Activation of Serum Response Factor by RhoA Is Mediated by the Nuclear Factor-κB and C/EBP Transcription Factors*

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The activity of the transcription factor NF-κB can be modulated by members of the Rho family of small GTPases (Perona, R., Montaner, S., Saniger, L., Sánchez-Pérez, I., Bravo, R., and Lacal, J. C. (1997) Genes Dev. 11, 463–475). Ectopic expression of RhoA, Rac1, and Cdc42Hs proteins induces the translocation of NF-κB dimers to the nucleus, triggering the transactivation of the NF-κB-dependent promoter from the human immunodeficiency virus. Here, we demonstrate that activation of NF-κB by RhoA does not exclusively promote its nuclear translocation and binding to the specific κB sequences. NF-κB is also involved in the regulation of the transcriptional activity of the c-fos serum response factor (SRF), since the activation of a SRE-dependent promoter by RhoA can be efficiently interfered by the double mutant IxBoS32A/S36A, an inhibitor of the NF-κB activity. We also present evidence that RapA and p50 NF-κB subunits cooperate with the transcription factor C/EBPβ in the transactivation of the 4 × SRE-CAT reporter. Furthermore, RhoA increases the levels of C/EBPβ protein, facilitating the functional cooperation between NF-κB, C/EBPβ, and SRF proteins. These results strengthen the pivotal importance of the Rho family of small GTPases in signal transduction pathways which modulate gene expression and reveal that NF-κB and C/EBPβ transcription factors are accessory proteins for the RhoA-linked regulation of the activity of the SRF.

Gene expression is regulated by the interplay of different transcription factors which bind to specific DNA recognition motifs and cooperate with the basal machinery to initiate transcription. During the last few years, an emerging body of evidence is revealing the importance of crossed interactions between members of distinct families of transcription factors to form higher complexes, enabling the accurate regulation of this process. The serum response element (SRE)† is a specific DNA sequence which is found in the promoter of several immediate-early genes (2). The prototypic c-fos SRE binds a ternary complex composed of a homodimer of p67SRF (serum response factor) and a third subunit, p62TCF (ternary complex factor), which belongs to the Ets family of accessory proteins. These TCF factors have the ability to bind a purine-rich motif 5’ to the SRF-binding site, known as the Ets recognition domain, only when SRF is bound to DNA, and include Elk-1, SAP-1, and SAP-2/ERP/NET proteins. The formation of this SRE binding-ternary complex requires the conserved B-box motif of the Ets subunits and sequences located in the core domain of the SRF protein (coreSRF), which is a region that is also responsible of its DNA binding and dimerization capabilities (2–8).

The SRE has shown to be necessary and sufficient for the rapid induction of the c-fos proto-oncogene in response to different external stimuli such as serum, growth factors, and phorbol esters (2, 9). Furthermore, this DNA motif is a point of convergence of different signal transduction cascades activated by an extensive range of agonists. The regulation of the activity of the SRE is mediated by two different signaling pathways. The first mechanism is elicited by the multiple phosphorylation of TCFs within their C-terminal transactivation domain. Such phosphorylation can be triggered by distinct families of mitogen-activated protein kinases. Actually, whereas the classical Ras-Raf-MEK-ERK cascade is responsible of the phosphorylation of TCFs proteins after growth factors or phorbol esters stimulation (2, 10–14), JNK/SAPK and p38 have also been shown to phosphorylate TCFs in response to certain cytokines and environmental stress conditions (15–17).

The second signaling pathway which controls an efficient transcriptional activation through the SRE site is mediated by the SRF. Hill et al. (18) showed that this TCF-independent regulation is modulated by members of the Rho family of small GTPases, RhoA, Rac1, and Cdc42Hs. Indeed, stimulation of the transcriptional activity of the SRF induced by serum, lysophosphatidic acid (LPA), and AlF4− (an activator of heterotrimeric G proteins) is signaled by RhoA in NIH 3T3 cells, since the expression of the C3 component of the Clostridium botulinum toxin efficiently blocks this effect. Fromm et al. (19) have also found that the Ga12 subfamily of heterotrimeric G protein α subunits is able to induce the SRE activity by a RhoA-dependent pathway. On the other hand, Rac1 GTPase plays an essential role in the activation of the c-fos SRE induced by certain agents such as the epidermal growth factor and hydrogen peroxide (20–21). Whereas Hill et al. (18) have described that Rac1 and Cdc42Hs GTPases can activate the SRF in a RhoA-independent manner, other authors have found a link between these signaling cascades (22).

The precise mechanism by which Rho proteins can modulate the transcriptional activation through the c-fos SRE is ill defined. It has been proposed that this regulation may be targeted by a second, unknown accessory protein, distinct from TCFs, which could interact with the DNA-bound p67SRF (3, 18, 23, 24). As other results suggest that the SRF contains differ-
ent sequences which can inhibit its own transactivation capa-
(bility (25), it is possible that such interaction could relieve the
transactivation domain from this inhibitory effect. And, more-
over, this putative recognition factor should be a critical target for
Rho family-mediated signal transduction pathways.

Our group has recently demonstrated that the Rho family of
small GTPases can efficiently induce the transactivation activity
of the nuclear factor-κB (NF-κB) (1, 26). The NF-κB complex
is mainly composed of two subunits of 50 and 65 kDa which are
retained in the cytoplasm by a third protein, IκB. These IκB
inhibitory proteins block the ability of the dimer to translocate to
the nucleus and activate gene expression (27–29). This trans-
scription factor has shown to play a relevant role in the control of
cell growth and apoptosis, along with different aspects of the
immune and inflammatory responses (30–41). RhoA, Rac1, and
Cdc42Hs are able to trigger the transactivation of the NF-κB
dependent-HIV promoter in different cell lines. The mecha-
nism involved is the conventional nuclear translocation of
RelA/p50 and p50/p50 dimers, by a mechanism that involves
the phosphorylation and proteolytic degradation of the inhibi-
tory subunit IκBα. Moreover, this activation is independent of
that induced by H-Ras/Raf cascade (1, 26).

Different interactions of the NF-κB dimers with other fam-
ilies of transcription factors have been widely reported (re-
viewed in Ref. 27). It has been described that RelA can act as an
accessory protein for the SRF and a physical association be-
 tween both subunits have been demonstrated in vitro (42). The
formation of this complex seems to be mediated through the Rel
homology domain of RelA and the DNA-binding domain of SRF,
which has shown to exert a negative effect on its transactiva-
tion ability. Furthermore, RelA functionally synergizes with
SRF in the transactivation of a reporter construct dependent
only on the SRE site, indicating that the direct or facilitated
interaction between both proteins may neutralize the inhibi-
tory functions of the core domain of SRF.

In the present study, we have investigated the implications of
RhoA-dependent activation of NF-κB in the regulation of
p67SFF function. We demonstrate that NF-κB modulates the
transcriptional activity of the SRF induced by this GTPase.
Furthermore, this mechanism may also involve a cross-talking of
RelA and p50 NF-κB subunits with the transcription factor
C/EBPβ, which is also able to bind to the SRF and regulate its
transactivation activity (43). Therefore, members of the NF-κB
and C/EBP families of transcription factors can interact and
behave as accessory proteins in the modulation of the tran-
scriptional activity of the SRF induced by the small GTPase
RhoA.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections—**Simian COS-7 fibroblast-like cells
were cultured in Dulbecco’s modified Eagle’s medium (DMEM)
supplemented with 10% fetal bovine serum and 1 μg/mL glutamine. Murine
NIH 3T3 fibroblasts were grown in DMEM with 10% newborn calf
serum. For transient expression assays, cells were transfected in
NIH 3T3 fibroblasts were grown in DMEM with 10% newborn calf

Gene Expression Analysis—Analysis of the NF-κB activity was per-
formed by transfection of the reporter containing the wild-type κB sites
of the HIV enhancer/promoter. (−453/−80) HIV-LUC contains the NF-
κB sites (46, 47). The promoters of the different reporters
contain the following specific motifs: 4 × SRE-CAT, (4X) (AGGATGTC-
CATATTAGGACATCT) the sequence of the SRE-binding site of the
human c-fos gene 5′ from a minimum promoter harboring the TATA box
(54); SRE-MUT-CAT promoter contains the mutations (AGGATGTC-
CATATTAGGACATCT) that prevents binding of SRE-dependent trans-
cription factor NF-κB to the SRE-binding site of the human c-fos gene 5′ from a minimum promoter containing the
TATA box, and 3D.A.CAT, a minimal κB promoter SRE with a TCF-
defective binding site inserted 5′ to a Xenoys C-actin TATA box (18).

**RESULTS**

**Rho Proteins Activate Transcription through κB- and SRE-
-binding Sites in COS-7 Cells—**Rho proteins are able to activate the
transcription factor NF-κB in diverse cell systems (1) and the serum response factors in NIH 3T3 fibroblasts (18). We
aimed to investigate whether activation of NF-κB by Rho pro-
teins is functionally related to the regulation of the SRE-de-
pendent transcriptional activity. We first identified a suitable
cell system to carry out both assays, NF-κB activation and
SRE-dependent transcription, under similar conditions. The
NF-κB-dependent (−453/−80) HIV-luciferase (HIV-LUC) plas-
mid was used (47) as a reporter for NF-κB activity. This
reporter contains two κB-binding sites within its enhancer re-

**Activation of SRF by NF-κB and c/EBPβ**
gion, which are mostly responsible for the transactivation of the HIV LTR. Fibroblast-like COS-7 cells were co-transfected with the cDNAs encoding for the constitutively activated forms of RhoA, Rac1, and Cdc42Hs (QL) proteins along with the HIV-LUC plasmid. As shown in Fig. 1A, an efficient transactivation of the HIV promoter could be readily observed. This effect was dependent on the NF-κB binding sites since the activation was completely avoided when a HIV-LUC reporter containing 3-base pair substitutions in each κB motif was used (data not shown). Under similar conditions, Rho proteins were also able to induce the transactivation of a 4×SRE-CAT reporter in the same cell line (Fig. 1B). The 4×SRE-CAT plasmid contains the CAT gene under the control of a minimum promoter composed of four copies of the sequence of the SRE of the human c-fos gene along with a TATA box (54). Similar results were obtained when both constitutively activated forms of two Rho family related exchange factors, Vav and Ost (55), were overexpressed (Fig. 1B). Although there is a clearly positive and reproducible response with a 3–4-fold induction by serum, this is reduced compared with other cell systems. We also were able to demonstrate a more than 10-fold activation by serum when the NIH 3T3 cell system was used instead of the COS-7 cell system using the same reporter vectors (data not shown). These results demonstrate that three members of the Rho family of GTPases mediate in the signal transduction pathways implicated in the activation of the transcription factor NF-κB and in the up-regulation of intracellular cascades which promote gene expression through the SRE-binding site in the simian fibroblast-like COS-7 cells.

Artificious regulation of the SRE-driven transcription due to the characteristic episomal replication of a transfection system based on COS-7 cells could affect the results. In order to eliminate this possibility we also investigated whether SRE-dependent transcription could be stimulated by Rho proteins in cells stably transfected with a plasmid carrying the SRE-binding site in a non-episomal vector. NIH 3T3 cells were stably transfected with the pSV2Neo vector along with the SRE-β-gal plasmid and selected for G418 resistance. Over 200 different resistant colonies obtained by serial dilution were isolated and tested for β-galactosidase activity after serum stimulation. Four of them that scored close to 100% staining when stimulated by serum were selected for further analysis. Cells were transiently transfected with the pCDNAIII-B derived expression vectors encoding for the constitutively activated forms of RhoA, Rac1, and Cdc42Hs GTPases and cells expressing the β-galactosidase were scored as positive. Efficiency of transfection for each plasmid preparation was normalized by parallel transfections using the β-galactosidase gene placed under control of the CMV promoter, allowing for constitutive expression of the β-galactosidase enzyme. As shown in Fig. 1C, the three GTPases were able to promote transactivation through the chromosomal SRE site in one of the selected clones by at least 2-fold. Cdc42Hs was much more efficient, suggesting additional signaling pathways activated by Cdc42 to those activated by RhoA and Rac1, in agreement with the results reported by Alberts et al. (56). Similar results were observed when the other three independent clones were used (data not shown). Thus, these results provide a strong support to the implication of Rho GTPases in signal transduction pathways which determine the activation of transcription due to the SRE sequences under physiological conditions and establishes that the COS-7 cell system is appropriate to carry out the proposed experiments.

NF-κB Activity Is Required for Transcriptional Activation of the SRE by RhoA—It has been previously reported that the different Rel/NF-κB proteins are able to interact physically and functionally with members of other families of transcription factors such as AP1, Sp1, Stat6, ATF, HMG/Y, the glucocorticoid receptor, TBP, TFIIIB, and C/EBP (57–66). Cross-talking between subunits of different families of transcription factors seems to be a relevant general event in the regulation of gene expression. In particular, previous studies suggested that NF-κB might also participate in the regulation of the transcriptional activity of the serum response factor (p67SRF), and a physical interaction between RelA and this protein had been observed in vitro (42, 67, 68). Thus, we explored whether the activation of NF-κB by Rho proteins could play any role in the cascades controlling transcription through the SRE-binding site.

For that purpose, an inhibitor of the NF-κB activity, the double mutant IκBαS32A/S36A was used. IκBα is a member of the IκB family, having the ability to retain the NF-κB complexes in the cytoplasm in their inactive state. The mutation in the residues Ser32 and Ser36 avoids the inducible proteolytic degradation of the protein in response to external stimuli (52, 69–73). As previously reported, activation of NF-κB by RhoA, Rac1, and Cdc42Hs GTPases is efficiently blocked by the overexpression of IκBαS32A/S36A (1). Simian COS-7 cells were co-transfected with the 4×SRE-CAT reporter and the expression vector corresponding to the constitutively activated RhoA along with increasing doses of the cDNA of the double mutant IκBαS32A/S36A. As shown in Fig. 2A, inhibition of the NF-κB activity efficiently interfered in the transcriptional activation of the SRE-dependent promoter induced by RhoA, in a dose-dependent manner. A similar assay coexpressing the activated version of each GTPase (RhoA QL, Rac1 QL, or Cdc42Hs QL) along with the IκBαS32A/S36A protein was then performed. While the transactivation of the SRE-dependent promoter induced by RhoA could be inhibited by IκBαS32A/S36A, it had no effect on the activation of the SRE due to Rac1 or Cdc42Hs overexpression (Fig. 2B). However, activation of the 4×SRE-CAT reporter by serum was not affected by the overexpression of the IκBαS32A/S36A mutant, an indication of a rather specific effect. Thus, the requirement of the NF-κB activity for the SRE transcriptional activation can be by-passed by alternative signaling pathways also promoted by Rac1 and Cdc42Hs.

We also explored whether the activation of the 4×SRE-CAT reporter by specific Rho-related exchange factors was affected by the expression of IκBαS32A/S36A. Ost and Vav proteins are two members of the Dbl family of specific exchange factors for the Rho family of small GTPases. In addition to the in vitro guanine nucleotide exchange activity exhibited by these proteins, several in vivo approaches have allowed to adscribe to these molecules certain specificity for the different Rho GTPases (reviewed in Ref. 74). In our assays, the Vav oncogene is able to activate the transcription factor NF-κB in a signaling pathway which involves Rac1 GTPase, but not RhoA or Cdc42Hs. On the contrary, activation of the HIV-LUC reporter due to Ost overexpression is efficiently inhibited by RhoA-Asn19 and Cdc42Hs-Asn17 dominant negative mutants, showing that RhoA and Cdc42, but not Rac1, mediate in the activation of NF-κB induced by the Ost exchange factor (26).

Next, COS-7 cells were co-transfected with the corresponding cDNAs of the constitutively activated Vav or Ost genes, along with the pRecCMV-IκBαS32A/S36A plasmid and the 4×SRE-CAT reporter. In agreement with the results described above, activation of the SRE due to Vav overexpression was not significantly inhibited by the expression of the IκBαS32A/S36A protein, showing that Rac1 activates transcription through the SRE-binding site by additional pathways (Fig. 2C). However, this inhibitor was able to block the SRE-dependent transcription induced by the Ost oncogene, suggesting the activation of complementary signals that make Ost dependent on
RhoA but not Cdc42 for the activation of SRF. From these experiments, we can conclude that activation of the SRE-binding site by RhoA critically involves a NF-κB-dependent cascade.

Expression of the IxBoS32A/S36A Mutant Blocks the Activation of SRE-dependent Transcription by Lysophosphatidic Acid—It has been described that activation of the SRE elicited by several extracellular agonists may be signaled by different Rho GTPases. Indeed, expression of the C3 component of the C. botulinum toxin is able to inhibit the transcriptional activity of the SRF induced by serum, LPA, and AIF4 (an activator of heterotrimeric G proteins), indicating that these stimuli activate SRF by RhoA-dependent pathways (18). It is also reported that this GTPase is involved in the endothelin-1-induced nuclear signaling to the c-fos SRE (75). On the other hand, the dominant negative mutant Rac1-Asn17 blocks the activation through this binding site in response to EGF and hydrogen peroxide (20, 21).

Since transactivation of the 4 × SRE-CAT induced by RhoA seemed to be dependent on the NF-κB activity (Fig. 2A), we investigated whether the expression of the IxBoS32A/S36A mutant had any effect in the activation of the SRE by LPA, a physiological activator of the RhoA signaling cascade. To that end, COS-7 cells were co-transfected with the pRcCMV or pRcCMV-IxBoS32A/S36A plasmid along with the (~453/~80) HIV-LUC or the 4 × SRE-CAT reporter. After removal of the precipitate, cells were treated with LPA for 5 h and total extracts were assayed for luciferase activity (NF-κB assay) or CAT activity (SRE assay). We observed that this physiological stimulant was able to efficiently induce the transactivation of both promoters in these cells (Fig. 3). Overexpression of the IxBoS32A/S36A mutant inhibited the transactivation of both NF-κB- and SRE-dependent reporters by LPA. These results indicate that activation of NF-κB is a relevant event in the physiological regulation of the SRE transcriptional activity induced by a RhoA-dependent extracellular stimulus.

RelA and p50 Subunits Cooperate with RhoA in the Transactivation through the SRE-biding Site—The mechanism by which NF-κB affected the transactivation through the SRE was then investigated. We had previously described that both RelA/p50 and p50/p50 dimers translocate to the nucleus due to RhoA overexpression (1). Thus, we explored whether these subunits could cooperate with RhoA in the activation of the SRE-depend-ent reporter. To that end, we used the corresponding expression vectors of the RelA and p50 transcription factors. Overexpression of both proteins was achieved by transient transfection of the corresponding plasmids into COS-7 cells (Fig. 4A). Both subunits were able to potentiate the transactivation of the HIV-LUC reporter induced by RhoA (Fig. 4B).

COS-7 cells were co-transfected with a suboptimal dose of the expression vector corresponding to RhoA QL and increasing concentrations of the cDNA of the RelA or p50 protein, along with the 4 × SRE-CAT plasmid. Expression of the RelA subunit along with the constitutively activated RhoA GTPase cooperated in the transactivation of the SRE-dependent reporter (Fig. 4C). Franzoso et al. (42) have previously shown that RelA but not p50 and p52 subunits bind to RhoA-activated SRF.

Fig. 1. Rho proteins activate transcription through the κB and the SRE binding motifs in COS-7 cells. A, Rho proteins induce the transcriptional activation of the HIV-LUC reporter. COS-7 cells were co-transfected with 0.5 μg of (~453/~80) HIV-LUC and 1 μg of pCMV-β-gal per 60-mm plate along with 3 μg of the plasmid pCDNAIII or the derived vectors expressing RhoA QL, Rac1 QL, or Cdc42 QL. Cells transfected with pCDNAIII were stimulated with TNFα (10 ng/ml) (vector + TNFα) 5 h before harvesting. Luciferase activity was determined 24 h after transfection. Data represent the mean of a single experiment performed in triplicate ± S.D. Results are expressed as fold induction considering as 1 the CAT activity of the cells transfected with the empty vector. Same results were observed in three independent experiments. B, Rho proteins induce the transcriptional activation of the 4 × SRE-CAT reporter. COS-7 cells were co-transfected with 3 μg of the plasmid pCDNAIII or the derived vectors expressing RhoA QL, Rac1 QL, or Cdc42 QL, pMEX or pMEX-vav, pCEV27, or pCEV27-lost. Cells transfected with pCDNAIII were stimulated with 20% fetal bovine serum (vector + 20% FBS) 5 h before harvesting. CAT activity was determined 24 h after transfection. Data represent the mean of a single experiment performed in triplicate ± S.D. Results are expressed as fold induction considering as 1 the CAT activity of the cells transfected with the empty vector. Same results were observed in three independent experiments. C, Rho proteins induce the β-galactosidase activity of SRE-β-gal stable transfectants. SRE-β-gal derived NIH 3T3 cells were transfected with 3 μg of the plasmid pCDNAIII or the derived vectors expressing RhoA QL, Rac1 QL, or Cdc42 QL and starved for serum for 48 h. Cells transfected with pCDNAIII were stimulated with 20% fetal bovine serum (vector + 20% FBS) 5 h before harvesting. β-Galactosidase activity was determined 48 h after transfection. Data represent the mean of a single experiment performed in triplicate ± S.D. Results are expressed as fold induction considering as 1 the β-galactosidase activity of the cells transfected with the corresponding empty vector. Similar data were observed in other three SRE-β-gal derived clones. Three independent experiments were performed for each derived clone with similar results.
FIG. 2. RhoA-induced transcriptional activation through the SRE site depends on NF-κB activity. A, dose-dependent inhibition of the RhoA-induced transcriptional activation of the 4 × SRE-CAT reporter by IpBoS32A/S36A. COS-7 cells were co-transfected with 3 μg of pCMV-β-gal and 2 μg of pRCMV or pReCMV-IpBoS32A/S36A along with either 0.5 μg of (−453/+30) HIV-LUC or 3 μg of 4 × SRE-CAT, per 60-mm plate. Cells were stimulated with (10 or 30 μM) LPA 5 h before harvesting. Luciferase and CAT activities were determined 24 h after transfection. Data represent the mean of a single experiment performed in triplicate ± S.D. Results are expressed as fold induction considering as 1 the activity of the cells transfected with the empty vector. Same results were observed in three independent experiments.

FIG. 3. LPA-induced transcriptional activation through the κB and SRE-binding sites is inhibited by the IpBoS32A/S36A mutant. COS-7 cells were co-transfected with 1 μg of pCMV-β-gal and 2 μg of pRCMV or pReCMV-IpBoS32A/S36A along with either 0.5 μg of (−453/+30) HIV-LUC or 3 μg of 4 × SRE-CAT, per 60-mm plate. Cells were stimulated with (10 or 30 μM) LPA 5 h before harvesting. Luciferase and CAT activities were determined 24 h after transfection. Data represent the mean of a single experiment performed in triplicate ± S.D. Results are expressed as percentage of maximal induction related to the stimulation obtained with each plasmid alone. Similar results were observed in three independent experiments.
scription factors are able to synergize efficiently in the transcriptional regulation of certain promoters and, particularly, physical interactions between C/EBPβ and RelA or p50 have been described (57, 68, 80–84). All these data and the results reported by Stein et al. (59) that suggested an increase of gene expression mediated by c-fos SRE in the presence of C/EBP and NF-κB transcription factors, prompted us to further investigate whether such cooperation was involved in the mechanism induced by RhoA regarding SRE activation.

Transcriptional activity assays were carried out co-transfecting the expression vector encoding for the constitutively activated form of RhoA along with the cDNAs of the RelA and the C/EBPβ proteins and the 4 × SRE-CAT reporter, which contains in its core the C/EBP-binding site (Fig. 5A). A cooperative effect in the promoter transactivation was observed when the RhoA GTPase was coexpressed with C/EBPβ alone. Moreover, the combined expression of RhoA, RelA, and C/EBPβ proteins potentiated further the transcriptional activation through the SRE-binding site. By contrast, the same promoter carrying a mutated SRE site but an intact C/EBPβ site was not functional after expression of the RelA or C/EBPβ proteins (Fig. 5B), an indication that both RelA and C/EBPβ were unable to stimulate transcription by themselves and require a functional SRE site. Similar results were obtained when RhoA and C/EBPβ were coexpressed along with the p50 subunit (Fig. 5C and data not shown). Thus, activation of RhoA triggers the translocation of NF-κB dimers to the nucleus, promoting their binding to the κB-specific sequences and also enabling the cooperation with the C/EBPβ protein in the transcriptional regulation of the SRE site in an SRF-dependent manner. Therefore, cross-talking between NF-κB and C/EBPβ transcription factors is an intracellular event regulated by a RhoA-dependent signal transduction pathway. These results also suggest that NF-κB and C/EBPβ can act as accessory factors for SRE-dependent transcription, which could liberate the SRF from its own inhibited transcriptional capability. On the contrary, a cooperation among H-Ras, NF-κB, and C/EBPβ was not observed (data not shown). Thus, the latter transcription factors may play a role in conjunction with the SRF in the TCF-independent pathway for the transcriptional activation through the SRE.

Inhibition of the RhoA-dependent Activation of the SRE-binding Site by a Specific Inhibitor of the C/EBP Family of Transcription Factors—The C/EBPβ gene encodes for three in-frame methionines which can potentially give rise to three translation products of 38, 35, and 20 kDa (rat or murine genes), p38 and p35 (LAP) are active proteins, while p20C/EBPβ (also known as LIP) behaves as a competitive inhibitor of transcription because it lacks the N-terminal transactivation domain (48). Sealy et al. (43) have reported that both p35 and p20 homodimers as well as p35/p20 heterodimers contribute to the SRE complex at the c-fos promoter in NIH 3T3 cells, while overexpression of p20C/EBPβ (LIP) is able to inhibit its activation by serum stimulation. In order to corroborate that C/EBPβ was implicated in the regulation of SRF by RhoA, we used LIP as a specific repressor of the activity of this transcription factor.

COS-7 cells were co-transfected with the 4 × SRE-CAT reporter and the expression vector corresponding to the constitutively activated RhoA, along with the cDNA for LIP (Fig. 6A).

with 3 μg of 4 × SRE-CAT, 1 μg of pCMV-β-gal, 1 μg of pCDNAIIIB or pCDNAIIIB-rhoA QL, along with 0.1 or 0.5 μg of pMEX, pMEX-RelA (C), or pMEX-p50 (D) per 60-mm plate. CAT activity was determined 24 h after transfection. Data represent the mean of a single experiment performed in triplicate ± S.D. Results are expressed as fold induction over the CAT activity obtained with the corresponding empty vector. Same results were observed in three independent experiments.

![Diagram](image-url)
Expression of such a specific inhibitor was able to block the transactivation of the SRE-dependent promoter due to either LPA stimulation or RhoA overexpression. Furthermore, the RhoA-dependent activation of the SRE binding motif inhibited by LIP was fully restored by co-transfection of the full-length active C/EBPβ. These results suggest that RhoA regulates the SRE through a signaling cascade which also involves the transcription factor C/EBPβ.

RhoA Affects the Levels of the C/EBPβ Protein—The above results indicate that RhoA is able to modulate the SRE-dependent transcription by a mechanism that depends upon the function of C/EBPβ. These results suggest that RhoA regulates the SRE through a signaling cascade which also involves the transcription factor C/EBPβ.

RhoA Affects the Levels of the C/EBPβ Protein—The above results indicate that RhoA is able to modulate the SRE-dependent transcription by a mechanism that depends upon the function of C/EBPβ. The activity of C/EBPβ is controlled at the transcriptional level, by direct activation of its expression (85–87) or by post-translational modifications (76, 88–90). When RhoA (QL) was transiently overexpressed in NIH 3T3 fibroblasts, an increase of the level of the C/EBPβ protein was observed (Fig. 6B). Under similar conditions, treatment with forskolin, an activator of PKA, also induced a time-dependent increase in the levels of the C/EBPβ protein, as described previously (87). Although we cannot exclude the possibility that RhoA could also affect C/EBPβ function by other mechanisms such as post-translational modifications, these results strongly support the evidence, provided in this study, of the regulation of the SRE through the participation of NF-κB, C/EBPβ, and SRF proteins.
DISCUSSION

Rho proteins regulate critical biological processes such as cell growth, transformation and metastasis, apoptosis, response to stress, and certain aspects of development (44, 45, 74, 91–103). Recently, our group has demonstrated that the human proteins RhoA, Rac1, and Cdc42Hs (three prototype members of the Rho family) activate NF-κB in diverse cell types (1) by at least two alternative pathways (26). Hill et al. (18) have recently demonstrated that in NIH 3T3 cells, the serum response factor (p67SRF) is regulated by Rho-dependent signaling pathways. This activation is achieved by mechanisms independent of the signal transduction cascades related to TCFs. Although it is not clear how Rho proteins modulate the transcriptional activation of the SRE, it has been suggested that this mechanism is dependent on a second accessory protein targeted by the Rho family of GTPases. Such a molecule could associate with the DNA-bound SRF and relieve the transactivation domain of the SRF from its own inhibitory regulation (3, 18, 23–25). In this study, we have explored this signaling pathway and found that the transcriptional activation through the SRE induced by RhoA, not Rac1 or Cdc42Hs, is dependent on the activity of the transcription factor NF-κB.

Three prototype members of the Rho family, RhoA, Rac1, and Cdc42Hs, induce the transcriptional activation of both NF-κB and a SRE-dependent promoter in the simian COS-7 system. NIH 3T3-derived clones stably transfected with a SRE-β-gal plasmid were used to demonstrate that RhoA, Rac1, and Cdc42Hs induce the transcriptional activation through a chromosomal SRE motif. Alberts et al. (56) have recently reported that RhoA, Rac1, and Cdc42 regulate transcriptional activation by SRF (18). At least RhoA and Cdc42 require also H4 hyperacetylation as an additional signal for the activation of SRF-regulated chromosomal templates. Furthermore, while RhoA does not provide by itself the cooperating signals required for the induction of H4 hyperacetylation, Cdc42 does. The partial discrepancies in the results of Alberts et al. (56) and ours may be due to differences in the sensitivity of the methodologies used, microinjection versus transient transfections. However, both reports are consistent with the fact that Cdc42 is more efficient than RhoA for the activation of SRF-regulated chromosomal templates, although the basis for this difference is currently unknown.

Previous reports showed that NF-κB could modulate the activity of p67SRF (42, 67). We investigated whether the activation of the SRF by Rho GTPases could involve a NF-κB-dependent cascade. Our results clearly demonstrate that the double mutant IκBαS32A/S36A was able to block the activation of the 4 × SRE-CAT reporter induced by RhoA. At the same time, activation of the 4 × SRE-CAT induced by the stimulation of COS-7 with LPA was also blocked by the ectopic expression of the IκBαS32A/S36A mutant. All these results demonstrate that NF-κB is critically involved in the signaling cascade triggered by RhoA to regulate SRE-dependent transcription. The cooperative effect observed when RhoA is co-expressed with the NF-κB subunits RelA or p50 also agrees with this hypothesis. Similar results were obtained with the 3D.A.CAT reporter which is lead by an SRE site defective for TCF binding, suggesting that NF-κB is involved in the regulation of the SRE by SRF. Indeed, Franzoso et al. (42) have found that RelA is able to physically interact in vitro with the DNA-binding region of SRF (coreSRF), through its Rel homology domain. Thus, NF-κB subunits could act as accessory proteins for the SRF, in order to regulate the activity of the SRE, liberating the negative effect exerted by the DNA-binding and dimerization domains, as other authors have previously suggested (18, 25, 42).

From the experiments shown here we cannot conclude whether RelA and p50 can bind to p67SRF as prototypical NF-κB dimers or as monomers, although NF-κB is supposed to bind to other transcription factors in its dimeric configuration (59). It has been described that p50 and SRF are not able to interact physically and that they do not cooperate functionally in SRE-driven transcription (42). In contrast, we have observed similar results by coexpression of RhoA along with the RelA or p50 subunits. RhoA promotes the translocation of both RelA/p50 and p50/p50 dimers to the nucleus (1). Thus, it can be proposed that RelA/p50 may be the NF-κB dimer involved in the regulation of SRF and that the physical interaction with such a factor could be mediated by the RelA subunit.

The region between residues 204 and 234 of RelA is involved in its interaction with SRF, while the dimerization between RelA and p50 is mediated by residues 222 to 231, all of them located in the Rel homology domain (42, 104). Thus, interaction of RelA with the SRF is feasible through residues 204 to 222. Furthermore, the positive effect observed on SRE-dependent transcription when p50 is overexpressed along with RhoA could be explained if the excess p50 subunit induced its dimerization with RelA and translocation to the nucleus, where it would contribute to the activation of the SRF. On the other hand, the region of p67SRF implicated in complexing with RelA overlaps the previously described domain which is responsible for neutralization of its own transcriptional activity, favoring the hypothesis that NF-κB acts as an accessory protein for SRF by affecting its functions.

Nevertheless, the IκBαS32A/S36A mutant was not able to block the activation of the 4 × SRE-CAT by Rac1 or Cdc42Hs or Vav, an exchange factor specific for Rac1 in vivo (26), suggesting that the requirement for the NF-κB activity can be bypassed through alternative mechanisms. This effect can be explained by the fact that both Rac1 and Cdc42Hs may regulate the activity of the SRE through the TCF-dependent pathway, since they activate JNK/SAPK and p38 cascades (1, 45, 97), and such kinases have been found to phosphorylate TCFs (15–17). The effects reported here were specific for the SRE site, since no competition was observed using an NF-κB site and was abolished by a mutated SRE construct.

We demonstrate that activation of the SRE by RhoA involves another factor, the C/EBPβ, which belongs to the family of CCAAT box enhancer-binding proteins, characterized by the presence of the bZIP motif (78–79). These results are in agreement with others previously described that suggest an increase of gene expression mediated by c-fos SRE in the presence of C/EBP and NF-κB transcription factors (59). These authors had observed a strong synergistic stimulation of a SRE-dependent reporter due to the joint overexpression of RelA(p65) NF-κB and C/EBPβ. Different C/EBP and NF-κB subunits are able to synergize for the transcriptional regulation of certain promoters and, particularly, physical interactions between C/EBPβ and RelA or p50, through their bZIP and Rel homology domains, respectively, have been described (57, 59, 68, 80–84, 105). It has been suggested that, at least, an additional factor could contribute to the C/EBP dimer bound neighboring to the SRE. Sealy et al. (43) have reported that approximately 50% of the C/EBP complex which is bound to 32P-labeled SRE DNA remains unaffected when competed by C/EBPβ antibodies, although they were not able to visualize any supershift when a RelA(p65)-specific antibody was used. In keeping with this, two proteins of 64 and 43 kDa have been described to associate with rNFIL-6 (C/EBPβ) by immunoprecipitation with a specific antibody, in cells stimulated with forskolin (76).

The p20/C/EBPβ (LIP) protein acts as a transdominant negative regulator of the C/EBP activity (48). It is known that the regulation of gene transcription by C/EBPβ involves a precise
balance of both activator and inhibitor forms (43, 48, 106). When p20/C/EBPβ (LIP) was transfected along with RhoA, the activation of the SRE was efficiently inhibited. Moreover, the full activity was recovered by overexpression of the full-length C/EBPβ, a demonstration of the specificity of the inhibitory effect by LIP. All these results support the idea of cooperation between NF-κB and C/EBPβ subunits in order to potentiate the transcriptional activation through the SRE, as shown schematically in Fig. 7. This cooperation is dependent on SRF binding to the SRE site since it was lost when a mutated SRE site was used and was not observed by expressing NF-κB or C/EBPβ alone or in combination, in the absence of RhoA.

We have also observed an increase in the level of the C/EBPβ protein induced by RhoA in NIH 3T3. Such an increase could be originated by the transcriptional activation of the C/EBPβ gene or the stabilization of its mRNA. From our experiments, we cannot conclude which of these mechanisms is regulated by RhoA. Regarding the transcription of the C/EBPβ gene, it is mostly controlled by two CREB sites situated close to the TATA box (87). The CREB protein is functionally regulated by phosphorylation, which can be mediated by PKA- or p38-linked kinases. The C/EBPβ gene, it is implicated in a similar process regarding the stabilization of its mRNA. From our experiments, we have demonstrated in this study for the interaction of SRF, RelA, p50, and C/EBPβ cannot be fully applied to Rac1 and Cdc42Hs, two important prototype members of the same family of Rho GTPases. Thus, each member of the Rho family of GTPases seems to serve specific signaling wirings that although controlling similar targets may exert different biological responses.

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