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Activation of SRF-Regulated Chromosomal Templates by Rho-Family GTPases Requires a Signal that Also Induces H4 Hyperacetylation

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Summary

Constitutively active forms of the small GTPases RhoA (RhoA.V14) and Cdc42 (Cdc42.V12) induce expression of extrachromosomal SRF reporter genes in microinjection experiments, but only Cdc42.V12 can efficiently activate a chromosomal template. Both SAPK/JNK-dependent or -independent signals can cooperate with RhoA.V14 to activate chromosomal SRF reporters, and it is SAPK/JNK activation by Cdc42.V12 that allows it to activate chromosomal templates. Cooperating signals can be bypassed by deacetylase inhibitors. Three findings show that histone H4 hyperacetylation is one target for cooperating signals, although it alone is not sufficient: (1) Cdc42.V12, but not RhoA.V14, induces H4 hyperacetylation; (2) cooperating signals use the same SAPK/JNK-dependent or -independent pathways to induce H4 hyperacetylation; (3) growth factor and stress stimuli induce substantial H4 hyperacetylation, detectable in reporter gene chromatin. These data establish a link between signal-regulated acetylation events and gene transcription.

Introduction

The serum response element (SRE) is a growth factor-regulated promoter element that is essential for the activation of immediate-early genes, such as c-*fos* and *Egr*-1, by extracellular signals. The c-*fos* SRE, which binds a transcription factor complex comprising SRF (serum response factor; Norman et al., 1988) and a member of the TCF family (ternary complex factor; Shaw et al., 1989), is a convergence point for several signal transduction pathways. TCF activity is regulated via phosphorylation of their C-terminal transcriptional activation domains by MAP kinases (for review and references, see Treisman, 1994; Price et al., 1996; Raingeaud et al., 1996). In contrast, SRF activity is regulated by a novel signal pathway that involves the Rho-family GTPase RhoA. This pathway, which acts independently of known MAPK pathways, is regulated by agents such as serum or LPA acting via heterotrimeric G protein–coupled receptors (Hill et al., 1995; J. Wynne and R. T., unpublished data). SRF activity can also be potentiated by intracellular expression of activated forms of both RhoA and two other Rho-family proteins, Cdc42 and Rac1; the latter GTPases act independently of RhoA, but their relevance to the response to extracellular signals has yet to be demonstrated (Hill et al., 1995).

Several observations suggest that in addition to the signal-induced potentiation of transcription factor activity, immediate-early gene transcription may require modification of chromatin components. First, growth factors and stress stimuli induce reversible histone H3 and HMG14 phosphorylation with kinetics similar to those of c-*fos* transcription (Mahadevan et al., 1991; Edwards and Mahadevan, 1992; Barratt et al., 1994a, 1994b). Second, regulation by several inducible promoter elements, including the SRE, is inhibited by microinjection of antibodies against the coactivator proteins p300 and CBP (Arias et al., 1994), and the TCFs SAP-1 and Elk-1 are among the many transcription factors whose activity can be stimulated by CBP in transfection assays (Janknecht and Nordheim, 1996a, 1996b). Although the molecular basis of these effects remains to be determined, both CBP and p300 are acetyltransferases (Bannister and Kouzarides, 1996; Ogryzko et al., 1996), and c-*fos* nucleosomes become hyperacetylated when transcription is activated (Allegra et al., 1987; Chen and Allfrey, 1987). The functional significance of these phenomena has remained unclear.

In this study, we set out to test whether activation of Rho-controlled signal pathways is sufficient for activation of the chromosomal c-*fos* gene, as is the case with Ras (Stacey et al., 1987). We used a microinjection approach as a short-term cell-based assay to minimize autocrine effects that may complicate the interpretation of transfection assays (see Cowley et al., 1994; Minden et al., 1994). We show that although the activated form of RhoA can activate microinjected templates, it is unable to activate either the chromosomal c-*fos* gene or integrated SRF-controlled reporter genes unless additional signaling pathways are activated. The cooperating signals may be either SAPK/JNK-dependent or -independent and can be substituted by treatment of cells with deacetylase inhibitors. Finally, we show that one target of the cooperating signals is the hyperacetylation of histone H4, which is also induced in reporter gene chromatin following growth factor stimulation. Our data provide evidence that acetylation events, including chromatin modifications, can be a prerequisite for activation of immediate-early gene expression.

Results

Differential Activation of Chromosomal and Extrachromosomal c-*fos* Genes by Rho-Family GTPases

We used a microinjection assay to test whether expression of the chromosomal c-*fos* gene can be induced by activated forms of RhoA (RhoA.V14), Cdc42 (Cdc42.V12), or Rac1 (Rac1.V12). Purified recombinant proteins, normalized for GTP binding activity, were injected into the cytoplasm of serum-deprived NIH3T3

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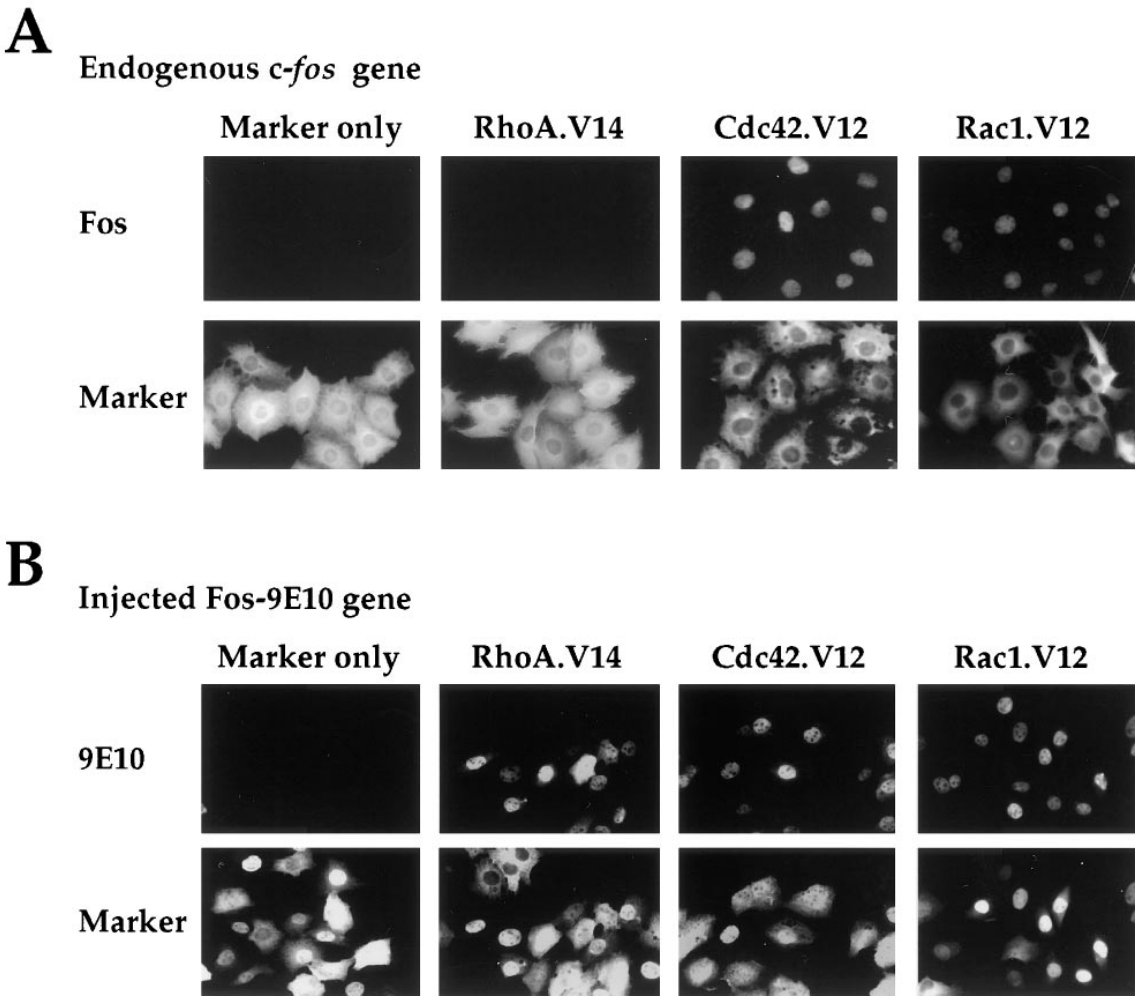

Figure 1. Differential Activation of Chromosomal and Extrachromosomal c-*fos* Genes by Rho-Family Proteins

(A) Cdc42.V12 and Rac1.V12, but not RhoA.V14, activate the chromosomal c-*fos* gene. RhoA.V14, Cdc42.V12, or Rac1.V12 proteins, together with marker guinea pig IgG, were injected into the cytoplasm of serum-deprived NIH3T3 cells. Two hours later, cells were fixed and Fos and marker proteins detected by indirect immunofluorescence.

(B) RhoA.V14 can activate a coinjected c-*fos* gene derivative. RhoA.V14, Cdc42.V12, and Rac1.V12 proteins were injected into the nuclei and cytoplasm of serum-deprived NIH3T3 cells together with a plasmid containing a 9E10-epitope-tagged human c-*fos* gene (pF711–9E10). Two hours later, cells were fixed and Fos.9E10 and marker proteins detected by indirect immunofluorescence.

cells together with a marker protein, and c-*fos* expression was assayed by indirect immunofluorescence 2 hr later. Surprisingly, Cdc42.V12 and Rac1.V12 induced c-*fos* expression but RhoA.V14 did not, although all proteins could induce appropriate cytoskeletal rearrangements (Figure 1A and data not shown). The rapidity of the response, and the lack of gene induction in neighboring uninjected cells, suggests that it is not mediated by autocrine pathways. Similar results were obtained following nuclear injection of Rho-family expression plasmids (data not shown). In contrast, all the GTPases could induce expression of an epitope-tagged c-*fos* gene (Fos-9E10) coinjected into the nuclei of NIH3T3 cells (Figure 1B; data not shown). Similar results were obtained when Fos-9E10 was coinjected with Rho-family expression plasmids (data not shown). Thus, the ability of RhoA.V14 to activate c-*fos* gene expression differs according to the context of the DNA template.

RhoA-Dependent Signals Are Necessary but Not Sufficient for Activation of a Chromosomal SRE

Differential activation of chromosomal and injected c-*fos* templates by Rho-family proteins might reflect a property of either the SRE itself or its interaction with other c-*fos* promoter elements. To address this issue, we constructed cell lines carrying chromosomal SRF-controlled reporter genes. One reporter line, SRE-lacZ, carries the bacterial *lacZ* gene controlled by a promoter comprising the RSV TATA box and three SRF binding sites; a second reporter cell line, SRE-FosHA, contains an epitope-tagged derivative of our previously characterized reporter gene 3D.AFos (Hill et al., 1995). Both SRE-lacZ and SRE-FosHA genes lack binding sites for TCF and respond to serum and LPA; they do not respond to stress stimuli such as UV irradiation or subinhibitory anisomycin concentrations, although a subset of the

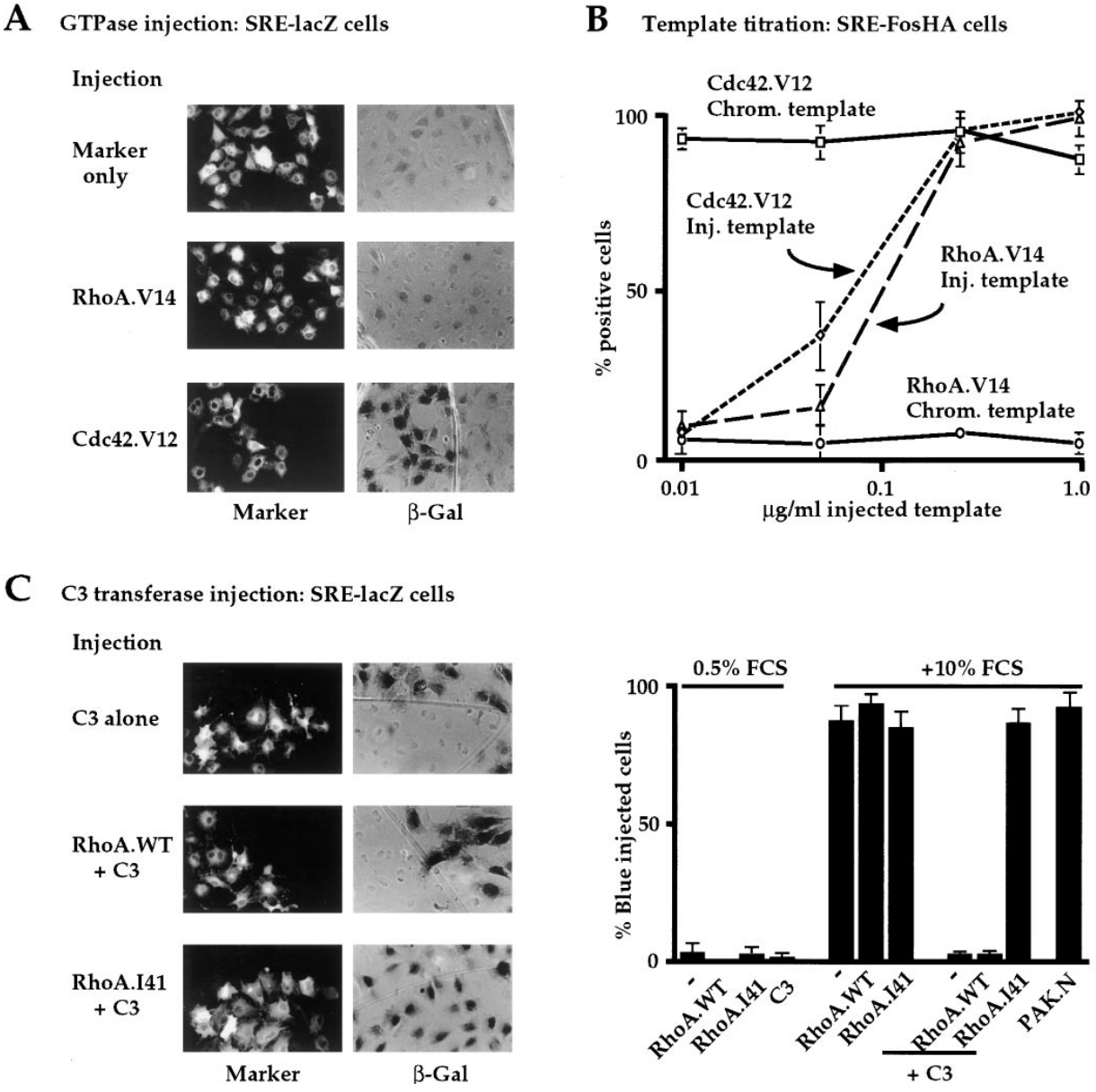

Figure 2. RhoA Signaling Is Necessary but Not Sufficient for Activation of the Chromosomal SRF-Controlled Templates

(A) Cdc42.V12 activates a chromosomal SRE while RhoA.V14 does not. RhoA.V14 and Cdc42.V12 proteins were injected into the cytoplasm of serum-deprived SRE-lacZ cells. Two hours after injection, cells were fixed and stained for marker and *lacZ* reporter activity. Refractive lines are from scores on the coverslip.

(B) An extrachromosomal SRE-FosHA template is equally responsive to Cdc42.V12 and RhoA.V14 proteins. Recombinant GTPases were injected into the nuclei and cytoplasm of SRE-FosHA cells together with various concentatrations of the SRE-Fos9E10 reporter plasmid. Cells were fixed 2 hr later, and marker proteins and the products of the injected and chromosomal reporter genes were detected by immunofluorescence with 9E10 and Y11 antibodies, respectively. Values represent mean ± SE of three independent experiments, 50–100 cells per treatment.

(C) RhoA but not Cdc42 mediates induction of the chromosomal SRF-controlled reporter. Left panel, ADP ribosylation–resistant RhoA (RhoA.I41) can mediate serum-induced signaling to C3 transferase–injected cells. C3 transferase and marker protein were injected into the cytoplasm of SRE-lacZ cells either alone or with wild-type RhoA or RhoA-I41 (both at 3 mg/ml) as indicated. Thirty minutes after injection, cells were stimulated with 10% FCS, and after another 2 hr fixed and stained for marker and *lacZ* activity. Representative fields of injected cells are shown; lines are scores on the coverslip. Right panel, summary of two experiments; PAK.N, injection of the Cdc42/Rac1-binding domain of PAK (mean ± half range; 100–200 cells injected for each treatment).

cells are induced by TPA treatment (data not shown). As with the chromosomal c-*fos* gene, neither chromosomal reporter gene was activated upon microinjection of RhoA.V14, although both were activated by Cdc42.V12 (Figures 2A and 2B). In contrast, both RhoA.V14 and Cdc42.V12 could activate coinjected SRE-lacZ and SRE-FosHA templates (Figure 2B). Since the two cell lines are independently derived transformants containing different basal promoter and reporter gene sequences, their identical behavior, both in these assays

and those presented below, must reflect properties of SRF itself.

We next considered the possibility that the differential response of the reporter genes to Rho-family proteins might arise because the SRF-controlled reporter genes are more sensitive to Cdc42.V12 than RhoA.V14, and a saturating amount of template is used in the coinjection experiments. We therefore performed a template titration experiment (Figure 2B). SRE-FosHA cells were injected with RhoA.V14 or Cdc42.V12, together with decreasing amounts of an SRE-Fos9E10 reporter gene (identical to the SRE-FosHA but with the 9E10 epitope; see Experimental Procedures), and expression of both the injected and chromosomal genes monitored simultaneously. Both RhoA.V14 and Cdc42.V12 activated the coinjected reporter template in a concentration-dependent manner, with half-maximal response occurring at about 100 ng/ml injected reporter DNA (about 2–20 copies; see Experimental Procedures). Similarly, when the concentrations of recombinant GTPases were titrated over a 20-fold range, reporter gene expression diminished in parallel (data not shown). Thus, the differential response of the chromosomal SRE to the activated Rho-family GTPases must reflect difference in their signaling properties rather than merely signal strength.

We previously showed that RhoA function is required for the response of a transfected SRF-controlled reporter gene to serum or LPA stimulation (Hill et al., 1995). To confirm that functional RhoA is indeed necessary for induction of a chromosomal SRE by external stimuli, SRE-lacZ cells were injected with the enzyme C3 transferase, which ADP-ribosylates and specifically inactivates RhoA. This treatment abolished serum- and LPA-induced SRE-lacZ reporter expression (Figure 2C); similar results were obtained when RhoA was inactivated by injection of the RhoA-binding domain of the RhoA effector kinase PKN (data not shown). Serum induction of the reporter gene was restored when cells were coinjected with both C3 transferase and the C3-resistant RhoA mutant RhoA.I41 (Figure 2C). In contrast, serum- or LPA-induced reporter gene expression expression was unaffected by expression of PAK.N, the GTPase-binding domain of the Cdc42/Rac1 effector kinase PAK (Manser et al., 1994), which specifically inhibits signaling by Cdc42 and Rac1 (Figure 2C; see below). Taken together with the inability of the constitutively active RhoA.V14 to activate the chromosomal reporter genes, these data show that RhoA is necessary, but not sufficient, for activation of the chromosomal SRE in response to serum and LPA stimulation.

The SAPK/JNK Pathway Cooperates with RhoA.V14 and Is Necessary for Induction of the Chromosomal SRE by Cdc42.V12

One interpretation of the observations described in the preceding sections is that Cdc42.V12, but not RhoA.V14, can activate a signaling pathway that is specifically required for the activation of chromosomal templates. We therefore sought to identify such signaling pathways. Intriguingly, previous studies have demonstrated that the activated forms of Cdc42 and Rac1, but not RhoA, can potentiate activity of stress-activated MAPK pathways in transfection experiments (Coso et al., 1995; Minden et al., 1995). We therefore tested whether activation of these pathways by stress stimuli such as UV irradiation or subinhibitory anisomycin concentrations would allow activated RhoA to induce chromosomal reporter gene expression. SRE-lacZ cells were injected with recombinant RhoA.V14, immediately stimulated with anisomycin or UV irradiation, and reporter gene activity assayed 2 hr later. Although neither anisomycin nor UV irradiation alone activated the chromosomal SRE-lacZ reporter gene, both these stimuli activated the reporter in the presence of RhoA.V14; in contrast, TPA treatment, which does not regulate stress-activated MAPKs in these cells, did not cooperate with activated RhoA to activate the reporter (Figure 3A). When SRE-lacZ cells were preinjected with expression plasmids producing either MEKK or v-src, both of which can activate the SAPK/JNK pathway (Devary et al., 1992; Yan et al., 1994), subsequent injection of RhoA.V14 protein activated reporter expression (Figure 3B).

We used specific inhibitors of different MAPK pathways to further investigate their involvement in the activation of the chromosomal SRF-controlled reporters. To inhibit the SAPK/JNK pathway, we injected either recombinant JNK.KA, a kinase-inactive mutant of p54SAPKβ, or an expression plasmid encoding SEK-AL, a nonactivatable SEK mutant (Yan et al., 1994). Both proteins inhibited activation of chromosomal SRF-controlled reporter genes by either Cdc42.V12 or the combination of RhoA.V14 and anisomycin (Figure 3C); in control experiments, both JNK.KA and SEK-AL prevented Cdc42.V12- and stress-induced phosphorylation of a coexpressed SAPK/JNK substrate, the Jun N terminus (data not shown; A. S. A. and R. T., submitted). In contrast, treatment of the cells with the small-molecule inhibitors PD98059 and SB203580, which inhibit the ERK and p38 MAPK pathways, respectively (Alessi et al., 1995; Cuenda et al., 1995), had no effect on the ability of anisomycin to cooperate with RhoA.V14 (Figure 3C, left panel). We also tested whether Cdc42 or Rac1 activity is required for SRF activation by RhoA.V14/anisomycin by coinjection of recombinant PAK.N, the N-terminal GTPase-binding domain of the Cdc42/Rac1 effector PAK (Manser et al., 1994). PAK.N had no effect on activation of the chromosomal reporter in this assay, although, as expected, it effectively blocked activation by Cdc42.V12 (Figure 3C, left panel). Conversely, titration of cellular RhoA by PKN.N, the RhoA-binding domain of its effector PKN, did not block activation of the reporter by Cdc42.V12, although it effectively blocked activation by RhoA.V14/anisomycin (Figure 3C, left panel).

The above results show that activation of the SAPK/JNK pathway is required for activation of the chromosomal SRF-controlled reporter gene by Cdc42.V12 or RhoA.V14/anisomycin. To confirm that SAPK/JNK activity is specifically required for only activation of chromosomal templates, we repeated the experiments using a coinjected SRE-Fos9E10 reporter gene, which is efficiently activated by both Cdc42.V12 and RhoA.V14: JNK.KA blocked activation by neither GTPase (compare Figures 3C and 3D). SAPK/JNK activation thus represents an additional signal specifically required for activation of chromosomal SRF reporter genes by RhoA.V14 and Cdc42.V12.

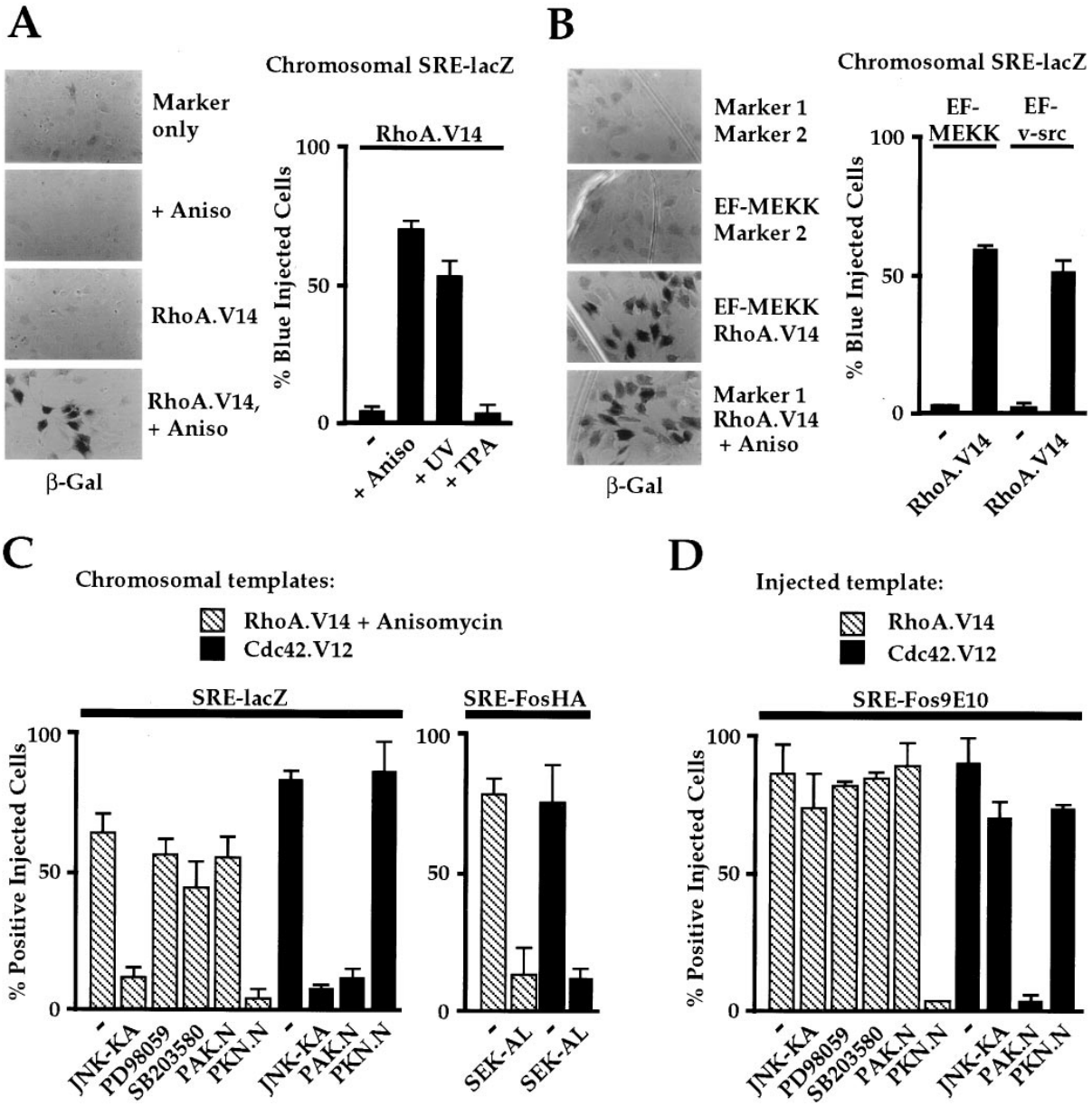

Figure 3. RhoA.V14 Can Cooperate with Stress Stimuli to Activate a Chromosomal SRE; Both This and Activation by Cdc42.V12 Require SAPK/JNK Activity

(A) Stress stimuli cooperate with RhoA.V14. RhoA.V14 protein was injected into the cytoplasm of serum-deprived SRE-lacZ cells, which were stimulated 20 min later as indicated and processed for marker and *lacZ* activity after a further 2 hr. Representative fields are shown. Bars represent the percentage of injected cells staining positive for *lacZ* from three independent experiments (mean ± SD, 50–100 cells injected for each inducing agent).

(B) MEKK1 and v-*src* cooperate with RhoA.V14. Expression plasmids encoding MEKK1 or v-*src* were injected into the nuclei of SRE-lacZ cells, together with marker 1 (rabbit Ig). After 2 hr, cells received a second, cytoplasmic injection of marker 2 (guinea pig Ig), with or without RhoA.V14 protein. After a further 2 hr, the cells were fixed and assayed for markers and *lacZ* activity. Bars represent the percentage lacZ-positive cells in two independent experiments (mean ± half range, 50–100 cells injected per experiment).

(C) Chromosomal SRE activation by anisomycin/RhoA.V14 and Cdc42.V12 involves the SAPK/JNK pathway. Left panel: RhoA.V14 or Cdc42.V12 and marker proteins were injected into the cytoplasm of serum-deprived SRE-lacZ cells either alone, together with PAK.N, PKN.N, or kinase-inactive p54SAPKβ (JNK.KA), or treated with 50 μM PD98059 or 10 μM SB203580 as indicated. RhoA.V14-injected cells were immediately treated with anisomycin. Two hours later, cells were fixed and assayed for marker and *lacZ* activity. Bars represent the percentage of *lacZ*-positive injected cells in three independent experiments (mean ± SE, 50–100 cells injected for each condition) except for PD98059 and SB203580 (mean ± half range of two experiments). Right panel: expression plasmids encoding RhoA.V14 or Cdc42.V12 together with kinase-inactive SEK (SEK-AL) were injected into the nuclei of serum-deprived SRE-FosHA cells as indicated; RhoA.V14-injected cells were immediately treated with anisomycin. Two hours later, cells were fixed and assayed for marker and FosHA expression. Bars represent the percentage of FosHA-positive injected cells in three independent experiments (mean ± SE, 50–100 cells injected for each experiment).

(D) SAPK/JNK activity is not required for activation of an extrachromosomal SRE. The SRE-Fos9E10 reporter gene was injected into the nuclei of SRE-lacZ cells, together with proteins as in (C) (left) and treated identically. Bars represent the percentage of 9E10-positive injected cells in three independent experiments (mean ± SE, 50–100 cells injected for each condition) except for PD98059 and SB203580 (mean ± half range of two experiments).

Other Signal Pathways Can Cooperate with Activated RhoA

To test whether the SAPK/JNK pathway is necessary for mitogen-induced activation of a chromosomal SRE, we examined whether microinjection of JNK.KA could inhibit the ability of SRE-FosHA cells to respond to serum or LPA stimulation. Inhibition of the SAPK/JNK pathway by injection of JNK.KA substantially inhibited the response to LPA stimulation but had no effect upon the ability of the reporter gene to respond to serum, suggesting that serum can induce a signal(s), distinct from SAPK/JNKs, which can also cooperate with RhoA (Figure 4A). We therefore examined other signal pathways associated with mitogen and stress stimuli.

SRE-FosHA cells were injected with expression plasmids encoding RhoA.V14 and other signaling molecules or their derivatives. In agreement with previous results, Ras.R12, an activated Ras protein, could not activate the SRF-controlled reporter genes although it efficiently activated c-*fos* transcription (Stacey et al., 1987; Hill et al., 1995; data not shown). However, Ras.R12 cooperated effectively with RhoA.V14, even though specific activation of the ERK pathway by expression of either activated Raf (Raf-CAAX; Leevers et al., 1994), activated MEK (MEK-EE; Cowley et al., 1994), or TPA, could not (Figure 4B). The ability of activated Ras.R12 to cooperate with RhoA.V14 was prevented by expression of SEK-AL, suggesting that Ras.R12 cooperation is mediated by the SAPK/JNK pathway (Figure 4B); consistent with this, Ras.R12 injection promoted phosphorylation of the Jun N terminus in control experiments (data not shown). Finally, we tested whether RhoA.V14 could cooperate with an activated mutant of another stress-activated kinase, MKK3 (MKK3E; Raingeaud et al., 1996). Injection of RhoA.V14 and MKK3E expression plasmids efficiently induced SRE-FosHA expression, which was insensitive to blockade of the SAPK/JNK pathway by both JNK.KA and SEK-AL (Figure 4B). Thus, in addition to the SAPK/JNK pathway, at least one other signal pathway can cooperate with RhoA.V14 to activate a chromosomal SRE (see Discussion).

Deacetylase Inhibitors Cooperate with RhoA.V14

One potential explanation for the differential signal requirements of extrachromosomal and integrated reporter genes is that signals that cooperate with RhoA.V14, but not RhoA.V14 itself, can induce a chromatin or transcription factor modification necessary only for transcription of a chromosomal template. Recent findings have implicated acetylation of both chromatin components and the transcriptional machinery in regulation of gene expression, so we tested whether deacetylase inhibitors (Yoshida et al., 1995) can cooperate with RhoA.V14 to induce chromosomal reporter gene expression.

SRE-lacZ cells were injected with an expression plasmid encoding RhoA.V14 and stimulated with deacetylase inhibitors, and activity of the reporter gene was assessed. Both sodium butyrate and the specific deacetylase inhibitor trichostatin A (TSA) could cooperate with RhoA.V14 to induce the chromosomal SRE in SRE-lacZ cells. Cooperation between RhoA.V14 and deacetylase

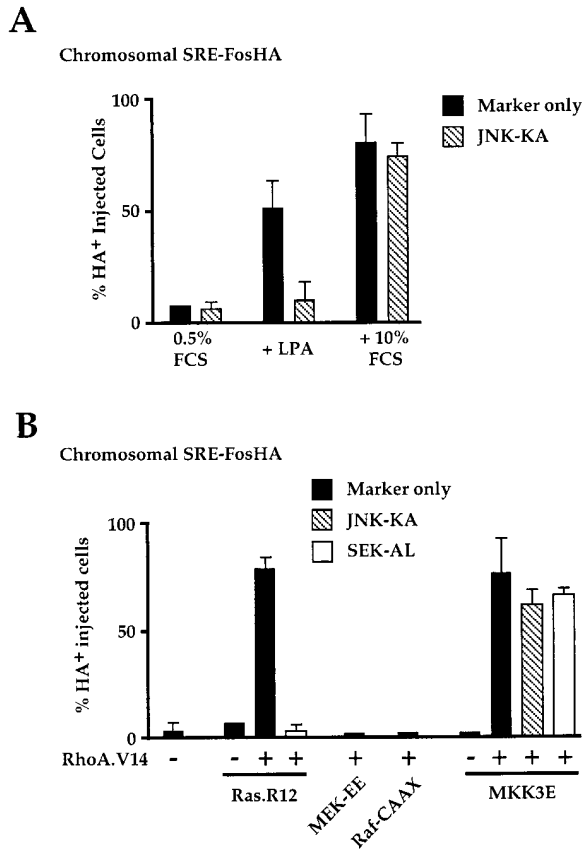

Figure 4. Other Pathways Can Cooperate with Activated RhoA

(A) The SAPK/JNK pathway is necessary for chromosomal SRE activation by LPA but not by serum. Kinase-inactive p54SAPKβ (JNK.KA) and marker proteins were injected into the cytoplasm of SRE-FosHA cells, which were stimulated 20 min later as indicated. Cells were then fixed and assayed for expression of FosHA and marker. Bars show percentage of FosHA-positive injected cells from two representative experiments (mean ± half range, 50–100 cells per treatment).

(B) RhoA can cooperate with MKK3E and Ras but not the ERK pathway. Expression plasmids were injected into the nuclei of serum-deprived SRE-FosHA cells, together with kinase-inactive p54SAPKβ protein (JNK.KA), as indicated. Four hours later, the cells were fixed and analyzed for marker and FosHA expression. Bars represent the percentage of 9E10-positive injected cells in three independent experiments (mean ± SE, 50–100 cells injected for each condition) except for Raf-RhoA.V14/RasR12/SEK-AL and RafCAAX (mean ± half range of two experiments).

inhibitors was not sensitive to blockade of the SAPK/JNK pathway by expression of SEK-AL, demonstrating that the inhibitors do not act via this pathway (Figure 5A). Similar results were obtained in SRE-FosHA cells (data not shown). We also investigated whether deacetylase inhibitors can cooperate with activated RhoA to induce expression of chromosomal immediate-early genes. Treatment of NIH3T3 cells with either butyrate or TSA alone was not sufficient to induce expression of either c-*fos* or *Egr*-1, another SRE-controlled gene; however, both inhibitors cooperated with activated RhoA.V14 to induce chromosomal c-*fos* or *Egr*-1 expression (Figure 5B). These data suggest that activation of RhoA.V14-induced signaling pathways together with an

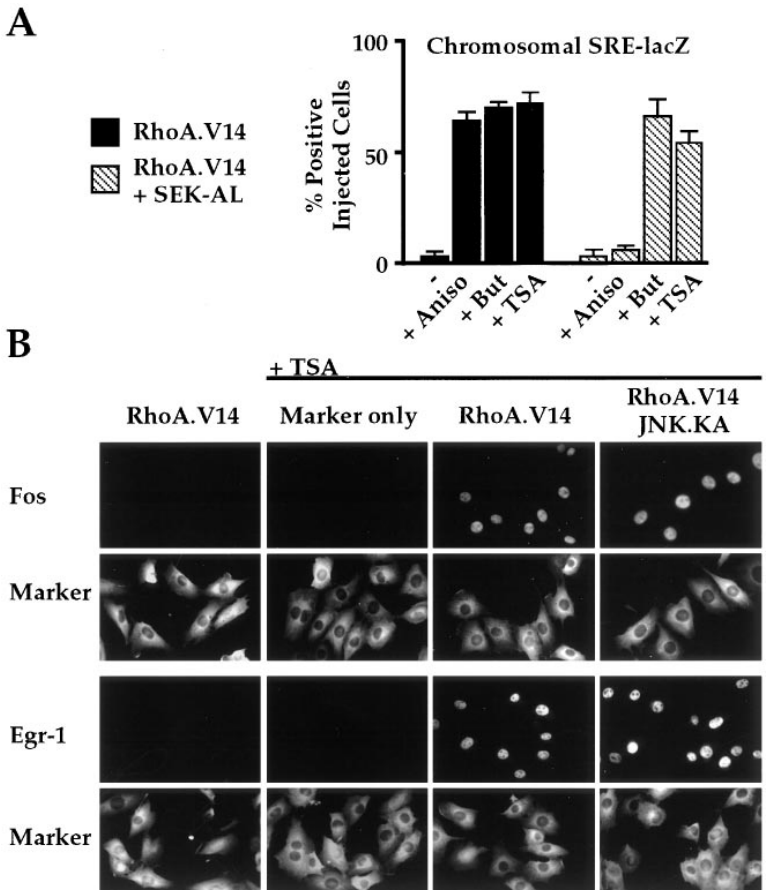

Figure 5. Deacetylase Inhibitors Cooperate with RhoA.V14 to Activate Chromosomal SRF-Controlled Gene Expression

(A) Deacetylase inhibitors do not act via the SAPK/JNK pathway. Expression plasmids encoding RhoA.V14 and kinase-inactive SEK (SEK-AL) were injected into the nuclei of SRE-lacZ cells as indicated. After 2 hr, the cells were treated with sodium butyrate (1 mM), trichostatin A (10 nM), or anisomycin, and after a further 2 hr fixed and assayed for *lacZ* activity and marker protein. Bars represent the percentage of *lacZ*-positive injected cells in three independent experiments (mean ± SE, 50–100 cells injected for each condition) except for TSA (mean ± half range, two experiments).

(B) Trichostatin A cooperates with RhoA.V14 to activate chromosomal c-*fos* (top panels) and *Egr*-1 (lower panels). Recombinant RhoA.V14 and kinase-inactive p54SAPKβ (JNK.KA) were injected into serum-deprived NIH3T3 cells as indicated, together with marker protein. After 2 hr, the cells were stimulated with TSA and stained for Fos, Egr-1, and marker proteins 2 hr later.

acetylation event is sufficient for induction of both chromosomal SRF-controlled reporter and immediate-early gene expression.

Extracellular Stimuli Induce Rapid Histone H4 Acetylation in Reporter Gene Chromatin

The observed cooperation between RhoA.V14 and deacetylase inhibitors led us to search for proteins whose acetylation status changes in response to extracellular signals. We therefore examined the effects of extracellular stimuli on histone acetylation status. Histones were partially purified from growing, serum-starved, or stimulated SRE-FosHA cells and fractionated on acid-urea gels to resolve different acetylated histone isoforms, which were detected by immunoblotting with antibodies raised against either acetylated tetrahymena histones H4 or H3 (Lin et al., 1989) or anti-acetyllysine (Hebbes et al., 1989). The tetrahymena H4 antibody cross-reacts specifically with acetylated mammalian H4, reacting preferentially with tetraacetylated H4, and to a lesser extent with triacetylated H4, but retaining only low reactivity for the diacetylated form (Meistrich et al., 1992; Perry et al., 1993); the anti-acetyllysine antibody R47 also preferentially reacts with hyperacetylated histone isoforms (Hebbes et al., 1989, 1994). Serum-starved cells exhibited substantially reduced acetylation of H4 compared with cycling cells (Figure 6A). In addition to the deacetylase inhibitors, several extracellular stimuli caused a rapid increase in hyperacetylation of H4. These include both stimuli that are sufficient to activate a chromosomal SRF-controlled reporter, such as serum or LPA, and stimuli that can cooperate with RhoA.V14 to do so, such as anisomycin and UV irradiation. However, TPA, which does not cooperate with RhoA.V14, also induced H4 acetylation (Figure 6A; see Discussion). Similar changes were detected using the anti-acetyllysine antibody (data not shown). In contrast, little change in H3 acetylation was detectable with either anti-acetyl H3 or anti-acetyllysine antibodies (Figure 6A; data not shown). Hyperacetylation of H4 was detectable within 15 min of stimulation and continued to increase for several hours; a substantial proportion of total H4 became acetylated, as revealed by examination of the histone preparations by direct staining of the gels (Figure 6B; see Discussion).

We next investigated whether extracellular signals increased the density of H4 hyperacetylation at the SRF-controlled reporter and c-*fos* genes by immunoprecipitation of cross-linked chromatin (Braunstein et al., 1993). Serum-deprived or stimulated cells were cross-linked using formaldehyde, and a crude nuclear lysate sonicated to generate DNA fragments 200–1000 base pairs in length. Following immunoprecipitation and reversal of cross-links, immunoprecipitated DNA was recovered and the presence of reporter gene sequences ascertained using the PCR. In this assay, immunoprecipitation is specific, since it is not observed with nonspecific IgG, and is dependent on prior cross-linking (Figure 6B and

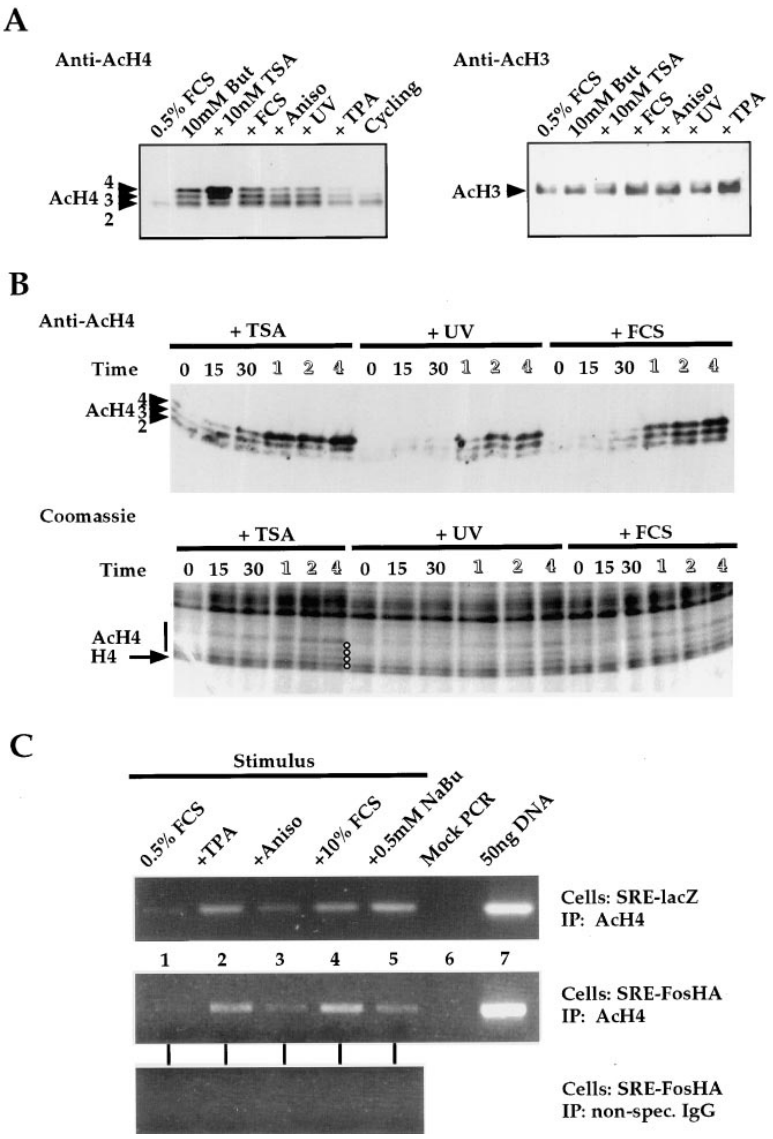

Figure 6. Extracellular Stimuli Induce Histone H4 Acetylation

(A) Extracellular signals cause histone hyperacetylation. SRE-FosHA cells were maintained in 0.5% FCS for 36 hr and stimulated for various times as indicated. Histones were extracted, fractionated on acid-urea gels, transferred to Immobilon membrane, and detected anti-acetyl H4 serum (left panel) or anti-acetyl H3 serum (right panel) (Lin et al., 1989). Similar results were obtained using anti-acetyllysine serum R47 (Hebbes et al., 1989; data not shown).

(B) Time course of H4 acetylation. Cells were stimulated as indicated for the times shown (minutes; hours indicated by open type). Top, acetylated histone H4 visualized by immunoblot. Bottom, duplicate gel run in parallel stained with Coomassie blue, acetylated H4 isoforms indicated by white circles (tetraacetylated H4 comigrates with an unidentified protein). Note that, as observed previously, approximately 50% of the H4 in resting cells is monoacetylated (see Hebbes et al., 1989, 1994).

(C) Extracellular signals induce H4 hyperacetylation at the SRF-controlled reporter gene chromatin. Lanes 1–5: cells were stimulated as indicated, cross-linked with HCHO, and nuclear lysates prepared. After precipitation with the indicated antibodies, reporter gene DNA was detected by PCR. Lanes 6 and 7: PCR controls with water (lane 6) or 50 ng genomic DNA from each cell line (lane 7).

data not shown). Moreover, under the conditions used for immunoprecipitation, treatment of starved cells with sodium butyrate substantially increases the yield of immunoprecipitated reporter gene DNA, indicating that the density of hyperacetylated H4 is increased by this treatment (Figure 6B, compare lanes 1 and 5). Hyperacetylation of H4 in reporter gene chromatin was induced by all stimuli that induced hyperacetylation of bulk H4 as assessed by immunoblot, including serum, anisomycin, and TPA, although induction by anisomycin was relatively weak (Figure 6B, lanes 1–4). Thus, extracellular signals cause rapid hyperacetylation of histone H4 in reporter gene chromatin.

Stimuli that Cooperate with RhoA for SRE Activation Utilize the Same Signal Pathways to Induce H4 Hyperacetylation

The above data show that extracellular stimuli induce rapid changes in the acetylation status of histone H4 at our reporter genes. We next wished to investigate the relationship between the signaling pathways involved in histone hyperacetylation and those that cooperate with activated RhoA to induce expression of chromosomal target genes. To do this, we developed an immunofluorescence assay for H4 hyperacetylation. Titration experiments with both the anti-acetyllysine and anti-acetyl H4 antibodies established staining conditions under which acetylated histone H4 immunoreactivity was virtually undetectable in serum-deprived cells but remained readily detectable in cells treated with a variety of stimuli, in agreement with the immunoblotting data (Figure 7A). Consistent with the relatively large amount of hyperacetylated H4 seen by direct staining of gels, staining was uniform: discrete foci were undetectable even upon examination by confocal fluorescence microscopy (Figure 7A and data not shown; see Discussion). Stimulus-induced H4 hyperacetylation was not affected following treatment with concentrations of actinomycin D sufficient to inhibit transcription, indicating that it occurs independently of ongoing transcription

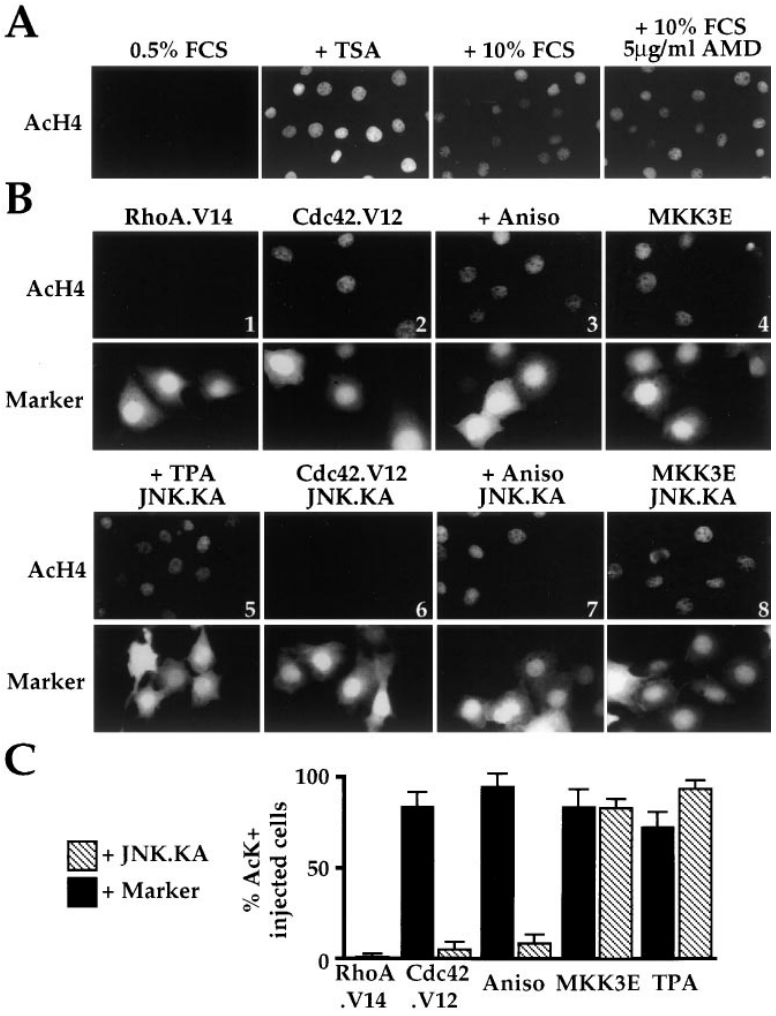

Figure 7. Cooperating Signals, but Not RhoA.V14, Induce H4 Hyperacetylation

(A) Immunofluorescence assay for histone hyperacetylation. Serum-deprived SRE-FosHA cells were either left alone or stimulated with TSA, serum, or serum with 5 mg/ml actinomycin D. After 1 hr, cells were fixed and histone acetylation detected by immunofluorescence using anti-acetyl H4 serum at high dilution. Similar results were obtained with anti-acetyl lysine serum (data not shown).

(B) Expression plasmids encoding RhoA.V14, Cdc42.V12, or MKK3E were injected into the nuclei of SRE-FosHA cells together with kinase-inactive p54SAPKβ (JNK.KA), as indicated, and marker protein; where indicated, cells were treated 20 min later with anisomycin or TPA. Two hours later, cells were fixed and stained for acetylated histone using anti-acetyl H4 serum at high dilution.

(C) Detection of hyperacetylated H4 by R47 anti-acetyllysine serum. Bars indicate the percentage of R47-positive injected cell (mean ± SE of three experiments, 50–100 cells injected for each condition, except for MKK3E, mean ± half range of two experiments).

(Figure 7A). In contrast, upon titration of the anti-acetyl H3 antibody, immunofluoresence signals in both serum-deprived and stimulated cells decreased in parallel, consistent with the immunoblotting data (data not shown).

We used the immunofluorescence assay to examine the abilities of Rho-family and other signaling molecules to induce H4 hyperacetylation in microinjected cells. Microinjection of RhoA.V14 did not induce H4 hyperacetylation (Figure 7B, panel 1). In contrast, H4 hyperacetylation was induced by Cdc42.V12, two stimuli that cooperate with RhoA.V14 to activate chromosomal SRF-controlled reporter gene, anisomycin, and MKK3E (Figure 7B, panels 2–4), and also upon activation of the ERK pathway by TPA, which does not (Figure 7B, panel 5). We next tested the dependence of induced H4 hyperacetylation on the SAPK/JNK pathway. Induction of H4 hyperacetylation by Cdc42.V12 and by anisomycin was effectively blocked by coinjection of JNK.KA (Figure 7B, compare panels 2 and 6, 3 and 7); similar results were obtained with UV irradiation (data not shown). In contrast, histone H4 hyperacetylation brought about by MKK3E and TPA was not blocked by coinjected JNK.KA (Figure 7A, panels 4 and 8; Figure 7C). These results show that several stimuli, but not RhoA.V14, can induce H4 hyperacetylation. Most importantly, stimuli that cooperate with activated RhoA, the same SAPK/JNK-dependent or -independent signaling pathways, are required for both H4 hyperacetylation and the transcriptional activation of a chromosomal reporter gene. These results therefore establish H4 hyperacetylation as a target for the cooperating signal pathways.

Discussion

Two Distinct Signals Are Required for Activation of a Chromosomal SRE

Our data show that activation of a chromosomally located SRF-controlled reporter gene is dependent on two kinds of signal, neither of which is by itself sufficient for activation (Figure 8). One signal, which is sufficient for activation of a transfected or microinjected template, can be supplied either by growth factors or by activated forms of either RhoA, Cdc42, or Rac1, and is independent of known MAPK pathways (Hill et al., 1995). The second signal, which is necessary but not sufficient for efficient activation of chromosomal templates, is induced by growth factors, stress stimuli, activated Cdc42, and TNFα (unpublished data), but not by activated RhoA.

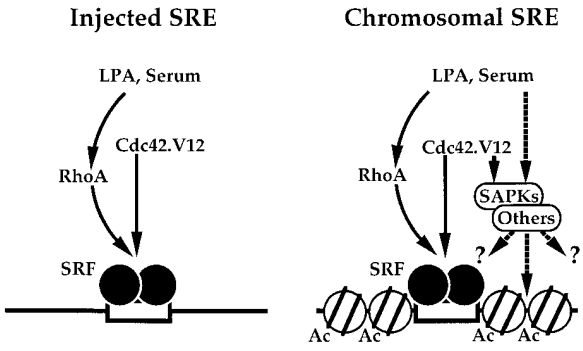

Figure 8. Signaling Pathways to Extrachromosomal and Chromosomal SRF Sites

DNAs are shown schematically as lines, with the SRF binding site an open box. The nucleosomal character of the chromosomal site is indicated by closed circles; absence of these symbols from the plasmid DNA does not imply the absence of nucleoprotein structures on this template. Signaling pathways from Rho-family proteins to SRF are indicated by solid lines, and the "chromosome-specific" signaling pathways are shown as dashed lines. Acetylation of H4 and other components, indicated by question marks, is indicated schematically by "Ac." Regulation may proceed either through regulation of acetyltransferases or deacetylases. For discussion, see text.

Treatment of cells with the deacetylase inhibitors sodium butyrate or trichostatin A bypasses the requirement for the second signal.

What is the molecular target for the cooperating signal pathway? The ability of deacetylase inhibitors to substitute for it strongly suggests that it controls an acetylation event specifically required for transcriptional activation by SRF in a chromosomal context. Our data strongly suggest that the signal pathways used by cooperating stimuli to induce transcription are the same as those used to induce hyperacetylation of histone H4. However, it is clear that H4 hyperacetylation per se cannot be sufficient. Thus, stimuli that cooperate with activated RhoA via the SAPK/JNK pathway, such as anisomycin and TNFα, also induce H4 hyperacetylation via the SAPK/JNK pathway, while activated MKK3 both cooperates with RhoA and induces H4 hyperacetylation independently of the SAPK/JNKs. Moreover, Cdc42.V12 but not RhoA.V14 induces H4 hyperacetylation in an SAPK/JNK-dependent manner, and it is the ability of Cdc42.V12 to activate the SAPK/JNK pathway that allows it to activate chromosomal templates. Nevertheless, H4 hyperacetylation is also induced following activation of the ERK pathway, which does not cooperate with RhoA.V14. It is therefore likely that the cooperating signals induce an additional acetylation event(s). Recent studies suggest that in addition to chromatin components, both basal and sequence-specific transcription factors are substrates for acetylases (Gu and Roeder, 1997; Imhof et al., 1997); however, it remains possible that cooperating and noncooperating signals induce functionally distinct patterns of H4 acetylation that cannot be distinguished by our antibodies.

Why should activated RhoA be sufficient for SRE and c-*fos* activation when templates are injected or transfected but not when the same templates are integrated into cellular DNA? In principle, this might arise from differential nuclear location of the two types of template; however, we favor the view that it reflects differences in chromatin structure. The nucleoprotein structure adopted by microinjected DNA has not been investigated, but several studies of transfected genes have shown that both their nucleoprotein structure and regulatory properties differ from those of chromosomal genes. Transfected DNA is assembled into repeating nucleosome-like structures (Innis and Scott, 1983; Cereghini and Yaniv, 1984), which exhibit abnormal repeat lengths and salt solubility (Jeong and Stein, 1994a, 1994b) and can be abnormally accessible to transcription factors (Archer et al., 1992). Moreover, even though the nucleoprotein structure of transfected DNA can be perturbed by deacetylase inhibitors (Innis and Scott, 1983), transfected and integrated DNAs often respond differently to such treatments (Bresnick et al., 1990; Schlake et al., 1994; Pennie et al., 1995; Van Lint et al., 1996). Our results provide further evidence that transfection or microinjection assays may underestimate the contribution of the nucleoprotein environment to the requirements for transcriptional activation.

Chromatin Modifications and Immediate-Early Gene Expression

H4 hyperacetylation can be induced in quiescent cells by a variety of extracellular stimuli, but not by activation of RhoA-controlled signal pathways. Immunofluorescence experiments revealed no evidence for the distinct foci of hyperacetylated H4 that might be expected if hyperacetylation were restricted to a relatively small number of active genes; consistent with this, accumulation of hyperacetylated isoforms of H4 was detectable even by direct staining of the histone gels. H4 hyperacetylation is detectable within 15 min of stimulation, but hyperacetylated H4 continues to accumulate for several hours, by which time both reporter and c-*fos* transcription has ceased. Signal-induced H4 hyperacetylation can occur even when transcription is blocked by actinomycin D. Moreover, stimuli that are not sufficient for transcriptional activation of the reporter genes can induce H4 hyperacetylation. Taken together, these observations suggest a model in which signal-induced H4 hyperacetylation represents a global response of the cell to stimulation, perhaps representing a prerequisite for transcription rather than a modification that is targeted to specific genes concomitant with their activation. Given the substantial basal histone acetyltransferase activity in serum-starved NIH3T3 cells, regulated changes in H4 acetylation status could be brought about either directly, by regulation of acetyltransferase or deacetylase activities themselves, or indirectly, by regulation of their access to substrate.

Recent studies have established direct links between the enzymes involved in histone acetylation and components of the transcriptional apparatus. Histone acetyltransferase activity is associated with TAFII250 (Mizzen et al., 1996) and coactivator complexes such as the yeast Ada and SAGA complexes (Grant et al., 1997) and the metazoan CBP/p300 complexes (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al.,

1996), while both yeast and mammalian deacetylases are found in transcriptional corepressor complexes (for references, see Pazin and Kadonaga, 1997). Moreover, both p53 and the basal factor TFIIE are substrates for the CBP/p300 coactivators (Gu and Roeder, 1997; Imhof et al., 1997). These coactivators have been implicated in the functions of both the SRE and other c-*fos* promoter elements (Arias et al., 1994; Janknecht and Nordheim, 1996a, 1996b). It is not yet clear whether the activity of CBP or p300 is regulated by signal transduction pathways, but their potential involvement in the phenomena presented here warrants further investigation.

We used chromatin immunoprecipitation experiments to confirm that H4 hyperacetylation is induced at our chromosomal SRF-controlled reporter genes in response to extracellular signals. We have also observed hyperacetylated H4 in c-*fos* gene chromatin under such conditions (O. G., unpublished data). A previous study, in which organomercurial agarose chromatography was used to purify hyperacetylated Balb/C 3T3 nucleosomes effectively (Allegra et al., 1987), also strongly suggested that c-*fos* chromatin becomes hyperacetylated when the gene is activated (Chen and Allfrey, 1987; Chen et al., 1990). In contrast, a more recent study of HL60 cells reported no increase in acetylation of the gene following TPA treatment (O'Neill and Turner, 1995). This discrepancy may reflect either cell-type differences, high basal levels of acetylase activity in these cells, or the antibodies used.

Extracellular signals such growth factors and stress stimuli also induce phosphorylation of chromatin components including histone H3 and HMG14, but the relation between this and immediate-early gene expression is unclear (Mahadevan et al., 1991; Edwards and Mahadevan, 1992; Barratt et al., 1994b). Staining with an antibody that recognizes phosphorylated H3 indicates that treatment with the deacetylase inhibitors does not induce H3 phosphorylation (A. S. A., unpublished data). These data suggest that H3 phosphorylation is not necessarily a prerequisite for activation by SRF, but it remains possible that under certain conditions phosphorylation of chromatin components can functionally substitute for H4 hyperacetylation, or that both hyperacetylation and phosphorylation of c-*fos* chromatin combine to promote efficient transcription in response to growth factor stimuli.

In this work, we exploited the fact that in isolation the SRF-controlled reporter gene is a target for only a subset of the signals that can activate the complex chromosomal c-*fos* promoter (see Hill et al., 1995; Price et al., 1996). For example, activation of the SAPK/JNK pathway is sufficient for c-*fos* activation, presumably because it activates other transcription factors such as the TCFs and CRE binding proteins (for references, see Price et al., 1996), but can only activate the SRF-controlled reporter in collaboration with activated RhoA. Our observations therefore establish an additional level at which extracellular signals might act in a combinatorial manner to control gene expression.

Experimental Procedures

Plasmids

DNA was manipulated by standard techniques; full plasmid details are available on request. Fos-9E10, SRE-FosHA, and SRE-Fos9E10 were made by insertion of 9E10 or HA epitope oligonucleotides at the c-*fos* exon 4 NcoI site in pF711 or p3D.AFos (see Hill et al., 1995). pSRE-lacZ/neo contains three copies of the c-*fosA* SRE, linked to the RSV basal promoter and bacterial *lacZ* coding sequences (J. Meinkoth and A. S. A., unpublished data). Expression plasmids were as follows: MKK3E (Raingeaud et al., 1996), Raf-CAAX (Leevers et al., 1994), MEKEE (Cowley et al., 1994), SEK-AL (Yan et al., 1994), v-src, RasR12, MEKK1 (Price et al., 1996), Rho-family proteins, and C3 transferase (Hill et al., 1995), and GST fusions containing C3, Cdc42, and RhoA sequences were derived from the corresponding expression plasmids (Hill et al., 1995). GST-PAK.N and GST-PKN.N contain residues 1–252 from PAK (Manser et al., 1994) and 1–511 from PKN, respectively (Palmer et al., 1994); pGEX. JNK.KA (p54SAPKβ K55A) was as described (Yan et al., 1994).

Fusion Proteins

Overnight cultures were diluted 1:10 to 50 ml, grown for 3 hr, lysed by sonication in 5 ml 25 mM Tris (pH 7.2), 100 mM NaCl, 5 mM $MgCl_2$ with protease inhibitors, adsorbed to glutathione-Sepharose 4B (0.5 ml), and washed extensively. Proteins were released by overnight incubation at 4°C with thrombin (5U; Sigma), which was removed by adsorption to *p*-aminobenzamidine-Sepharose 6B (0.5 ml; Sigma), and protein concentration determined by dye-binding assay (Biorad).

Reporter Cell Lines and Cell Culture

Reporter cell lines were generated following transfection of NIH3T3 cells with either SRE-FosHA and pSV2neo or SRE-lacZ/neo by calcium phosphate precipitation. Thirty G418-resistant colonies were grown up from each transformation. Two lines in which more than 95% of the cells responded to serum stimulation, SRE-FosHA and SRE-lacZ, were selected for analysis. Cells were passaged in DME/10% FCS/0.3 mg/ml G418. Unless otherwise stated, cells were transferred to DME/0.5% serum for 36 hr before injection and/or stimulation with FCS, 10% (v/v); LPA, 10 μM; TPA, 25 ng/ml; UV, 50 J/m^2; anisomycin, 50 ng/ml; SB203580, 10 μM; PD98059, 50 μM; sodium butyrate, 10 mM; trichostatin A, 10 nM.

Microinjection and Reporter Gene Assays

Cells were grown on glass coverslips and injected using a Zeiss 5171 semiautomated machine using pulled glass capillaries (Alberts et al., 1993). For each treatment, 50–100 cells per experiment were injected. Reporter DNAs were injected at 10 μg/ml, and expression plasmid DNAs at 50 μg/ml unless otherwise stated. Assuming microinjection of 10%–50% of the nuclear volume of ~0.5 fl, we estimate that 10 μg/ml corresponds to 200–1000 copies per nucleus. Proteins were injected at the following concentrations in the needle: RhoA.V14, 1.0 mg/ml; Cdc42.V12, 0.5 mg/ml (equal GTP binding activity to RhoA.V14), JNK.KA, 3.0 mg/ml; PAK.N, 3.0 mg/ml; PKN.N, 3.0 mg/ml; C3 transferase, 50 μg/ml; rabbit or guinea pig marker immunoglobulins 5 mg/ml. Reporter gene expression analysis by indirect immunofluorescence was with polyclonal pan-Fos, 9E10 monoclonal, and for HA, either 12CA5 monoclonal (ICRF) or Y11 polyclonal (Santa Cruz). In situ β-galactosidase assays were performed as described previously (Alberts et al., 1994).

Immunofluorescence

Staining was essentially as described previously (Alberts et al., 1994). Cells were washed and fixed in 4% (v/v) formaldehyde in PBS for 10 min at room temperature. Following extraction in 0.3% Triton X-100, cells were incubated with primary antisera in 0.5% NP-40, 1% fish gelatin, 5% whole donkey serum (Sigma) in PBS. Primary antibodies were used as follows: R47 anti-acetyllysine (Hebbes et al., 1989), 1:300; anti-acetylated H4 (Lin et al., 1989; Meistrich et al., 1992; Perry et al., 1993), 1:8000; 12CA5, 15 μg/ml; Y11, 1:100; 9E10, 10 μg/ml; Fos, 1:100. Secondary antibodies, all donkey, were either anti-rabbit/Texas red, anti-mouse/FITC, or anti-mouse/lissamine-rhodamine; marker Ig was detected with anti-guinea pig/AMCA or FITC (Jackson/Stratech, UK) diluted 1:200 in 0.1% Tween 20 in PBS.

Images were either recorded using a Zeiss Axiophot fluorescence microscope (X20 or X40; 1.4 NA) with Kodak TMAX film (ASA 400) or captured with similar objectives using a Nikon Microphot-FX

microscope and a model C5310–11 CCD-camera (Hamamatsu Photonics, UK). Captured images were processed in PICT format using Adobe Photoshop.

Histone Preparation and Immunoblotting

Histone extracts were prepared by acid extraction with 0.25 M HCl, extracted into phenol, and recovered by acetone precipitation as described (Nicolas, 1990). Proteins were dissolved in 8 M urea, 5% acetic acid, 5% β-mercaptoethanol, fractionated on 15% acrylamide/5% acetic acid/4 M urea gels, and transferred to Immobilon-P in 0.1% acetic acid/10% methanol overnight at 40 V. Membranes were incubated with R47 (1:1000), anti-acetylated H4 (1:1000), or anti-acetylated H3 (1:7000) in PBS, 5% nonfat-dried milk, 0.1% Tween 20 at room temperature, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (DAKO), and histones detected by chemiluminescence (ECL; Amersham, UK).

Chromatin Immunoprecipitation

The method of Braunstein and coworkers (Braunstein et al., 1993) was adapted as follows. Cells from a 9 cm dish (5×10^5 cells) were cross-linked by addition of 1% HCHO to the medium for 10 min. Crude nuclei prepared by hypotonic lysis were resuspended in 100 μl SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), sonicated under conditions that reduced DNA length to between 200 and 1000 base pairs, and debris removed by centrifugation. The chromatin solution was diluted 10-fold in IP buffer (Braunstein et al., 1993) and precleared for 45′ at 4°C on protein A beads preadsorbed with sonicated single-stranded DNA. The chromatin solution was then incubated with anti-AcH4 antibody (0.2 μl; Lin et al., 1989) for 2 hr at 4°C, and immune complexes collected with protein A beads preadsorbed with sonicated single-stranded DNA. Following washes and elution (Braunstein et al., 1993), cross-links were reversed by heating at 65°C for 4–5 hr; DNA was recovered by phenol extraction and ethanol precipitation. Specific sequences in the immunoprecipitates were detected by PCR under conditions in which product yield was dependent on input DNA dose, using primers specific for the SRE-lacZ or SRE-FosHA reporter genes. The basal level of acetylated H4 necessitated immunoprecipitation using subsaturating levels of antibody.

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