Ras regulates the association of serum response factor and CCAAT/enhancer-binding protein β*

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The serum response element (SRE) is a promoter element essential for transcriptional activation of immediate early genes, such as c-fos and early growth response-1, by mitogenic signals. Several transcription factors bind the SRE, including the serum response factor (SRF), the ternary complex factor, and the CCAAT/enhancer-binding protein β (C/EBPβ). The C/EBPβ mRNA encodes three translation products of 38, 35, and 20 kDa. p35-C/EBPβ activates transcription of the SRE in an SRF-dependent fashion, whereas p20-C/EBPβ, which initiates at an internal in-frame methionine, lacks a transactivation domain and inhibits transcription. We show that SRF and C/EBPβ interact in vivo through the DNA binding domain of SRF and the C terminus of C/EBPβ common to p35 and p20. Therefore, like the ternary complex factor, C/EBPβ may be recruited to the SRE not only by binding to the DNA, which is not a high affinity site, but also by protein-protein interactions with SRF. Strikingly, in both the mammalian two-hybrid assay and in vivo immunoprecipitations, the association of SRF and p35-C/EBPβ but not p20-C/EBPβ is dramatically stimulated by activated Ras. Furthermore, mutation of the threonine within a mitogen-activated protein kinase consensust motif in the C terminus of C/EBPβ eliminates the response to Ras. These results suggest a new mechanism by which mitogenic signals may influence transcription activity of the SRE by selectively promoting protein-protein interactions between SRF and the transactivator p35-C/EBPβ.

The c-fos proto-oncogene is a prototypic member of the set of “immediate early” genes whose transcription is rapidly activated in the absence of protein synthesis by mitogenic signals (1). Within the promoter of the c-fos gene is a 26-bp regulatory sequence, the serum response element (SRE), that mediates the rapid and transient induction of the c-fos gene by serum and growth factors (2). The SRE binds a ubiquitous transcription factor, the serum response factor (SRF), which is essential and required for serum and growth factor activation of SRE (3–5). SRF is an obligatory member of a multiprotein complex that occupies the SRE and serves as a convergence point for multiple signal transduction pathways. Thus, growth factors that activate the Ras-Raf-extracellular signal-regulated kinase cascade and phorbol esters target a family of transcription factors containing an N-terminal Ets domain that form a ternary complex with SRF and SRE DNA (6). c-fos promoter mutants that cannot bind the ternary complex factor (TCF) remain responsive to serum induction through a second TCF-independent pathway that still requires SRF and can involve certain Rho family GTPases (5, 7, 8). The role of accessory factors in the TCF-independent pathway has yet to be completely defined.

We have observed that maximal TCF-independent serum induction of the SRE requires SRF and another transcription factor, C/EBPβ (9, 10). C/EBPβ, which binds to the SRE overlapping and immediately 3′ to SRF, is a member of the basic leucine zipper family of transcription factors (11, 12). The C/EBPβ gene contains 3 in-frame methionines that give rise to three translation products of 38, 35, and 20 kDa (13). (Values are for the murine or rat genes; the human and avian genes are slightly larger.) The N-terminal half of p38 and p35 contains a strong transactivation domain, whereas p20-C/EBPβ lacks this transactivation domain and acts as an inhibitor of transcription. Many fibroblast cell types, such as NIH 3T3 cells, express both the long (35 kDa) and short (20 kDa) forms of C/EBPβ at approximately equal levels (14). Both p35 and p20 homodimers as well as p35-p20 heterodimers bind to the SRE with equivalent affinity. Moreover, in transient transfection assays p20-C/EBPβ completely blocks serum induction of the c-fos SRE, whereas p35-C/EBPβ potentiates activity approximately 20–30-fold (10). Interestingly, transactivation by p35-C/EBPβ is dependent upon SRF binding to the c-fos SRE. Moreover, we have shown that C/EBPβ is able to bind SRF through protein-protein interactions in vitro (10). Taken together, these results suggest the requirement for SRF binding in p35-C/EBPβ transactivation of the SRE reflects some physical interaction between these two transcription factors that is essential for transactivation.

In this study we confirm that SRF and C/EBPβ associate in vivo using both a mammalian two-hybrid assay for demonstrating protein-protein interactions and coimmunoprecipitation studies. The domains of each protein that are sufficient for their interaction in vivo are comparable to the domains previously identified in vitro (10) and include the DNA binding domain of SRF (amino acids 133–265) and the C terminus of C/EBPβ (amino acids 152–297) common to p35 and p20. Strikingly, the association of SRF and p35-C/EBPβ but not p20-C/EBPβ is dramatically stimulated by activated Ras. Furthermore, mutation of the threonine within a MAP kinase consensus motif PGTP in the C terminus of C/EBPβ eliminates the
response to Ras. These results indicate that C/EBPβ-SRF protein-protein interactions may be one target for TCF-independent signaling pathways to the SRE.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—HeLa cells (kindly provided by Dr. R. Chalkley, Vanderbilt University) were maintained in Dulbecco’s modified Eagle’s medium and nutrient mixture F-12 (Life Technologies, Inc.) containing 10% donor calf serum with iron (Life Technologies, Inc.), 0.24% sodium bicarbonate, 25 units of penicillin G sodium/ml, and 25 mg of streptomycin sulfate/ml. NIH 3T3 fibroblasts, from the American Type Culture Collection, were grown in Dulbecco’s modified Eagle’s medium with 10% calf serum (Colorado Serum Company), 0.22% sodium bicarbonate, and 4 mM L-glutamine, penicillin, and streptomycin. COS-7 cells (kindly provided by Dr. S. Hann, Vanderbilt University) were maintained in the same medium as described for NIH 3T3 fibroblasts except that 10% fetal bovine serum (Hyclone) instead of calf serum was added. HeLa and COS-7 cell transfections were performed using the calcium phosphate technique (15). Cells were plated at a density of 5 × 10⁵ cells/dish 1 day before transfection. The cells were exposed to the CaP-DNA precipitate for 8 h. The medium was removed and replaced with complete medium for an additional 36 h before harvesting. NIH 3T3 cell transfections were performed using a CalPhos Maximizer (CLONTECH) as described by the manufacturer. Cells at 60–70% confluence were exposed to the CaPO₄-DNA precipitate for 8 h before the medium was removed and replaced with complete medium for 24 h. Cells were then serum-deprived for 36–40 h in Dulbecco’s modified Eagle’s medium supplemented as above except containing 0.5% calf serum before harvesting. Cell extracts were prepared, and chloramphenicol acetyl transferase (CAT) assays were performed on extracts containing equivalent cell protein as described previously (16).

Two-hybrid Assay—The pGAL4, pVP16, and pG5CAT plasmids used are from the Mammalian MATCHMAKER Two-Hybrid Assay Kit (CLONTECH). pGAL4-SRF and pGAL4-SRF(133–265) were constructed by polymerase chain reaction amplification of a 1563-bp fragment (encoding amino acids 1–508 of SRF) or a 420-bp fragment (encoding amino acids 133–265 of SRF), respectively, from pGEM3.5 (gift of R. Treisman) as described previously (10). The amplified fragments (CLONTECH) as described by the manufacturer. Cells at 60–70% confluence were exposed to the CaPO₄-DNA precipitate for 8 h before the medium was removed and replaced with complete medium for 24 h. Cells were then serum-deprived for 36–40 h in Dulbecco’s modified Eagle’s medium supplemented as above except containing 0.5% calf serum before harvesting. Cell extracts were prepared, and chloramphenicol acetyl transferase (CAT) assays were performed on extracts containing equivalent cell protein as described previously (16).

RESULTS

C/EBPβ and SRF Associate in Vivo—To extend the validity of the C/EBPβ-SRF protein interactions previously demonstrated by GST pulldown assays in vitro (10), we have also developed a mammalian “two-hybrid” system to monitor the possible association of SRF and p35-C/EBPβ (also called LAP) or p20-C/EBPβ (also called LAP) in vivo. Initially, we prepared a construct in which the DNA binding domain of Gal4 (amino acids 1–80) is fused to SRF. When pGal4-SRF is transfected into HeLa cells along with pG5CAT (CLONTECH), a CAT reporter gene-driven by five copies of the Gal4 DNA binding site linked to the E1B promoter, at best only a 1.5-fold increase transcription is observed with the average of four experiments being 1.1-fold (Fig. 1). Thus, although SRF contains a C-terminal transactivation domain, it is not particularly potent in this assay. Other investigators have also observed very low activity with Gal4-SRF (17). However, a much larger increase in transactivation is observed when pCMV-LAP (encoding p35-C/EBPβ) or p20-C/EBPβ is coexpressed with Gal4-SRF and the pG5CAT reporter. At the highest level of p35-C/EBPβ, an average 7.5-fold increase in CAT activity was observed with up to a 15-fold increase in some experiments. This increase is not observed when p35-C/EBPβ alone is expressed with pG5CAT as expected, because Gal4 sites, not C/EBPβ sites, are upstream of the promoter driving the CAT gene. The results presented in Fig. 1 indicate that SRF, when affixed to the DNA through a Gal4 DNA binding domain, can recruit p35-C/EBPβ to the promoter whereby the transactivation domain of p35-C/EBPβ, either alone or in concert with the transactivation domain of SRF, results in a strong increase in CAT activity. In the strictest sense, this approach does not constitute a two-hybrid assay, because only one hybrid protein, Gal4-SRF, was employed. Because p35-C/EBPβ intrinsically contains a strong activation
domain, we did not need to append another. However, because the approach follows the concept of the two-hybrid assay and because we do in one instance (Fig. 4) use two-hybrid proteins Gal4-SRF and VP16-p20-C/EBPβ, we will for simplicity sake refer to this experimental approach as the two-hybrid assay.

Next we extended the two-hybrid system to examine possible mitogenic regulation of SRF-p35-C/EBPβ interactions in NIH 3T3 cells. In these experiments we used a Ras protein with an activating mutation at codon 12 (Ras.V12) to simulate a variety of growth-promoting signals. As shown in Fig. 2A, dramatic increases (190–240-fold) in p53CAT reporter gene expression are observed when pGal4-SRF and pCMV-LAP (encoding p35-C/EBPβ) are cotransfected with an expression construct for activated Ras, compared with a 10–13-fold increase in the absence of Ras. This result suggests that the interaction of SRF and p35-C/EBPβ could be regulated in vivo by a Ras-dependent signaling pathway. It is also possible that activated Ras promotes the “unmasking” of a dormant transactivation domain in p35-C/EBPβ as has been observed in some cell types. Because p35-C/EBPβ, when overexpressed, activates the c-fos SRE nearly equivalently in quiescent as well as stimulated cells (10), it does not appear that the transactivation domain of p35-C/EBPβ necessarily requires unmasking by a signal-dependent pathway in NIH 3T3 cells. However, to address this possibility directly we repeated the experiment in Fig. 2A with a Gal4-LAP construct. pGal4-LAP activated p53CAT but no stimulation by activated Ras was observed (data not shown). In Fig. 2B we demonstrate that the DNA binding domain of SRF (amino acids 133–265) is sufficient for both the interaction with p35-C/EBPβ in vivo and stimulation of this interaction by activated Ras, although the magnitude of the effect is somewhat smaller, particularly in the case of activation by Ras (note the difference in y axis scales in Fig. 2, A and B). Nonetheless, given the DNA binding domain of SRF is instrumental in TCF-independent serum activation of the SRE (5, 7), this result is highly significant.

The Association of SRF and C/EBPβ Is Stimulated by Activated Ras in Vivo—Although the two-hybrid system is a standard and well accepted approach for analyzing protein-protein interactions in vivo, we cannot exclude the possibility that the stimulation by activated Ras observed in Fig. 2 is because of other DNA binding factors and/or general transcription factors that have been modified to make them more responsive to the effects of SRF/p35-C/EBPβ on the promoter. We therefore sought direct evidence, via coimmunoprecipitation, that SRF and p35-C/EBPβ associate in vivo and that their interaction is stimulated by activated Ras. COS cells were transfected with an expression vector for a 6x histidine-tagged version of SRF carrying the φ10 (T7 tag) epitope sequence in the presence or absence of pCMV-LAP (encoding p35-C/EBPβ). Because endogenous COS cell C/EBPβ proteins are larger in size (42 and 45 kDa, similar to the human C/EBPβ gene, NFIL6), the exogenous p35 rat C/EBPβ is easily distinguished (compare Fig. 3, lanes 8 and 9). Because p35-C/EBPβ is overexpressed, to avoid overexposure of the p35-C/EBPβ signal, the film exposure in lane 8 is shorter than in lane 9 (10 versus 60 s). This difference is reflected in the correspondingly lower signal of endogenous p42/45 and p20-C/EBPβ in lane 8 compared with lane 9. Cell lysates were incubated with T7 tag antibody-agarose beads, and the immunoprecipitated proteins were subjected to immunoblotting with C/EBPβ antibody. As shown in Fig. 3, p35-C/EBPβ coprecipitated with tagged SRF on the antibody beads but only when activated Ras was included in the cotransfections (lane 5). We are aware of the caveats of using overexpressed proteins in this type of assay, because the high level of expression may promote their fortuitous association. However, in this case nonspecific interactions are rendered less likely by the fact that the SRF-p35-C/EBPβ interaction is only observed in the presence of activated Ras.

The Association of SRF and p35-C/EBPβ, but Not SRF and p20-C/EBPβ, Is Stimulated by Activated Ras in Vivo—In Fig. 2 we determined that the DNA binding domain of SRF is sufficient for association with C/EBPβ in vivo, in agreement with the GST pulldown experiments (data not shown). We also wanted to determine the domain of C/EBPβ sufficient for interaction with SRF in vivo. Because p20-C/EBPβ lacks a transactivation domain, we used a chimeric VP16-p20-C/EBPβ protein in the two-hybrid assay shown in Fig. 4. Gal4-SRF was able to recruit VP16-p20-C/EBPβ to the
The association of SRF and p35-C/EBPβ, but not SRF and p20-C/EBPβ, is stimulated by Ras in vivo. A, NIH 3T3 cells were transfected with 5 μg of pG5CAT alone or with 2 μg of pGal4-SRF, 1 or 2 μg of pCMV-LAP, 5 μg of VP16-p20-C/EBPβ, or 10 μg of VP-16 as indicated. Total DNA in each transfection was adjusted to 19 μg with pUC19. Cells were serum-deprived for 40 h prior to harvesting. CAT activity was measured as described in Ref. 16. B, same as A except that 2 μg of an activated Ras expression vector were included. Data are the average of 6–16 determinations. Average standard error was 52% for A and 40% for B.

FIG. 4. Ras-activated association with SRF requires threonine 235 in C/EBPβ. NIH 3T3 cells were transfected with 5 μg of pG5CAT alone, 2 μg of pGal4-SRF, or 2 μg of pGal4-SRF and 1 or 2 μg of pCMV-NFIL6 or pCMV-NFIL6(235T-A) as indicated in the absence or presence of 2 μg of an activated Ras expression vector. Total DNA in each transfection was adjusted to 11 μg with pUC19. Cells were serum-deprived for 40 h prior to harvesting. CAT activity was measured as described in Ref. 16. Data are the average of four determinations in duplicate; average standard error was 24%.

DISCUSSION

In this report we have presented evidence that C/EBPβ and SRF associate in vivo and this protein-protein interaction is regulated by Ras-dependent signaling pathway(s). These transcription factors interact in vivo through the DNA binding domain of SRF, which is known to be required for TCF-independent signaling to the SRE (5, 7) and the C terminus of C/EBPβ common to p20 and p35-C/EBPβ. The two-hybrid assay in mammalian cells and in vivo communoprecipitations were used to show that activated Ras dramatically stimulates the association of p35-C/EBPβ and SRF. These data support the involvement of a physical association of C/EBPβ and SRF in transcriptional activation of the SRE, which is stimulated by Ras-dependent signaling pathways. However, future development of a mutant C/EBPβ unable to bind SRF will be necessary to confirm the role of C/EBPβ-SRF protein-protein interactions in SRE function.

Interestingly, activated Ras completely failed to stimulate the association of Gal4-SRF and VP16-p20-C/EBPβ in the two-
hybrid assay; in fact, a 3–4-fold inhibition was observed. Unfortunately, we have been unable to confirm the lack of Ras-stimulated association of SRF and p20-C/EBPβ in the coimmunoprecipitation studies because of an unacceptably high level of nonspecific binding of p20-C/EBPβ (or VP16-p20-C/EBPβ) to the antibody-agarose beads. We presume this is because of the highly charged nature of p20-C/EBPβ and are currently working on developing satisfactory precautions to prevent this. Nonetheless, our observations with the two-hybrid assay suggest a new model for SRE function in which protein-protein interactions with the DNA binding domain of SRF are a critical target for TCF-independent mitogenic regulation because they are key to determining which isoform of C/EBPβ stably occupies the SRE. Our data indicate that p35 and p20-C/EBPβ are recruited to the SRE not only by binding to the DNA, which is not a high affinity site, but also by protein-protein interactions with SRF. If SRF and p20 homodimers occupy the SRE, no transactivation occurs. When SRF and p35 homodimers or a p35/20 heterodimer occupy the SRE, transactivation results. Mitogenic signals that activate Ras dramatically enhance the interaction of SRF selectively with p35-C/EBPβ, ultimately resulting in efficient transactivation.

This mechanism is compatible with the fact that both p35 and p20 are located in the nucleus in similar amounts in serum-deprived or -stimulated NIH 3T3 cells. Moreover, the distribution of homodimers and heterodimers comprising C/EBPβ does not appear to change in gel shift assays performed with nuclear extracts from serum-deprived or -stimulated cells (data not shown), suggesting that direct regulation of p20-C/EBPβ or p35-C/EBPβ DNA binding activity is unlikely. In vivo footprinting experiments have not found detectable changes in protein occupancy of the SRE upon serum stimulation (18). However, the exchange of p35-C/EBPβ for p20-C/EBPβ would be compatible with this observation in that both proteins share the same DNA binding domain and thus would be expected to exhibit extremely similar, if not identical, footprints on SRE DNA.

The Ras-dependent signaling pathway(s), which selectively promote the interaction of p35-C/EBPβ with SRF, have yet to be defined. Although Raf was the first effector protein located downstream of Ras to be identified, it is now clear that there are multiple Ras effector proteins (for review, see Ref. 19). Moreover, Ras can activate another family of small GTPases, the Rho family GTPases (for review, see Ref. 20). Recent studies on MAP kinase signaling pathways have uncovered the presence of at least three distinct MAP kinase pathways that can be activated by Ras and Rho family GTPases. The terminal kinases in these three pathways are the extracellular signal-regulated kinases, Jun N-terminal kinases/stress-activated protein kinases, and p38 kinases (for review, see Refs. 21 and 22). The effect of Ras on p35-C/EBPβ-SRF protein-protein interactions was not observed with the Thr235-Ala C/EBPβ mutant, suggesting that phosphorylation of threonine 235 strongly enhances the interaction of p35-C/EBPβ with SRF. Threonine 235 is contained in a MAP kinase consensus sequence conserved among C/EBPβ genes from all species, and this site is known to be an in vitro target for partially purified MAP kinase (23). It will be interesting to investigate which of the terminal MAP kinase(s) may phosphorylate C/EBPβ at Thr235 in response to mitogenic signals in vivo and identify the upstream kinases that link this phosphorylation event to Ras activation.

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