ERK2- and p90Rsk2-dependent Pathways Regulate the CCAAT/Enhancer-binding Protein-β Interaction with Serum Response Factor*

Mary Hanlon§§, Thomas W. Sturgill¶, and Linda Sealy‡‡

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The serum response element (SRE) of the c-fos promoter is a convergence point for mitogenic signaling pathways. Several transcription factors regulate SRE, including serum response factor (SRF), ternary complex factors, and CCAAT/enhancer-binding protein-β (C/EBPβ). C/EBPβ can interact with both SRF and the ternary complex factor family member Elk-1, but only in response to activated Ras. Transactivation of the SRE by C/EBPβ is also greatly stimulated by Ras. The Ras effectors that signal to C/EBPβ are unknown. In this report, we demonstrate that a consensus MAPK site in C/EBPβ is necessary for Ras stimulation of both C/EBPβ-SRF interaction and transactivation of the SRE by C/EBPβ. To dissect signaling pathways activated downstream of Ras, different Ras effector constructs were analyzed. We show that activated forms of Raf and phosphatidylinositol 3-kinase stimulate C/EBPβ-SRF interaction. We also show a novel selectivity for the MAPK family member ERK2, where dominant-negative ERK2, but not dominant-negative ERK1, blocks Ras stimulation of C/EBPβ-SRF interaction. In addition, recombinant C/EBPβ is phosphorylated by ERK2, but not by ERK1, in vitro. Finally, we demonstrate a requirement for p90Rsk2 in regulation of C/EBPβ-SRF interaction. These data show that multiple Ras effectors are required to regulate C/EBPβ and SRF association.

c-fos is a member of the family of immediate-early genes, and its transcription is rapidly induced in response to mitogenic signals (1). The serum response element (SRE) in the c-fos promoter is a convergence point for several signaling pathways that regulate induction of the c-fos gene (reviewed in Ref. 2). The SRE is located ~300 base pairs (bp) upstream of the transcriptional start site and is necessary for serum induction of c-fos (3). Serum response factor (SRF) is a 67-kDa protein that was found to be necessary, but not sufficient, for serum induction of the SRE (4, 5). SRF is constitutively bound to the SRE in both the absence and presence of mitogenic stimulation, as demonstrated by in vivo footprinting analysis (6). This suggests that it is a transcriptional complex of SRF and its accessory proteins that is regulated rather than regulation of SRF DNA binding.

Both Ras- and Rho-dependent signaling pathways have been shown to regulate the SRE. Ras is a member of the family of small GTPases and is a key mediator of intracellular signaling cascades (reviewed in Ref. 7), including the Raf and phosphatidylinositol 3-kinase (PI3K) pathways. The Raf pathway activates the extracellular signal-regulated kinases (ERKs), ERK1 and ERK2 (reviewed in Ref. 8). These two ERK isoforms are thought to have overlapping substrates in vivo and in vitro. There are some reports, however, suggesting that ERK1 and ERK2 may have distinct substrates in vivo. For example, the ERK1 knockout mouse has a defect in thymocyte maturation, even though ERK2 is expressed in the mutant thymocytes (9). This would suggest that ERK1 and ERK2 are not completely redundant in vivo. PI3K effectors include PI3K-dependent kinases, protein kinase B, Rac, Cdc42, p70S6K, and p90Rsk2. p90Rsk2 is an interesting effector of PI3K because there is evidence that full activation requires phosphorylation by PI3K-dependent kinase-1 as well as the ERKs (reviewed in Ref. 10). Thus, the Ras-activated signaling cascades do not function in completely linear pathways.

Extensively studied targets of the Ras pathway at the SRE are the ternary complex factors (TCFs). The TCFs are members of the Ets family of transcription factors and have a role in regulating the SRE in concert with SRF (reviewed in Ref. 11). The TCFs are inactive in the absence of mitogenic signaling, but are activated by phosphorylation of consensus mitogen-activated protein kinase (MAPK) sites in their C-terminal transactivation domains. This phosphorylation is accomplished by all three MAPK families: the ERKs, the c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases, and p38 kinase (12–14).

Rho-dependent signaling pathways have also been shown to regulate the SRE independently of the TCFs. This pathway involves the Rho family members RhoA, Rac1, and Cdc42 (15). Activation of this pathway is dependent on SRF binding to DNA through its own DNA-binding domain (16, 17). It is hypothesized that this pathway is targeting an unknown accessory factor of SRF (16).

CCAAAT/enhancer-binding protein-β (C/EBPβ) is another
Signaling Pathways Regulating the C/EBPβ-SRF Interaction

transcription factor that has a role in regulating the c-fos SRE (18, 19). Our laboratory has previously shown that the activator isoform of C/EBPβ, p35-C/EBPβ (also known as liver-enriched activator protein (LAP)), activates a SRE-driven reporter construct, whereas the repressor isoform, p20-C/EBPβ (also known as liver-enriched inhibitory protein), represses serum stimulation of this reporter (19). We have also shown that p35-C/EBPβ can interact with SRF and the TCF family member Elk-1 in vivo, but these interactions are dependent on the presence of activated Ras (20, 21). It is unknown, however, which signaling pathways act downstream of Ras to stimulate p35-C/EBPβ interaction with transcription factors at the SRE.

This report has analyzed the signaling pathways that are activated in response to Ras to stimulate the interaction of p35-C/EBPβ with SRF and subsequently stimulate transcriptional activation of the SRE. We show that a conserved MAPK site in C/EBPβ is necessary for Ras-stimulated C/EBPβ-SRF interaction as well as C/EBPβ transactivation of the SRE. Furthermore, we show that activated forms of Raf and PI3K can stimulate the C/EBPβ-SRF interaction. We also demonstrate that dominant-negative ERK2, but not ERK1, inhibits Ras stimulation of the C/EBPβ-SRF interaction. Recombinant C/EBPβ is also selectively phosphorylated in vitro by ERK2, but not by ERK1. We also show a requirement for p90 Rsk2 in regulating p35-C/EBPβ interaction. These data strongly suggest that multiple Ras effectors are regulating C/EBPβ and SRF to stimulate their interaction, resulting in enhanced transactivation of the SRE.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—NIH 3T3 fibroblasts (from American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium with 10% calf serum (Colorado Serum Co.), 2% sodium bicarbonate, 25 units/ml penicillin G sodium, and 25 mg/ml streptomycin. NIH 3T3 transfections were performed using NovoFector (Venn Nova) or Trans-IT LT1 (Pan Vera) as described previously (21). Cell extracts of equivalent protein concentration were prepared and assayed for chlamydocin acetyltransferase (CAT) reporter activity as previously described (22). For drug-treated samples, SB 202190 (10 μM) for chloramphenicol acetyltransferase (CAT) reporter activity as previously described (22). For drug-treated samples, SB 202190 (10 μM) for chloramphenicol acetyltransferase (CAT) reporter activity as previously described (22).

Immunoblotting—Whole cell extracts from NIH 3T3 cells containing equivalent protein concentration were analyzed by electrophoresis on a SDS-10% polyacrylamide gel, and gelatin incorporation was detected by autoradiography.

RESULTS

An Intact MAPK Site in C/EBPβ Is Necessary for Ras Stimulation of the C/EBPβ-SRF Interaction—Threonine-35 (numbering for the human protein) of C/EBPβ, located in a consensus MAPK phosphorylation sequence, has been shown to be phosphorylated in vivo by activated Ras and also to be phosphoryl-
Fig. 1. Mutation of Pro\textsuperscript{189} in the p35-C/EBP\beta MAPK site abolishes Ras-stimulated interaction with SRF. NIH 3T3 cells were transfected with 1 \(\mu\)g of pG5CAT either alone or with 0.5 \(\mu\)g of pGAL4-SRF, CMV-LAP (encoding p35-C/EBP\beta), or pcDNA3-p35-C/EBP\beta(P189G) as indicated in the absence and presence of 2 \(\mu\)g of pCMV-Ras.V12. Total DNA in each transfection was adjusted to 3 \(\mu\)g with CMV-4. Cells were serum-deprived for 40 h prior to harvesting, and CAT activity was measured. Data are the average of four determinations; error bars represent S.E.

Fig. 2. Mutation of Thr\textsuperscript{235} in the NFIL6 MAPK site abolishes Ras-stimulated transactivation of the SRE. NIH 3T3 cells were transfected with 0.4 \(\mu\)g of SRE-CAT either alone or with 0.4 \(\mu\)g of CMV-NFIL6 or CMV-NFIL6(T235A), as indicated, in the absence and presence of 2 \(\mu\)g of pCMV-Ras.V12. Cells were serum-deprived for 40 h prior to harvesting, and CAT activity was measured. Data are the average of three determinations; error bars represent S.E.

rectly as evidenced by DNA binding. We reason that the mutant was unable to respond to Ras in this assay due to the inability of the protein to be phosphorylated by the MAPKs.

**Thr\textsuperscript{235} of C/EBP\beta Is Necessary for Ras-stimulated Transactivation of the SRE**—Due to the fact that mutation of the MAPK site in C/EBP\beta abolishes Ras stimulation of its interaction with SRF, we extended our studies to determine if the MAPK site was also necessary for transactivation of the SRE by C/EBP\beta in response to Ras. To address this question, we used a CAT reporter gene driven by one copy of a TCF mutant SRE upstream of the Rous sarcoma virus long terminal repeat minimal promoter (19). This reporter is unable to bind the TCF family members, which are known targets of Ras. Therefore, this oblates any effect of Ras that could be signaling through the TCF family members.

As shown in Fig. 2, when the TCFmut-CAT reporter construct was transfected together with an expression construct for human C/EBP\beta (NFIL6), there was a 7-fold increase in CAT activity in the absence of Ras. When activated Ras was also cotransfected, this stimulated the CAT activity to 19-fold. This was expected since Ras greatly stimulates the interaction between SRF and C/EBP\beta, which would result in enhanced transactivation. Interestingly, the transactivation of a mutant C/EBP\beta construct containing a mutation of Thr\textsuperscript{235} in the MAPK site to alanine (NFIL6(T235A)) was not stimulated by Ras. Therefore, mutating the MAPK phosphorylation site in C/EBP\beta abolished the ability of Ras to stimulate transactivation. These data also agree with a previous study showing that Ras does not stimulate the interaction of the NFIL6(T235A) mutant with SRF in the mammalian two-hybrid assay (20). Together, these data demonstrate that C/EBP\beta Thr\textsuperscript{235} is required in mediating Ras stimulation of the C/EBP\beta interaction with SRF and transactivation of the SRE.

**Analysis of Signaling Pathways Regulating the C/EBP\beta-SRF Interaction**—We next wanted to analyze the signaling pathways that are downstream of activated Ras in stimulating the interaction between C/EBP\beta and SRF. Ras has many known effectors, but the above data suggest that Ras is stimulating an MAPK pathway to target C/EBP\beta. We tested several Ras effectors to determine if they could regulate the C/EBP\beta-SRF interaction in the mammalian two-hybrid assay.

Fig. 3A shows that when p35-C/EBP\beta was cotransfected with Gal4-SRF and the pG5CAT reporter construct into NIH 3T3 cells in the presence of an activated Ras construct, there was a large increase in CAT activity that was normalized to 100%. As
Members of the Rho family of small GTPases are down-
regulated by Ras. There was no significant increase in CAT activity of
the reporter alone or the reporter and Gal4-SRF in the pres-
ence of activated Raf or PI3K. In the presence of both PI3K and
the MAPK family members inhibits Ras stimulation of the
p35-C/EBPβ-SRF interaction and suggest that MAPKs
are working downstream of Ras to target C/EBPβ.

Dominant-negative ERK2, but Not ERK1, Blocks Ras Stimula-
tion of the C/EBPβ-SRF Interaction—Since MKP-1 inhibits
all three MAPK family members, the next step in our analysis
was to inhibit specific MAPKs to determine what effect this
would have on the interaction between SRF and p35-C/EBPβ.
We therefore tested the requirement for the three MAPK
family members in stimulation of the p35-C/EBPβ-SRF interaction by Ras.

We first tested the importance of the ERK family members
by utilizing dominant-negative ERK1 and ERK2 constructs in the
two-hybrid assay. When the pG5CAT reporter was trans-
ferred with Gal4-SRF and p35-C/EBPβ into NIH 3T3 cells, there
was a large stimulation in CAT activity in the presence of
activated Ras to 47-fold over basal levels (Fig. 5A). When
expression vector encoding a kinase-inactive and dominant-
negative (DN) ERK1 was cotransfected with the above
constructs, there was no significant inhibition by the DN ERK1
construct. Therefore, we did not observe an inhibition by DN
ERK1, as we observed with MKP-1 shown above (Fig. 4). Inter-
estingly, when DN ERK2 (32) was cotransfected with Gal4-

SIGNIFICANT PATHWAYS REGULATING THE C/EBPβ-SRF
INTERACTION

To determine if the MAPKs play a role in this Ras stimula-
tion, we utilized an MKP-1 construct that can inactivate all
three families of MAPKs (25). We tested this construct's ability
to inhibit Ras stimulation of the interaction between SRF and
p35-C/EBPβ. When MKP-1 was transfected with Gal4-SRF,
p35-C/EBPβ, and activated Ras, MKP-1 blocked Ras stimula-
tion from 95- to 18-fold (Fig. 4). These data show that inacti-

shown to be independent of the TCFs (15). These data show
that p35-C/EBPβ is also not a target of the TCF-independent
Rho pathway.

MKP-1 Inhibits Ras Stimulation of the C/EBPβ-SRF Inter-
action—Because the Ras signaling pathway appears to be
working through an MAPK cascade, we tested whether inhibi-
tion of the MAPKs would inhibit Ras stimulation of the
C/EBPβ-SRF interaction. To address this question, the mam-
alian two-hybrid assay was again used. As shown in Fig. 4,
cotransfection of p35-C/EBPβ, Gal4-SRF, and the Gal4 re-
porter construct resulted in an average 3-fold increase in CAT
activity in the absence of Ras, and Ras stimulated the C/EBPβ-
SRF interaction to 95-fold over basal levels.

Domino-negative ERK2, but Not ERK1, Blocks Ras Stimula-
tion of the C/EBPβ-SRF Interaction—Since MKP-1 inhibits
all three MAPK family members, the next step in our analysis
was to inhibit specific MAPKs to determine what effect this
would have on the interaction between SRF and p35-C/EBPβ.
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was a large stimulation in CAT activity in the presence of
activated Ras to 47-fold over basal levels (Fig. 5A). When
an expression vector encoding a kinase-inactive and dom-
inant-negative (DN) ERK1 (32) was cotransfected with the above
constructs, there was no significant inhibition by the DN ERK1
construct. Therefore, we did not observe an inhibition by DN
ERK1, as we observed with MKP-1 shown above (Fig. 4). Inter-
estingly, when DN ERK2 (32) was cotransfected with Gal4-

also shown in Fig. 3A, activated constructs of Raf (Raf-CAX)
and PI3K (p110-CAAX), two known Ras effectors, stimulated
the interaction of the two proteins. Raf was a stronger activa-
tor, stimulating to ~70% the level of Ras. PI3K also stimulated
the interaction of the proteins, to ~35% of the level observed
with Ras. There was no significant increase in CAT activity of
the reporter alone or the reporter and Gal4-SRF in the pres-
ence of activated Ras or PI3K. In the presence of both PI3K and
Raf, there was an additive effect on the stimulation over adding
either effector alone. This suggests that the Raf and PI3K
effectors are activating distinct pathways to stimulate the in-
teraction between SRF and C/EBPβ.

Members of the Rho family of small GTPases are down-
stream of PI3K, and the SRE is a target of RhoA-, Cdc42-, and
Rac1-dependent signaling cascades (15). Stimulation of SRE
transactivation by the Rho family has been shown to be SRF-
dependent, possibly targeting an unknown accessory factor
(15–17). We next tested members of the Rho family to deter-
mine if they regulated the interaction between SRF and p35-
C/EBPβ in the two-hybrid assay (Fig. 3B). Activated forms of
RhoA (RhoA.V14), Cdc42 (Cdc42.V12), and Rac1 (Rac1(QL))
had no stimulatory effect on the p35-C/EBPβ-SRF interaction.
Likewise, dominant-negative Rac1 (Rac1.N17) did not inhibit
Ras stimulation. Rho family signaling to the SRE has been

Fig. 4. MKP-1 inhibits Ras stimulation of the p35-C/EBPβ-SRF
interaction in vivo. NIH 3T3 cells were transfected with 1 μg of
pG5CAT either alone or in the presence of 0.5 μg of pGAL4-SRF, 0.5 μg
of CMV-LAP (encoding p35-C/EBPβ), 1 μg of pG5-p110-
CAAX, or 1 μg of pCMV-Ras.V12 as indicated. B, NIH 3T3 cells
were transfected with 1 μg of pG5CAT either alone or in the presence of 0.5
μg of pGAL4-SRF, 0.5 μg of CMV-LAP (encoding p35-C/EBPβ), 1 μg
of pcDNA3-RhoA.V14, 1 μg of pEF/myc/V12Cd42hs, 1 μg of pcDNA3-Rac1(QL),
or 1 μg of pCEV-N2’Rac1 as indicated. Cells in both A and B were serum-deprived for 40 h prior to harvesting, and CAT activity was measured (22). Data are the average of four to five
determinations; error bars represent S.E.
SRF, p35-C/EBPβ, and Ras, there was a large inhibition of CAT activity, which decreased from 47- to 11-fold. This observed difference between the ERKs was surprising since ERK1 and ERK2 are rarely thought of as having distinct substrates.

Since the dominant-negative constructs provided us for expression of untagged proteins, expression of the DN ERKs cannot be measured by Western blot analysis. To control for the possibility that the proteins are expressed at different levels, we utilized the fact that both ERK1 and ERK2 stimulate transcriptional activation of the TCF family member Elk-1 (11). We therefore tested the dominant-negative ERK constructs for their ability to inhibit Ras-stimulated Elk-1 transactivation of the SRE. As shown in Fig. 5B, when the wild-type SRE-CAT reporter was transfected with an Elk-1 expression construct, there was no increase in CAT activity in the absence of Ras, but Ras stimulated Elk-1 transactivation to 4.5-fold over basal levels. When a DN ERK1, DN ERK2, or an MKP-1 construct was cotransfected with the reporter, Elk-1, and activated Ras, there was a large inhibition of CAT activity, which decreased from 47- to 11-fold. This observed difference between the ERKs was surprising since ERK1 and ERK2 are rarely thought of as having distinct substrates.

Due to the differing effects of the DN ERK constructs observed in Fig. 5A, we determined whether C/EBPβ 1) was a direct substrate of the ERKs and 2) would be differentially phosphorylated by purified ERK1 and ERK2 in vitro. Fig. 6 shows that when a bacterial p35-C/EBPβ protein was incubated with activated ERK1 (lane 1) or ERK2 (lane 2) in the presence of [γ-32P]ATP and Mg2+, the C/EBPβ protein was only phosphorylated by ERK2. Both ERK1 and ERK2 phosphorylated a control substrate, MBP (lanes 3 and 4), showing that both kinases were active. These data show that p35-C/EBPβ is selectively phosphorylated by ERK2, supporting the two-hybrid data and suggesting that ERK2 is targeting p35-C/EBPβ to stimulate its interaction with SRF.

Dominant-negative p90Rsk2 Inhibits Ras Stimulation of the C/EBPβ-SRF Interaction—To further analyze the signaling cascades that regulate the C/EBPβ-SRF interaction, we next tested the requirement for p90Rsk2 in mediating Ras stimulation in the two-hybrid assay. This effector was an attractive candidate for several reasons. First, p90Rsk2 is activated by the ERKs, and we have shown that ERK2 is necessary for Ras stimulation of the C/EBPβ-SRF interaction (Fig. 5A). Second, there is evidence that p90Rsk2 is also activated by the PI3K pathway (10), and we also showed that activated PI3K can stimulate the C/EBPβ-SRF interaction (Fig. 5A). Third, p90Rsk2 has previously been shown to phosphorylate C/EBPβ (33) and SRF (28). Phosphorylation of C/EBPβ by p90Rsk2 is necessary for hepatocyte proliferation in response to transforming growth factor-α (33). The in vivo relevance of SRF phosphorylation by p90Rsk2 (28) is unknown. Finally, experi-
ments using fibroblasts from RSK2 knockout mice strongly implicate RSK2 in regulation of the SRE (34). For these reasons, we tested a dominant-negative p90\(^{Rsk2}\) construct in the two-hybrid assay to determine if p90 Rsk2 is necessary for Ras stimulation of the C/EBPβ–SRF interaction.

Fig. 7A shows that when the pG5CAT reporter construct was cotransfected with Gal4-SRF and p35-C/EBPβ, there was a large increase in CAT activity to 15-fold, as we have observed before. Cotransfection of a dominant-negative p90\(^{Rsk2}\) construct in which the critical lysine in the amino-terminal kinase domain is mutated to alanine (Rsk2(K100A)) inhibited Ras stimulation to an average of 3-fold over basal levels. Therefore, like ERK2 described above, p90\(^{Rsk2}\) appears necessary for Ras stimulation of C/EBPβ–SRF interaction.

Since there is evidence that p90 Rsk2 can be a downstream effector of both the Raf/ERK and PI3K pathways, we next determined if the DN p90\(^{Rsk2}\) construct would inhibit either Raf or PI3K stimulation of the p35-C/EBPβ–SRF interaction in the two-hybrid assay. Transfection of a DN p90\(^{Rsk2}\) construct inhibited stimulation of the p35-C/EBPβ–SRF interaction by Raf (Fig. 7B), but not by PI3K (Fig. 7C). These data suggest that activation of p90\(^{Rsk2}\) by Ras to regulate the p35-C/EBPβ interaction with SRF is mainly through the Raf/ERK pathway.

The Consensus p90\(^{Rsk2}\) Site in SRF, but Not p35-C/EBPβ, Is Necessary for Ras Stimulation of the Protein-Protein Interaction—As stated above, both SRF and C/EBPβ have previously been shown to be phosphorylated by Rsk2. SRF is phosphorylated at Ser\(^{103}\) (28), whereas C/EBPβ has been shown to be phosphorylated at Ser\(^{105}\) (33). We obtained constructs of both SRF and C/EBPβ containing a mutation of the serine in the Rsk2 site to alanine. We tested these mutants in the two-hybrid assay to determine if p90 Rsk2 was targeting both SRF and C/EBPβ (Fig. 8A). NIH 3T3 cells were transfected with 1 μg of pG5CAT either alone or in the presence of 5 μg of pGAL4-SRF, 0.5 μg of CMV-LAP (encoding p35-C/EBPβ), 1 μg of pCMV-Ras.V12, or 1 μg of pK3H-p90-Rsk2(K100A) as indicated. B and C, NIH 3T3 cells were transfected with 1 μg of pG5CAT, 0.5 μg of pGAL4-SRF, 0.5 μg of CMV-LAP (encoding p35-C/EBPβ), 1 μg of pEXV-Raf-CAAX (B), or 1 μg of pSG5-p110-CAAX (C) in the absence and presence of 1 μg of pK3H-p90-Rsk2(K100A). Cells in A–C were serum-deprived for 40 h prior to harvesting, and CAT activity was measured (22). Data are the average of three determinations; error bars represent S.E.
C/EBPβ and that loss of the site results in an inhibition of the activation.

We next tested a C/EBPβ-Ala105 construct (29), which contains the mutation of the serine residue in the Rsk2 site (Ser105) to alanine, in the two-hybrid assay. Fig. 8C shows that mutation of the Rsk2 site in p35-C/EBPβ had no significant effect on the ability of Ras to stimulate the mutant protein’s interaction with SRF. Therefore, it appears that the p90Rsk2 kinase is primarily targeting SRF, but not p35-C/EBPβ, to stimulate the interaction of the proteins.

**DISCUSSION**

In this report, we have analyzed the signaling pathways that regulate the interaction of the SRF and C/EBPβ transcription factors. We have demonstrated that Ras signaling pathways are important in regulating the interaction of the two proteins and that Ras is targeting the MAPK site of C/EBPβ to stimulate its transactivation of the SRE as well as its interaction with SRF in the two-hybrid assay. We have also shown that activated forms of both Raf and PI3K stimulate the interaction of C/EBPβ and SRF, but that the Rho family of GTPases plays no role. Furthermore, dominant-negative constructs of p90Rsk2 and ERK2, but not dominant-negative ERK1, inhibit stimulation of the C/EBPβ-SRF interaction by Ras in the mammalian two-hybrid assay. Importantly, C/EBPβ is an in vitro substrate for ERK2, but not ERK1. Finally, p90Rsk2 appears to target SRF, but not p35-C/EBPβ, as mutation of the Rsk2 site in SRF inhibits Ras stimulation of the interaction of the proteins.

The Rho family of GTPases has been shown to signal to the SRE by a TCF-independent mechanism (15). This pathway is dependent on SRF, and the DNA-binding domain of SRF was found to be the minimal domain necessary to mediate Rho signaling (16, 17). Since C/EBPβ interacts with the DNA-binding domain of SRF (20), it made an attractive candidate for the unknown target of the Rho pathway. However, in this report, we have shown that activated forms of RhoA, Rac1, and Cdc42 have no effect on the regulation of the C/EBPβ-SRF interaction. Therefore, the Rho family does not seem to be targeting C/EBPβ, at least in stimulating its interaction with SRF. This also agrees with a previous study showing that C/EBPβ synergizes with Elk-1 in transactivating the SRE in response to Ras (21). Therefore, our studies indicate that C/EBPβ is not in a suggested TCF-independent signaling pathway activated by the Rho family (15), but rather is working in concert with Elk-1 in response to Ras-dependent signals.

The fact that we saw specific effects of ERK2, in both the ability of dominant-negative ERK2 to inhibit Ras stimulation of the C/EBPβ-SRF interaction and the ability of ERK2 to phosphorylate C/EBPβ in vitro, was surprising. ERK1 and ERK2 are thought to have overlapping substrates in vitro and in vivo; and therefore, differential phosphorylation by the two kinases was unexpected. Interestingly, a study by Hochholdinger et al. (35) shows that individually, dominant-negative forms of ERK1 and ERK2 inhibit induction of a c-fos promoter reporter construct. This implies that ERK1 and ERK2 are both necessary for c-fos induction. If the kinases were completely redundant, then inhibiting one kinase would not be expected to have an effect on gene induction. Other examples of differences between ERK1 and ERK2 have been reported: the ERK1 knockout mouse has a defect in thymocyte maturation (9); MEK partner-1 binds specifically to ERK1 and not ERK2 (36); v-Raf selectively activates ERK2 in Rat-6 fibroblasts (37); and the urokinase-type plasminogen activator promoter is specifically activated by ERK1 (38). Based on these examples, ERK1 and ERK2 are most likely not completely redundant in vivo. Our studies show another important difference between the ERKs: ERK2, but not ERK1, specifically phosphorylates C/EBPβ in vitro and is necessary for Ras-stimulated interaction of C/EBPβ with SRF in vivo.

These studies also suggest a requirement for p90Rsk2 in Ras stimulation of the p35-C/EBPβ-SRF interaction in vivo. Rsk2(K100A) was a strong dominant-negative in the mammalian two-hybrid system, as effective as DN ERK2. This result was interesting due to the facts that both SRF (28) and p35-C/EBPβ (33) are known substrates of p90Rsk2, and p90Rsk2-dependent pathways have recently been shown to target the SRE (34). We further show that the Rsk2 site in SRF (Ser105) is necessary for the complete Ras stimulation. Although SRF has previously been shown to be a substrate of p90Rsk2 (28), an in vivo function for this phosphorylation is not known. We propose that a functional consequence of this phosphorylation is to promote interaction with p35-C/EBPβ in response to growth-promoting signals. We did not observe any significant inhibition of Ras stimulation of the interaction between a C/EBPβ Rsk2 site mutant (C/EBPβ-Ala105) and SRF. Since the p90Rsk2 site in C/EBPβ is not required for Ras stimulation, this suggests that p90Rsk2 is regulating the interaction of the proteins primarily through phosphorylation of SRF.

We also observed that DN p90Rsk2 can inhibit stimulation of the p35-C/EBPβ-SRF interaction by Raf, but not by PI3K. Therefore, it appears that the activation of p90Rsk2 by Ras in our system is through the Raf/ERK pathway. We did see a stimulation of the p35-C/EBPβ-SRF interaction by PI3K, but we currently do not know the kinase(s) targeting the proteins in vivo. We have studied many downstream effectors of PI3K, including Rac (Fig. 3B), Cdc42 (Fig. 3B), and protein kinase B (data not shown), but none of these proteins had a role in regulating the interaction of p35-C/EBPβ-SRF. We are currently examining other potential PI3K effectors in our studies.

We have not yet been able to observe a ternary complex between SRF and p35-C/EBPβ at the SRE in vitro. A likely explanation for this lies in the complexity of the signaling pathways targeting p35-C/EBPβ. ERK2 and p90Rsk2 are important downstream effectors of Ras in our studies, and ERK2 phosphorylates p35-C/EBPβ (Fig. 6), whereas p90Rsk2 phosphorylates SRF (28). We cannot exclude the possibility that additional phosphorylations of p35-C/EBPβ and SRF, such as those mediated by downstream effectors of PI3K, are necessary to regulate the interaction of these two proteins. Furthermore, based on the study showing that p35-C/EBPβ and Elk-1 synergize in Ras-stimulated transactivation of the SRE (21), Elk-1 may also need to be included to observe a quaternary complex of p35-C/EBPβ, SRF, and Elk-1 at the SRE. Therefore, due to the complexity of factors and the essential modifications necessary for interaction, further studies are needed to optimize...
conditions for a ternary or quaternary complex to be formed in vitro.

Overall, our studies suggest that the formation of a complex between SRF and C/EBPβ at the SRE is a highly regulated event. Complex formation is dependent on signals from at least ERK2 and p90Rsk2 and possibly other kinases. These studies, along with a previous study showing that C/EBPβ, Elk-1, and SRF are constitutively bound at the SRE, based on in vivo footprinting analysis (6). Footprinting studies also indicate occupancy of SRF, so either an inactive p35-C/EBPβ isoform or the repressor p20-C/EBPβ isoform could be present since both isoforms could interact with SRF in the absence of mitogenic signaling by Ras (20). However, in response to Ras-dependent signaling pathways, either ERK1 or ERK2 or both phosphorylate Elk-1, whereas p35-C/EBPβ is phosphorylated by ERK2. p90Rsk2 also phosphorylates SRF. Other kinases may also target these transcription factors as well, such as kinases downstream of PI3K. Thus, the convergence of these growth-promoting signals on factors at the SRE is necessary for a competent transcriptional complex to form and to allow maximal induction of c-fos.

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