chem.* 275, 37429–37435
50. Spencer, J. A., and Misra, R. P. (1996) *J. Biol. Chem.* 271, 16535–16543
51. Belaguli, N. S., Schildmeyer, L. A., and Schwartz, R. J. (1997) *J. Biol. Chem.* 272, 18222–18231
52. Moisseyeva, E. P., Weller, P. A., Zhidkova, N. I., Corben, E. B., Patel, B., Jasin, T., Kotelianisky, V. E., and Critchley, D. R. (1997) *J. Biol. Chem.* 268, 4318–4325
53. Spencer, J. A., and Misra, R. P. (1999) *Oncogene* 18, 7319–7327
54. Spencer, J. A., Major, M. L., and Misra, R. P. (1999) *Mol. Cell. Biol.* 19, 3977–3988
55. Jhun, B. H., Rose, D. W., Seely, B. L., Rameh, L., Cantley, L., Saltiel, A. R., and Olefsky, J. M. (1994) *Mol. Cell. Biol.* 14, 7466–7475
56. Hu, Q., Klippel, A., Muslin, A. J., Fanti, W. J., and Williams, L. T. (1995) *Science* 268, 100–102
57. Yamauchi, K., Holt, K., and Pessin, J. E. (1993) *J. Biol. Chem.* 268, 14397–14400
58. Beltman, J., Erickson, J. R., Martin, G. A., Lyons, J. F., and Cook, S. J. (1999) *J. Biol. Chem.* 274, 3772–3780
59. Kumagai, N., Morii, N., Fujisawa, K., Nemoto, Y., and Narumiya, S. (1993) *J. Biol. Chem.* 268, 24355–24358
60. Wang, Y., and Prywes, R. (2000) *Oncogene* 19, 1379–1385