A multifunctional DNA-binding protein that promotes the formation of serum response factor/homeodomain complexes: identity to TFII-I

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The human homeodomain protein Phox1 interacts functionally with serum response factor (SRF) to impart serum responsive transcriptional activity to SRF-binding sites in a HeLa cell cotransfection assay. However, stable ternary complexes composed of SRF, Phox1, and DNA, which presumably mediate the transcriptional effects of Phox1 *in vivo*, have not been observed *in vitro*. Here, we report the identification, purification, and molecular cloning of a human protein that promotes the formation of stable higher-order complexes of SRF and Phox1. We show that this protein, termed SPIN, interacts with SRF and Phox1 *in vitro* and *in vivo*. Furthermore, SPIN binds specifically to multiple sequences in the *c-fos* promoter and interacts cooperatively with Phox1 to promote serum-inducible transcription of a reporter gene driven by the *c-fos* serum response element (SRE). SPIN is identical to the initiator-binding protein TFII-I. Consistent with this hypothesis, SPIN exhibits modest affinity for a characterized initiator sequence *in vitro*. We propose that this multifunctional protein coordinates the formation of an active promoter complex at the *c-fos* gene, including the linkage of specific signal responsive activator complexes to the general transcription machinery.

[Key Words: Homeodomain; serum response factor; transcription; *c-fos*; TFII-I]

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Interactions between the MADS box protein serum response factor (SRF) and homeodomain proteins may serve as a model for understanding how cell type-specific genes are activated in response to generic signals. We have shown previously that SRF interacts with the human homeodomain protein Phox1 and that this interaction imparts to reporter genes the potential to respond to extracellular signals by facilitating the binding of SRF and its signal responsive accessory protein Elk-1 to the serum response element (SRE) derived from the *c-fos* gene (Grueneberg et al. 1992, 1995). Furthermore, Phox1 facilitates the binding of SRF to a subset of variant SRE sequences that may represent imperfect SREs found in the promoters of cell type-specific genes (Grueneberg et al. 1995). This interaction suggests that homeodomain proteins can direct signal transduction pathways to cell type-specific genes and provides a possible mechanistic

connection between signal transduction and cell identity.

The Phox1 protein was originally identified in a yeast selection for its ability to interact with the yeast SRF homolog MCM1 to activate a cell type-specific and pheromone-inducible reporter gene (Grueneberg et al. 1992). The Phox1 cDNA contained a homeobox related most closely to that of the *Drosophila paired* gene. In a mobility shift assay using *Escherichia coli*-produced proteins, Phox1 interacts with SRF to enhance the rate at which SRF binds to and dissociates from the SRE. The enhancing activity resides within the homeodomain of Phox1 and is shared by closely related homeodomains of the *paired* class but not by more distantly related homeodomains. Surprisingly, we could never detect stable ternary complexes composed of SRF, Phox1, and the SRE using bacterially produced proteins.

Here we show that novel SRF/Phox1 complexes can be formed in HeLa extracts, which suggests that other cellular proteins may be required to promote complex formation. Using a biochemical approach, we have purified, characterized, and cloned the encoding gene for a protein that interacts with Phox1 and SRF and promotes the for-

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mation of this complex. The encoded protein, called SPIN (SRF-Phox1 Interacting protein), has multiple activities, including the ability to bind specifically to multiple sequences in the *c-fos* promoter and to act cooperatively with Phox1 and SRF to impart serum-inducible expression to a *c-fos*-driven reporter gene. The observation that this protein is identical to the transcription factor TFII-I (A.L. Roy, H. Du, P.D. Gregor, C.D. Novina, E. Martinez, and R.G. Roeder, in prep.), which interacts with the basal transcription machinery, suggests that SPIN/TFII-I acts by coordinating the formation of a multiprotein complex at the *c-fos* promoter and linking specific signal responsive activation complexes to the general transcription machinery (Roy et al. 1991; Manzano-Winkler et al. 1996).

Results

Novel Phox1-dependent SRF/SRE complexes are formed in HeLa extracts

Previously, we found that *E. coli*-produced Phox1 and SRF interact to enhance the rate at which SRF binds to the SRE (Grueneberg et al. 1992). Furthermore, Phox1 promotes serum stimulation of an SRE-containing reporter gene in HeLa cells, suggesting that SRF and Phox1 interact functionally on the SRE in vivo (Grueneberg et al. 1995). Surprisingly, however, using purified recombinant proteins, we were never able to detect stable ternary complexes containing SRF, Phox1, and the SRE in vitro. To determine whether Phox1 could form stable complexes with endogenous SRF from HeLa cells, transfection experiments were performed using either the full-length Phox1 expression plasmid or a plasmid expressing an 80-amino-acid fragment of Phox1 that includes the homeodomain. Transfected cells were starved for serum for 24 hr, whole-cell extracts were prepared, and mobility-shift assays were performed. To maximize the detection of any high-molecular-weight complexes, the mobility-shift reactions were analyzed on 3.5% polyacrylamide gels.

Figure 1 shows that expression of the full-length Phox1 protein led to a slight reduction in mobility of the endogenous SRF/SRE complex in HeLa extracts (lanes 2,11), compared to the empty expression plasmid (lanes 1,10) and the smaller homeodomain fragment (lanes 3,12). This observation suggests that overexpression of Phox1 in HeLa cells can lead to the formation of a novel complex on the SRE. That the mobility of the SRF/SRE complex was unaffected by the homeodomain fragment suggests either that this fragment is too small to change the mobility of the complex or that regions outside the homeodomain are required for stable complex formation with SRF.

To confirm that this novel complex contains SRF, we performed additional assays using a monoclonal antibody against SRF (lanes 7–9). Addition of the SRF antibody supershifted both the standard SRF/SRE binary complex (lane 7) and the novel Phox1-dependent complex (lane 8), suggesting that SRF was present in the

Figure 1. Formation of a novel Phox1-dependent SRF/SRE complex in HeLa cells. Mobility-shift assay measuring SRF/SRE interactions from HeLa cell extracts in the presence of exogenous Phox1. Lanes 1–9 utilize the wild-type SRE probe; lanes 10–12 utilize the Elk-1 mutant probe (pm18; Graham and Gilman 1991). (Lanes 1,4,7,10) Cells are transfected with an empty expression plasmid; (lanes 2,5,8,11) cells are transfected with an expression plasmid producing the full-length Phox1 protein; (lanes 3,6,9,12) cells are transfected with an expression plasmid producing a truncated Phox1 protein, containing the homeodomain. Lanes 4–6 were incubated with a Phox1 monoclonal antibody; lanes 7–9 were incubated with an SRF monoclonal antibody.

novel complex. In contrast, monoclonal antibody to Phox1 abolished the formation of the novel complex and restored the SRF/SRE complex seen in control cells (lane 5). Despite its inhibitory effect on the novel SRF complex, the Phox1 antibody enhanced and supershifted the Phox1/SRE binary complex (dark band, lane 5). This observation suggests that formation of this novel complex requires Phox1 surfaces distinct from those required for DNA binding. We conclude that Phox1 is required for formation of this novel SRF-containing complex, although we cannot be certain that it is actually present in this complex.

The response of the *c-fos* SRE to mitogen-activated protein kinase (MAPK) signals requires a family of Ets domain proteins, termed ternary complex factors, which bind cooperatively with SRF to an adjacent Ets site (Shaw et al. 1989; Dalton and Treisman 1992; Gille et al. 1992; Hipskind et al. 1991; Marais et al. 1993). This ternary complex can be seen immediately above the SRF/SRE complex in Figure 1, lanes 1–9. We found previously that Phox1 can promote the recruitment of the ternary complex factor Elk-1 to the SRE in vivo (Grueneberg et al. 1995). Consistent with this observation, this ternary

complex is also shifted in the presence of Phox1 (lane 2). Formation of the ternary complex can be abolished by nucleotide substitutions in the Ets site (lane 10). Using this probe, the shifting of SRF/SRE complex in the presence of Phox1 could still be seen (lane 11). Thus, expression of Phox1 promotes the formation of two novel SRF-containing complexes—one that contains an Elk1-like factor and one that does not.

Because we had never observed such complexes previously using purified SRF and Phox1, we infer that either (1) SRF and/or Phox1 undergo post-translational modifications in HeLa cells that are required for complex formation, or (2) an additional protein in HeLa cell extracts is required for complex formation. Because SRF and Phox1 produced in rabbit reticulocyte lysates, which are generally competent for most post-translational modifications, also fail to form stable complexes (data not shown), we favor the hypothesis that a third protein is involved.

Purification of a HeLa cell protein that interacts with bacterially produced SRF and Phox1

We used conventional chromatographic methods to identify a potential cofactor for SRF and Phox1 in HeLa cell extracts. The purification scheme is shown in Figure 2A. After each purification step, fractions were monitored for their ability to promote the formation of a novel complex in the presence of *E. coli*-produced Phox1 and SRF. Figure 2B shows that the addition of Q-Sepharose fractions 16–20 altered the mobility of the protein–DNA complex obtained with bacterially produced SRF in the presence of Phox1 (lanes 12,16,20). The same fractions do not have a significant effect on the mobility of SRF alone (lanes 11,15,19) or Phox1 alone (lanes 10,14,18). Thus, as in crude HeLa extracts, formation of the novel complex requires both SRF and Phox1. Because the SRF and Phox1 proteins used in this experiment were produced in *E. coli*, we conclude that a distinct protein present in HeLa extracts is required for the formation of stable SRF/Phox1 complexes. Furthermore, these observations suggest that the activity in the Q-Sepharose fractions is the same as that detected in the crude extracts of transfected HeLa cells.

Interestingly, these fractions also contained a protein with intrinsic DNA-binding activity that recognized the SRE-containing probe (lanes 9,13,17,21). Thus, these fractions contain both an SRE-binding activity and an activity that promotes the formation of higher-order SRF-containing complexes on the SRE.

To visualize the protein(s) responsible for the supershifting and DNA-binding activities, the Q-Sepharose fractions were analyzed by SDS-PAGE (Fig. 2C). The same Q-Sepharose fractions (lanes 4–8) that exhibited supershifting and DNA-binding activities contained a predominant protein with apparent relative molecular mass (M_r) of 120 kD. This protein (p120) was most abundant in fractions 19–21 (lanes 5–7). To confirm that this band correlates with the SRF/Phox1-interacting activity, we examined its ability to interact directly with SRF and

Phox1 in the absence of added DNA. For these experiments we employed Q-Sepharose fraction 22, in which the amount of p120 is relatively low, enabling the other proteins in the fractions to serve as specificity controls. We incubated fraction 22 with glutathione *S*-transferase (GST) fusion proteins carrying the Phox1 homeodomain or the core domain of SRF. Figure 2D shows that p120 was selectively retained on both GST–Phox1 and GST–SRF beads, relative to other proteins in the fraction (lanes 5 and 6, respectively). In contrast, a control GST fusion protein, containing the SH2 domain of phospholipase C- γ (PLC γ) (Rhee 1991), failed to bind p120 (lane 7).

Although p120 was the major protein retained on the GST–Phox1 and GST–SRF beads, other minor bands can be seen in these lanes. To rule out these proteins as candidates for the supershifting activity, we repeated this experiment with Q-Sepharose fraction 20, which is much more highly enriched for both p120 (see Fig. 2C, lane 6) and supershifting activity (see Fig. 2B, lane 20). If any of these minor bands were responsible for this activity, they should again be present in the GST–Phox1 and GST–SRF eluate. However, only p120 was detectably retained from fraction 20 on GST–Phox1 and GST–SRF beads (data not shown). Thus, we provisionally conclude that p120 is responsible for the supershifting activity in the Q-Sepharose fractions.

To determine whether the DNA-binding activity in this fraction copurified with the supershifting activity, eluted material from the GST–Phox1 beads and GST–PLC γ beads was assayed for its ability to bind an SRE probe. Figure 2E shows that the GST–Phox1 eluted material contained an activity that recognized the SRE (lane 1), whereas the GST–PLC γ eluate did not (lane 2). Taken together, these results suggest that p120 interacts directly with SRF and Phox1 in the absence of DNA and that p120 or a tightly associated protein has intrinsic DNA-binding activity. We provisionally refer to p120 as SPIN.

*SPIN binds to multiple sites in the *c-fos* promoter*

To determine whether SPIN specifically binds to the SRE, we performed DNase I footprinting analysis on a probe containing *c-fos* promoter sequences from –362 to –266. Surprisingly, we observed two footprints on this probe, one on the SRE and the other on a site 25 bp upstream (data not shown). The upstream site corresponds to the *c-sis*/platelet-derived growth factor (PDGF)-inducible element (SIE), which cooperates with the SRE in the *c-fos* promoter to mediate induction by polypeptide growth factors, serum, and TPA (Hill and Treisman 1995). The SIE binds a protein SIF (*c-sis*-inducible factor), which is composed of Stat1 and Stat3 (Hayes et al. 1987; Wagner et al. 1990; Fu and Zhang 1993; Sadowski and Gilman 1993; Sadowski et al. 1993). To confirm that SPIN binds specifically to these sites and to map the sites at nucleotide resolution, we performed dimethylsulfate (DMS) interference analysis. Figure 3A shows a DMS interference assay of Q-Sepharose-purified

Figure 2. Identification of a human protein required for the formation of the novel Phox1-dependent SRF/SRE complex. (A) Purification scheme for identifying a HeLa cell protein that supershifts SRF/SRE complexes in the presence of Phox1. (B) HeLa cell protein peaks in fractions 16–20 to supershift *E. coli*-produced SRF in the presence of Phox1. Mobility-shift assays using *E. coli*-produced Phox1 in the absence (lane 2) and presence (lanes 6, 10, 14, 18, 22, 26) of HeLa fractions; *E. coli* produced SRF in the absence (lane 3) and presence (lanes 7, 11, 15, 19, 23, 27) of HeLa fractions; and both proteins together in the absence (lane 4) and presence (lanes 8, 12, 16, 20, 24, 28) of HeLa fractions on the wild-type SRE probe. (C) A 120 kD protein copurifies with the Phox1-dependent SRF/SRE supershift. The protein fractions were monitored by 4%–20% SDS-PAGE revealing an abundant 120 kD protein that correlates with the supershifting activity. SDS-PAGE analysis shows phosphocellulose B fraction (lane 2) and Q-Sepharose fractions (lanes 3–10), visualized by coomassie blue. (D) A 120 kD protein from fraction 22 interacts directly with GST-Phox1, GST-SRF, but not with GST-PLCγ. The GST fusion proteins, including GST-Phox1, GST-SRF, and GST-PLCγ, were incubated with glutathione-agarose beads and washed several times to remove the background lysate proteins. The GST-Phox1, GST-SRF, and GST-PLCγ proteins (lanes 2, 3, and 4, respectively) were incubated with 20 μl of fraction 22 (lane 8) and washed several times, and the bound proteins were eluted off at 600 mM KCl. The 120 kD protein was eluted off the Phox1 beads (lane 5), the SRF beads (lane 6), but not the PLCγ beads (lane 7). The proteins were detected by 12% SDS-PAGE and stained with coomassie blue. (E) Purified 120 kD protein binds to an SRE-containing probe. Mobility-shift assay monitoring DNA-binding activity in the GST-Phox1 eluate (lane 1) and the PLCγ eluate (lane 2) using a 31-bp oligonucleotide probe containing the wild-type SRE.

SPIN on the coding and noncoding stands of each probe. The wild-type SIE probe (lanes 1–4) contains a wild-type SIE and a mutant SRE, whereas the wild-type SRE probe (lanes 5–8) contains a wild-type SRE and a mutant SIE.

Mutant probes were used to confine binding to a single site on each probe. Consistent with the DNase I footprinting assay, the methylated guanines that interfered with SPIN–DNA interactions corresponded to residues

in the vicinity of the SIE (lanes 2,4) and the SRE (lanes 6,8). The two sequences bound by SPIN are clearly related: 5'-GTCAAT-3' at the SIE and 5'-GTCCAT-3' at the SRE, where boldface type indicates the single discrepant position. These results show that SPIN is a sequence-specific DNA-binding protein that recognizes sequences in the vicinity of the SIE and the SRE in the *c-fos* promoter.

To determine whether SPIN binds to these sites with similar affinity, radiolabeled SPIN/SIE complexes were incubated with cold competitor oligonucleotides containing either SIE or SRE sequences. Figure 3B shows that SPIN bound to the SIE probe (lanes 1,6,11) in the absence of unlabeled competitor. Binding of SPIN was competed by the addition of excess unlabeled wild-type SIE (lanes 2,7,12) or wild-type SRE (lanes 4,9,14) but not by mutant SIE (lanes 3,8,13) or mutant SRE (lanes 5,10,15) oligonucleotides. These results suggest that SPIN binds specifically and with similar affinity to both the SIE and the SRE in the *c-fos* promoter.

Isolation of cDNA clones encoding SPIN

SPIN was purified by the fractionation scheme shown in Figure 2A, separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was stained with amido black, and the 120 kD band was excised. A 22-amino-acid peptide was generated by digestion with cyanogen bromide and sequenced by Edman degradation. The peptide sequence was used to design a 56-nucleotide guess-mer and run against the Genbank expressed sequence tag (EST) database (Lathe 1985). A

match was found and nucleotides in the wobble position of the guess-mer were modified to generate a perfectly homologous hybridization probe. The probe was used to screen a HeLa cell λ gt11 cDNA library, and 40 positive clones were obtained after three rounds of screening. The cDNAs isolated from three separate clones contained the same open reading frame (ORF) but differed in the 3'-untranslated region. The sequence encodes a 957-amino-acid protein with a predicted M_r of 108 kD. The 22-amino-acid peptide isolated from the purified protein was present in the predicted sequence of the encoded protein (underlined in Fig. 4A). There were no significant matches to any proteins in the database and no recognizable DNA-binding domain, suggesting that SPIN uses a novel structure for DNA recognition. The most significant feature of the sequence is an 88-amino-acid motif that is present six times, as four highly conserved repeats and two less well-conserved repeats (underlined in Fig. 4A).

SPIN is identical in sequence to the initiator binding protein TFII-I (A.L. Roy, H. Du, P.D. Gregor, C.D. Novina, E. Martinez, and R.G. Roeder, in prep.). Like the activity purified from HeLa cells, TFII-I binds specifically to the initiator Inr elements and activates transcription of a TATA[–]/Inr⁺ promoter in vitro. To determine whether Q-Sepharose-purified SPIN also binds to initiator elements, we performed a mobility-shift assay on a probe containing the Inr from the adenovirus major late (AdML) promoter (Manzano-Winkler et al. 1996). Figure 4B shows that SPIN bound to the AdML Inr element (lane 5) in addition to the SIE (lane 1) and the SRE (lane 3). However, under our conditions, competition analysis showed that SPIN binds to the SIE and SRE with

Figure 4. The SPIN cDNA and the TFII-I cDNA are identical; SPIN binds to an Inr element. (A) Clones encoding the SPIN protein were isolated from a human HeLa cell cDNA library. The SPIN ORF predicts a peptide of 957 amino acids that identifies a protein of 108 kD in its unmodified state. The peptide generated by Edman degradation is underlined and labeled; the six repeats are underlined and also labeled. (B) Native SPIN protein from HeLa cells binds to the AdML Inr element. Mobility-shift assays were performed using fraction 19 and 31-bp oligonucleotide probes containing the wild-type SIE (lane 1), m67 SIE, (lane 2), wild-type SRE (lane 3), pm12 SRE (lane 4), or AdML (lane 5).

slightly higher affinity than to the AdML Inr element (data not shown).

The TFII-I protein contains SPIN activities

To further establish the relationship between SPIN and TFII-I, we analyzed the SPIN-containing Q-Sepharose fractions with an antiserum raised against a synthetic peptide derived from TFII-I (C.D. Novina, V. Cheriya, and A.L. Roy, in prep.). The resulting blot showed that the SPIN/TFII-I antiserum recognized a 120 kD band in the same Q-Sepharose fractions that contained the supershifting and DNA-binding activities of SPIN (Fig. 5A, lanes 3–7; cf. to Fig. 2C).

To determine whether the SPIN/TFII-I antibody recognized the SIE/SRE DNA-binding activity in Q-Sepharose-purified SPIN, we added the antibody to a mobility-shift assay. Figure 5B shows that the SPIN/SIE complex was abolished by the SPIN/TFII-I antibody (lane 5) but not by preimmune serum (lane 4). The effect of the antibody is specific for SPIN/SIE complexes because it did not abolish binding of the Phox1 homeodomain to the SRE (lane 8). Interpretation of this experiment is somewhat complicated by the presence of contaminating protease activity in the SPIN/TFII-I antiserum, which partially cleaves all of the control proteins we have tested, creating novel high-mobility complexes on mobility-shift gels, as seen in lanes 5 and 8. Nevertheless, the antiserum specifically and reproducibly abolished SPIN DNA-binding activity at concentrations at which it had no effect on DNA-binding activity of control proteins.

To further establish that the cloned SPIN/TFII-I gene encodes a protein with the same DNA-binding specificity as the SPIN activity in HeLa cells, mobility-shift assays were performed with recombinant protein expressed in mammalian cells. The full-length protein was produced in COS cells as a GST fusion under the control of the EF-1 α promoter (Tanaka et al. 1995). The GST-SPIN protein is easily distinguished from endogenous

SPIN by its increased molecular mass (C.D. Novina, V. Cheriya, and A.L. Roy, in prep.). Figure 5C shows that purified GST-SPIN binds to the *c-fos* SIE site (lane 1) and less strongly to the mutant site (lane 2). These results confirm that the isolated SPIN cDNA encodes the 120 kD sequence-specific DNA-binding protein and that this activity also resides in TFII-I.

To establish that recombinant SPIN also interacts with Phox1 and SRF, we transfected COS cells with a mammalian expression plasmid producing GST-SPIN and either a Phox1 or an SRF expression plasmid. Nuclear extracts from transfected cells were incubated with SPIN/TFII-I antiserum, and immunoprecipitated complexes were probed with an antibody against epitope tags present at the amino termini of Phox1 and SRF. Figure 5D shows that full-length Phox1 coprecipitated with SPIN, as indicated by the presence of a 33-kD Phox1 band (lane 2) not seen in extracts of cells transfected with the empty expression plasmid (lane 1). Both full-length SRF (lane 7) and the SRF core domain (lane 8) also coprecipitated with SPIN. Thus, SPIN interacts with both Phox1 and SRF in nuclear extracts.

To assess the specificity of this interaction, we also examined three Phox1 mutants carrying amino acid substitutions in solvent-exposed side chains of homeodomain helices one and two. These residues have been shown previously to be required for the activity of Phox1 at the SRE in vivo but not for binding of Phox1 to the SRE in vitro, suggesting that they participate in protein-protein interactions required for in vivo activity (Gruneberg et al. 1995). One of the three Phox1 mutants, ED, which carries substitutions in glutamate and aspartate residues on homeodomain helix two, failed to coprecipitate with SPIN (lane 3), despite being expressed at levels equal to the other proteins (data not shown). Thus, recombinant SPIN interacts specifically with both Phox1 and SRF, and its interaction with Phox1 requires surface-exposed amino acids previously implicated in the activity of Phox1 at the SRE in vivo.

Figure 5. TFII-I protein contains SPIN activity. (A) SPIN/TFII-I antisera recognize the 120 kD protein in HeLa cells. The same protein fractions from Fig. 2C were separated by 12% SDS-PAGE, transferred to PVDF membrane, immunoblotted with a polyclonal antibody to SPIN/TFII-I, and visualized by chemiluminescence. The 120 kD immunoreactive band is present in phosphocellulose B fraction (lane 1) and a subset of Q-Sepharose fractions (lanes 2–9). (B) SPIN/TFII-I antisera disrupt the SPIN/SIE complex. A mobility-shift assay monitoring SPIN protein (fraction 19) binding to a 31-bp oligonucleotide probe containing the SIE site (lanes 3–5), or *E. coli*-produced Phox1 homeodomain binding to a 31-bp oligonucleotide probe containing the SRE (lanes 6–8). Lanes 5 and 8 were incubated with SPIN/TFII-I antibody; lanes 4 and 7 with preimmune serum; lanes 3 and 6 represent the no-antibody control. The SPIN/TFII-I antibody (lane 1) and the preimmune serum (lane 2) have no effect on probe alone. (C) Recombinant GST-SPIN binds to the same sequence as the native protein from HeLa cells. Mobility-shift assays monitoring the binding of recombinant GST-SPIN protein, produced in mammalian cells and purified to homogeneity (C.D. Novina, V. Cheriya, and A.L. Roy, in prep.), to the 31-bp oligonucleotide probes containing the wild-type SIE site (lane 1) or the m67 SIE site (lane 2). (D) Transfected Phox1 and SRF can be immunoprecipitated from COS cells with SPIN/TFII-I antibodies. COS cells were cotransfected with an expression plasmid producing GST-SPIN and expression plasmids producing the wild-type Phox1 protein (lane 2), mutant Phox1 protein substituting amino acids ED to AA at positions 32 and 33 of the homeodomain (lane 3), mutant Phox1 protein substituting amino acids RR to AA at positions 36 and 37 of the homeodomain (lane 4), mutant Phox1 protein substituting amino acids ER to AA at positions 21 and 22 of the homeodomain (lane 5), GST-SPIN (lane 6), SRF (lane 7), SRF core containing the DNA-binding domain (lane 8), or with an empty expression plasmid (lane 1). Transfected extracts were immunoprecipitated with SPIN/TFII-I antibody, analyzed by 12% SDS-PAGE, and visualized with the monoclonal antibody 12CA5, which recognizes the HA epitope located at the amino terminus of each protein.

SPIN/TFII-I cooperates with Phox1 to impart serum-responsive transcription to the *c-fos* SRE

Our biochemical data suggest that SPIN mediates the formation of productive SRF/Phox1 complexes on sequences such as the *c-fos* SRE. To test this hypothesis in vivo, we introduced expression vectors producing Phox1 and/or SPIN into HeLa cells together with a chloramphenicol acetyl transferase (CAT) reporter gene under the control of a basal *c-fos* promoter and a single copy of the *c-fos* SRE. Figure 6 shows that, as shown previously (Grueneberg et al. 1995), expression of Phox1 in HeLa cells leads to a 17-fold enhancement of serum-induced reporter gene activity (lane 2). Expression of SPIN had no effect on this activity at these concentrations, suggesting that SPIN on its own was incapable of promoting the formation of a signal responsive complex on the SRE (lane 1). However, the combination of Phox1 and SPIN was significantly more effective than Phox1 alone, suggesting that SPIN promotes the formation of Phox1-dependent signal responsive complexes on the SRE (lane 3). This effect was abolished on a reporter gene containing mutations that prevent SRF binding, suggesting indi-

Figure 6. SPIN interacts with SRF and Phox1 in mammalian cells and potentiates the transcriptional response. HeLa cells were cotransfected with expression plasmids producing the GST-SPIN protein (lane 1), wild-type Phox1 protein (lane 2), wild-type Phox1 and GST-SPIN (lane 3), mutant Phox1 substituting amino acids ED to AA at positions 32 and 33 of the homeodomain (lane 4), and Phox-ED and GST-SPIN (lane 5). Extracts were prepared from the cells 48 hr after transfection and assayed for CAT activity. The final numbers represent fold induction where activated transcription is divided by the basal transcription resulting from the blank expression plasmid.

rectly that SRF is also required for the formation of these complexes.

In contrast to wild-type Phox1, the mutant Phox1 derivative ED, which fails to bind SPIN, was unable to activate the SRE *in vivo* (Fig. 6, bar 4; see also Grueneberg et al. 1995). Furthermore, the activity of the ED mutant was not enhanced by coexpression of SPIN (lane 5). Thus, a direct interaction between Phox1 and SPIN is apparently required for SPIN to exert its enhancing activity on serum inducibility of the SRE *in vivo*. We conclude that SPIN promotes the interaction of multicomponent signal responsive complexes composed of Phox1 and SRF.

Discussion

We have been studying the interaction of the human homeodomain protein Phox1 with SRF. Phox1 was originally identified in a yeast screen through its ability to interact functionally with the yeast SRF homolog MCM1 (Grueneberg et al. 1992). We found that Phox1 exhibited a similar functional interaction with SRF in that it permitted certain SRF-binding sites to respond to incoming signal transduction information in mammalian cells (Grueneberg et al. 1995). These observations led us to propose that one aspect of the cell identity function harbored by many homeodomains may be the ability to endow cell-specific genes with the capacity to respond to extracellular signals and that this activity may be a key component of the cell identity function.

One difficulty with this hypothesis was that we lacked information on the biochemical organization of these putative SRF/homeodomain complexes. In particular, we could not reconstitute from purified proteins any stable SRF/Phox1 complexes that would be expected to mediate the activity we observed in transfected cells. Although Phox1 could influence the kinetics of DNA binding by SRF *in vitro*, strongly suggesting that these proteins interact directly, the interaction appeared to be highly transient in the absence of any other cofactors.

The idea that a missing cofactor might be required for stable complex formation was first suggested by our observation that extracts from yeast expressing Phox1 generated an MCM1-containing mobility-shift complex with distinct mobility from the complex generated by MCM1 alone (D.A. Grueneberg, unpubl.). Again, however, this complex could not be reconstituted with purified proteins, and preliminary reconstitution experiments with fractionated yeast extracts suggested that an additional activity was required to promote the formation of this novel complex. Here, we have purified a similar activity from human cells. We find that this protein has multiple activities, including the ability to interact with both SRF and Phox1, to bind specific sequences in the *c-fos* promoter, and to cooperate with Phox1 and SRF to mediate serum-inducible transcriptional activity at the SRE. Furthermore, we have discovered that this protein is identical to the transcription factor and initiator-binding protein TFII-I, suggesting that it may have a broader role in the organization of

multiprotein transcription factor complexes, particularly at developmentally regulated genes.

Interaction of SPIN/TFII-I with SRF and Phox1

Three distinct lines of evidence suggest that SPIN interacts with both SRF and Phox1. First, we can detect direct and independent interactions of SPIN with SRF and Phox1 using two experimental approaches. Native SPIN is selectively bound from complex protein fractions by GST fusions of both SRF and Phox1. And both SRF and Phox1 can be coimmunoprecipitated with SPIN following cotransfection in COS cells. Second, the novel SRF-containing complex is formed only when SRF, Phox1, and SPIN are all present. Third, SPIN significantly enhances the ability of Phox1 to impart serum-responsive transcriptional activity to the *c-fos* SRE. This activity requires amino acid residues in Phox1 that are also required for the direct interaction of Phox1 with SPIN, and it also requires an intact SRF binding site. Thus, we believe that formation of this active transcription complex requires the interaction of SPIN with both SRF and Phox1. Whether that interaction is simultaneous or sequential, or stable or transient, remains unclear.

The interaction of SPIN with Phox1 appears to require surfaces of the Phox1 homeodomain not directly involved in DNA binding. This idea is supported by the observation that a monoclonal antibody to Phox1 abolishes formation of the SPIN-dependent complex but does not affect DNA binding by Phox1. Furthermore, amino acid substitutions on the solvent-exposed surface of homeodomain helix two, previously shown not to affect DNA binding (Grueneberg et al. 1995), abolish both the interaction of Phox1 with SPIN in the coimmunoprecipitation assay and the ability of SPIN to augment the transcriptional activity of Phox1 at the SRE *in vivo*.

It remains difficult to envision the precise physical organization of the novel SRF/SRE complex that we observe on mobility-shift gels. Clearly, the reduction in mobility of this complex relative to the putative SRF/SRE binary complex is much less than would be expected for the incorporation of a 120-kD protein. When mobility-shift assays are performed using a *c-fos* promoter probe containing both the SIE and the SRE, which allows independent co-occupancy of the probe by SPIN and SRF, respectively, a complex of considerably slower mobility is observed (data not shown). This complex is clearly different from the one that forms on an isolated SRE in the presence of SPIN, SRF, and Phox1. This observation could mean that the action of SPIN is transient—either catalytic in a classic sense or merely unstable on the mobility-shift gels. An alternative hypothesis is that SPIN, SRF, and Phox1 are all present in the novel complex observed on the mobility-shift gels, but that the effect of this cooperative interaction is to dramatically compact the complex, increasing its mobility in the gel. A third possibility stems from the observation that SPIN binds only to one-half of the SRE dyad; in joining this complex, SPIN may displace one subunit of an SRF dimer.

Interaction of SPIN/TFII-I with DNA

As we purified SPIN activity, we noted the copurification of a DNA-binding activity that recognized the *c-fos* SRE. The purified protein and recombinant SPIN exhibit high-affinity binding to two critical regulatory elements of the *c-fos* promoter—the SRE, which mediates the response of *c-fos* to multiple signal transduction pathways, and the SIE, which is a binding site for STAT (signal transducer and activator of transcription) proteins (Gilman 1988; Treisman 1990; Fu and Zhang 1993; Sadowski et al. 1993; Zhong et al. 1994; Hill and Treisman 1995). The two bound sequences are closely related and are contained within the Inr consensus sequence T/CT/CANT/AT/CT/C (Javahery et al. 1994). SPIN, as purified from HeLa cells in our hands, also binds to the AdML Inr sequence, although it does so with noticeably lower affinity relative to the SRE and SIE sites.

Inspection of the promoter sequence of *c-fos* reveals at least two other sequences that match the binding consensus for SPIN. One of these sites is located at the transcriptional initiation site of the gene, and the other is located just downstream of the cAMP responsive element. Although these sites have not been tested directly for binding, this observation suggests that SPIN may make multiple contacts with the *c-fos* promoter. Whether these sites are bound independently by monomeric SPIN or coordinately by a higher-order multimer is not known. But the presence of multiple helix-loop-helix-like motifs in the TFII-I sequence suggests that multimeric forms of the protein may be common. It is of particular interest that a SPIN-binding site is closely associated with each of the major signal-responsive elements in the *c-fos* promoter.

Organization of a multiprotein complex at the c-fos promoter

SPIN can potentially interact with many sites in the *c-fos* promoter and with both upstream activator proteins and basal transcription factors. If even a subset of these interactions can be performed simultaneously by a single SPIN protein or multimer, it is likely that SPIN imposes a specific and restricted architecture on the *c-fos* promoter. This architecture may be critical to the unusual demands placed on this promoter. The *c-fos* gene is poised for rapid and high-magnitude transcriptional response to extracellular signals (Greenberg and Ziff 1984; Greenberg et al. 1985). The gene reaches maximal transcriptional activity within minutes of external stimulation (Greenberg and Ziff 1984; Kruijer et al. 1984; Greenberg et al. 1985). The promoter receives multiple signaling inputs through a set of transcription factors that are independently modulated by distinct signaling pathways (Graham and Gilman 1991; Marais et al. 1993; Hill et al. 1994). Furthermore, it is evident that the *c-fos* promoter can integrate incoming signaling information, as suggested, for example, by the observation that a promoter in which both the SRE and the SIE are intact responds more robustly than a promoter in which one of

the elements is missing (Hill and Treisman 1995). In this specific example, SPIN binding in the vicinity of both sites could coordinate communication between these signaling elements and relay integrated signaling information to the basal transcriptional machinery. Furthermore, the observation that SPIN is itself phosphorylated on serine, threonine, and tyrosine residues (C.D. Novina, V. Cheriya, and A.L. Roy, in prep.) and its recently reported association with the protein-tyrosine kinase Btk (Yang and Desiderio 1997) suggests additional mechanisms through which this protein may participate directly in the processing of signal transduction information into a transcriptional response.

Materials and methods*Mammalian expression plasmids*

For transient transfection assays in HeLa and COS cells, full-length Phox1 amino acids 1–217, Phox1 homeodomain amino acids 58–138, full-length SRF amino acids 1–508, and SRF core amino acid 92–222 were expressed from the cytomegalovirus (CMV)-based vector pCGN (Tanaka and Herr 1990). The Phox1 mutant constructs were described previously (Grueneberg et al. 1995). The pCGN vector contains the influenza virus hemagglutinin (HA) epitope tag inserted at the amino terminus of each effector plasmid (recognized by the 12CA5 antibody; Field et al. 1988). The full-length GST-SPIN (same as GST-TFII-I) containing amino acids 1–957 was expressed from the EF1 α vector pEBGI (C.D. Novina, V. Cheriya, and A.L. Roy, in prep.).

Transient transfection assay

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All transfections were performed using calcium phosphate coprecipitation as described (Grueneberg et al. 1995). Cells were seeded on 10-cm² plates and transfected at 30% confluence. For the CAT assay, transfection cocktails contained 0.6 μ g of wild-type Phox1, a mutant Phox1 derivative ED, or GST-SPIN expression plasmids along with 2 μ g of the reporter plasmid containing a single *c-fos* SRE upstream of a *c-fos*-CAT fusion gene containing *fos* sequences from –56 to +109. All other transfection cocktails contained 4.8 μ g of Phox1 and SRF expression plasmids and 3.2 μ g of the GST-SPIN expression plasmid. In all cases, the total DNA concentration was adjusted to 20 μ g with pUC119 DNA. The cells were incubated with the transfection precipitates for 16 hr, washed three times with phosphate-buffered saline (PBS), and incubated for 24 hr in DMEM containing 15% FBS. For the mobility-shift assays, HeLa cells were harvested and resuspended in 70 μ l of IP buffer [0.1% NP-40, 25 mM HEPES at pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. For the coimmunoprecipitation assay, COS cells were harvested and resuspended in 200 μ l of hypotonic buffer. For the CAT assay, HeLa cells were harvested and resuspended in 100 μ l of PBS. The CAT assay was done as described previously (Gorman et al. 1982).

E. coli expression plasmids

For *E. coli* expression, the full-length SRF protein was expressed from pT7f1.A (Kuret et al. 1988), a derivative of pET11c (Studier et al. 1990). SRF was used as a crude *E. coli* lysate. All other proteins were produced as GST fusions from the vector pET-GST, including Phox1 amino acids 5–217, Phox1-homeodomain

amino acids 58–138, and PLC γ amino acids 547–659. The GST fusion proteins were purified on glutathione-agarose (Sigma) (Smith and Johnson 1988) and the GST moiety was cleaved off the GST-Phox1 and GST-Phox1-homeodomain proteins with thrombin (Sigma) (Gearing et al. 1989).

Mobility-shift and DMS interference assays

For the mobility-shift assay using transfected cells, 10-cm² plates were harvested, resuspended in 70 μ l of IP buffer, and sonicated twice with 2-sec pulses. The extracts were centrifuged for 20 min to remove cell debris, and 2 μ l of the supernatant was used in DNA-binding assays. Assays contained 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol (DTT), 5% glycerol, 50 μ g/ml of poly[d(G-C)]–[d(G-C)]. Assays containing the bacterially produced proteins and the purified fractions are identical to the above, except they contain 75 mM NaCl, 15 μ g/ml of poly[d(G-C)]–[d(G-C)], 5 μ g of bovine serum albumin (BSA), and 3 mM MgCl₂. Reactions were incubated in the absence of probe for 10 min at room temperature. Following the addition of probe (20,000 cpm), the reactions were incubated for another 10 min. Analysis was typically on a 5% polyacrylamide gel (39:1 acrylamide/bis ratio) in 0.5 $\times$ TBE (Tris borate EDTA) buffer, except for Figure 1, which required a 3.5% polyacrylamide gel.

Mobility-shift probes used in Figures 1 and 2B contained the SRE sequence derived from the –56 $\times$ SRE *fos*-CAT fusion gene, where the SRE is positioned at –56 in the mouse *c-fos* promoter and contains sequences from –56 to +109 (Berkowitz et al. 1989; Gilman et al. 1986). Probes were synthesized in a PCR reaction using ³²P-end-labeled T3 and +23 primers, which flank the SRE sequence. All other mobility-shift probes were generated by annealing 31-bp oligonucleotides: the wild-type *c-fos* SRE 5'-AGCTTACAGGATGTCCATATTAGGACATCTG-3' (Gilman et al. 1988); the pm12 SRE 5'-AGCTTACAGGATGTGGATATTACCACATCTG-3' (Gilman et al. 1988); the wild-type *c-fos* SIE 5'-AGCTGCCGCGAGCAGTTCCCGTCAATCCCTC-3' (Hayes et al. 1987); the m67 SIE 5'-AGCTGCCGCGAGCATTTCCTCGTAAATCCCTC-3' (Wagner et al. 1990); and the AdML Inr 5'-AGCTTACAGGATGTCTCACTCTCTACATCTG-3' (Manzano-Winkler et al. 1996). Probes were labeled by a Klenow reaction with all four [α -³²P]deoxynucleotide triphosphates and gel purified before use.

Probes used for the DMS interference assay incorporated *c-fos* promoter sequence –362 to –266, which were radiolabeled on either strand in reactions containing one ³²P-end-labeled primer and one cold primer. The DMS interference protocol was described previously (Attar and Gilman 1992).

Purification of SPIN from HeLa extracts

HeLa cells were grown to 10⁶ cells/ml in spinner flasks containing Joklik's modified Eagle's medium supplemented with 5% calf serum. For the purification of SPIN, HeLa cell nuclear extracts were prepared according to Dignam et al. (1983). Approximately 0.5 liter of nuclear extract was selectively precipitated using ammonium sulfate [(NH₄)₂SO₄] from 18% to 32%. The precipitate was resuspended in 100 ml of buffer D [20 mM HEPES (pH 7.9), 0.2 mM EDTA, 20% glycerol, 3 mM DTT, 0.5 mM PMSF] containing 90 mM KCl and was subsequently dialyzed against the same buffer for 3 hr at 4°C. The 18%–32% (NH₄)₂SO₄-precipitated material (1.1 gram protein) was loaded on a P11 (Whatman) phosphocellulose column (8 mg of protein/ml of packed resin). The column was washed with 3 column volumes of buffer D + 100 mM KCl (P11-A fraction) and then step eluted with 3 column volumes of buffer D + 350 mM KCl

(P11-B fraction), followed by 3 column volumes of buffer D + 500 mM KCl (P11-C fraction) and buffer D + 1 M KCl (P11-D fraction). The P11-B fraction contained SPIN activity and was diluted with 2 volumes of buffer Q0 [20 mM HEPES (pH 7.9), 0.5 mM EDTA, 5% glycerol, 0.1% Tween-20, 1 mM DTT, 0.5 mM PMSF, 1 mM sodium metabisulfite, 1 mM benzamidine, 1 μ M pepstatin A] to reduce the KCl concentration and was loaded onto a Mono-Q HR 10/10 column (Pharmacia). The column was washed with 40 ml of buffer Q100 (Q0 containing 100 mM KCl) and the bound material was eluted with an 80-ml linear gradient of 100 mM to 1 M KCl in buffer Q. Fractions were collected (BMQ fractions, 1 ml each) and were tested for SPIN activity by mobility-shift assay. The peak of SPIN activity eluted between 180 and 250 mM KCl (fractions BMQ 16–BMQ 20).

Generation of SPIN peptides

Mono-Q fractions 19 and 20 were combined and resuspended in 2 $\times$ SDS sample buffer, heated to 90°C for 5 min, and subjected to 10% SDS-PAGE. The polypeptides were transferred to PVDF membrane by wet transfer in a buffer containing 25 mM Tris base, 192 mM glycine, and 20% methanol. The membrane was stained with amido black to visualize the 120 kD band, which was excised and treated with 150 mM cyanogen bromide (CNBr) in 70% formic acid for 5 hr at 55°C. The resulting peptides were analyzed using an ABI 494 Procise sequencer revealing a strong proline residue at cycle three. The CNBr digestion was repeated, and the sequencer was stopped at cycle two exposing a peptide with a proline residue at position 3 from the amino terminus. The membrane was subsequently treated with *o*-phthalaldehyde (OPA), blocking all CNBr-generated peptides except the proline-containing peptide, and sequencing commenced from the proline at position 3 out to position 22 (Brauer et al. 1984).

cDNA library screening

The peptide generated by microsequencing was converted into a guess-mer probe and searched against the Genbank EST data base to generate an exact match probe (Lathe 1985). The oligonucleotide probe 5'-GATGATGATTATTCTCCACCGTCTAAGAGACCAAAGGCCAATGAGCTACCGCAGCC-3' was used to screen an oligo(dT) plus random-primed λ gt11 cDNA library (Clontech), derived from human HeLa cells. Approximately 300,000 plaques were lifted onto Hybond-N membrane (Amersham) and probed with the 5'-end-labeled oligonucleotide probe. The filters were soaked for 4 hr at 58°C in prehybridization solution 2 (Clontech) containing 6 $\times$ SSPE, 5 $\times$ Denhardt's solution, 0.25% SDS, and 100 μ g/ml of herring sperm DNA. Hybridization was carried out in the same solution; however, the radiolabeled probe was included and the reaction was extended for another 16 hr. Filters were rinsed in 2 $\times$ SSC with 0.05% SDS for 1 hr at 37°C, followed by a more stringent wash at 58°C for 15 min. After three rounds of successive screening, 40 positive clones were obtained and the DNA isolated from three revealing 3.0-, 3.4-, and 4.0-kb cDNAs. All three cDNAs were amplified in a PCR reaction using λ gt11 5' and 3' insert amplifying primers (Clontech) and cloned into a TA cloning vector (Invitrogen). Two of the cDNA clones were sequenced on both strands to generate a putative ORF, which, when compared to the sequence of TFII-I (A.L. Roy, H. Du, P.D. Gregor, C.D. Novina, E. Martinez, and R.G. Roeder, in prep.), revealed our sequence was missing 90 amino acids from the amino terminus.

Generation of TFII-I antibodies and immunoblotting

Polyclonal antibodies were raised against the synthetic peptide: GKRRVREFNFEWNARITDLR generated from the deduced

amino acid sequence of the TFII-I cDNA (C.D. Novina, V. Cheriya, and A.L. Roy, in prep.). For the coimmunoprecipitation assay, the SPIN/TFII-I antibodies were purified using protein A-Sepharose, enriching for the IgG fraction.

All immunoblots were blocked in 5% BSA/PBS for 1 hr at room temperature and washed three times in Western wash buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, and 0.2% NP-40. The dilution of the primary antibody was typically 1:2500, and the secondary antibody was 1:1500. The primary antibodies, SPIN/TFII-I and 12CA5, and secondary anti-rabbit or anti-mouse horseradish peroxidase conjugate antibodies were incubated in 2.5% BSA/PBS for 1 hr at room temperature and washed five times in Western wash buffer. The immunoreactive proteins were visualized by chemiluminescence (NEN).

Nuclear extracts and coimmunoprecipitation assay

For the coimmunoprecipitation assay transfected-COS cells were harvested and resuspended in 200 μ l of hypotonic buffer containing 20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na_3VO_4 , 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 0.125 μ M okadaic acid, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 1 μ g/ml each of leupeptin, aprotinin, and pepstatin (Sadowski and Gilman 1993). The cells were incubated on ice for 15 min and lysed by five passages through a 25-gauge needle. The nuclei were collected by centrifugation and resuspended in 100 μ l of the above buffer containing 420 mM NaCl and 10% glycerol, for the extraction of nuclear proteins. For the immunoprecipitation assay, the nuclear extracts were precleared in a reaction containing 50 μ l of nuclear extract, 350 μ l of hypotonic buffer, and 50 μ l of protein A-Sepharose. Following centrifugation to remove the protein A-Sepharose, 7 μ l of purified TFII-I antibody was added to the reaction and incubated for 4 hr at 4°C. The immune complexes were recovered by the addition of 50 μ l of protein A-Sepharose and washed five times in hypotonic buffer containing 57 mM NaCl and 0.1% NP-40. After the final wash, 2 $\times$ SDS sample was added, and the beads were heated to 90°C for 5 min, analyzed by SDS PAGE, and immunoblotted with 12CA5 antibodies that recognize the HA epitope fused to the amino terminus of each exogenously produced protein. The immunoreactive proteins were visualized by chemiluminescence.

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A multifunctional DNA-binding protein that promotes the formation of serum response factor/homeodomain complexes: identity to TFII-I

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