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Sequence-Specific Targeting of Nuclear Signal Transduction Pathways by Homeodomain Proteins

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Cells translate extracellular signals into specific programs of gene expression that reflect their developmental history or identity. We present evidence that one way this interpretation may be performed is by cooperative interactions between serum response factor (SRF) and certain homeodomain proteins. We show that human and *Drosophila* homeodomain proteins of the *paired* class have the ability to recruit SRF to DNA sequences not efficiently recognized by SRF on its own, thereby imparting to a linked reporter gene the potential to respond to polypeptide growth factors. This activity requires both the DNA-binding activity of the homeodomain and putative protein-protein contact residues on the exposed surfaces of homeodomain helices 1 and 2. The ability of the homeodomain to impart signal responsiveness is DNA sequence specific, and this specificity differs from the simple DNA-binding specificity of the homeodomain in vitro. The homeodomain imparts response to a spectrum of signals characteristic of the natural SRF-binding site in the c-*fos* gene. Response to some of these signals is dependent on the secondary recruitment of SRF-dependent ternary complex factors, and we show directly that a homeodomain can promote the recruitment of one such factor, Elk1. We infer that SRF and homeodomains interact cooperatively on DNA and that formation of SRF-homeodomain complexes permits the recruitment of signal-responsive SRF accessory proteins. The ability to route extracellular signals to specific target genes is a novel activity of the homeodomain, which may contribute to the identity function displayed by many homeodomain genes.

Throughout development, cells respond to extracellular cues with specific patterns of gene expression. The specificity inherent in this process must reside at least in part in the nucleus, because most cytoplasmic signal transduction pathways are composed of ubiquitously expressed proteins shared by many receptors. In *Saccharomyces cerevisiae*, for example, haploid cells of opposite mating types interpret the activation of an identical signal transduction pathway into distinct patterns of gene expression (2, 38). This interpretation occurs at the level of transcription by routing of the incoming signal to cell-type-specific genes (54).

Similar information processing must occur in animal cells. In *Drosophila melanogaster*, several receptor tyrosine kinases have common signal transduction machinery but elicit different responses in the receiving cells (5, 12, 13; for a review, see reference 41). In mammalian cells as well, both receptor tyrosine kinases and noncatalytic receptors of the cytokine class use shared signal transduction pathways and transmit generic signals that are differentially interpreted by the receiving cell. For example, the nerve growth factor receptor, encoded by the *trk* gene, induces terminal differentiation in PC12 cells, but it induces mitogenesis in fibroblasts and meiotic maturation in *Xenopus* oocytes (10, 39). Thus, distinct receptors elicit common intracellular signals that must be interpreted by the receiving cell in the context of its developmental history or identity.

How is generic signal transduction information interpreted into a cell-type-specific program of gene expression? We have proposed that this interpretation occurs by collaboration of transcription factors of two classes—homeodomain and MADS box proteins (19). Homeodomain proteins have roles in establishing cell and segmental identity in animals and are thought to exert this function by acting as transcription factors (37, 51). MADS box proteins are less well understood at a biological level. In plants, several genes of this class exhibit homeotic functions in flower development (9, 50). Two relatively well understood MADS box proteins, the yeast protein encoded by the *MCM1* gene and the mammalian serum response factor (SRF), are transcription factors that link genes to signal transduction pathways (59). Mcm1 is required for the pheromone activation of cell-type-specific genes in yeast cells, whereas SRF is required for the activation of the proto-oncogene c-*fos* and other immediate-early genes by growth factors and cytokines. Both Mcm1 and SRF are known to interact with proteins of the homeodomain family (19, 27). The physical association of proteins of the homeodomain and MADS box families is consistent with the idea that these proteins cooperate to translate incoming signaling information into cell-type-specific patterns of gene expression.

We previously described a genetic selection in yeast cells for human cDNAs encoding proteins that cooperate with Mcm1 to activate a cell-type-specific, pheromone-inducible reporter gene in yeast cells (19). We isolated cDNAs encoding a novel human *paired* class homeodomain protein, Phox1. We found that Phox1 interacts in vitro with both Mcm1 and SRF. Phox1 enhances the binding of SRF to its cognate site in the c-*fos* gene, the serum response element (SRE). Under in vitro conditions with bacterially expressed proteins, the enhancement was primarily kinetic and stable complexes of SRF and Phox1 were not detected. The enhancement activity resided within the homeodomain of Phox1 and was shared with related

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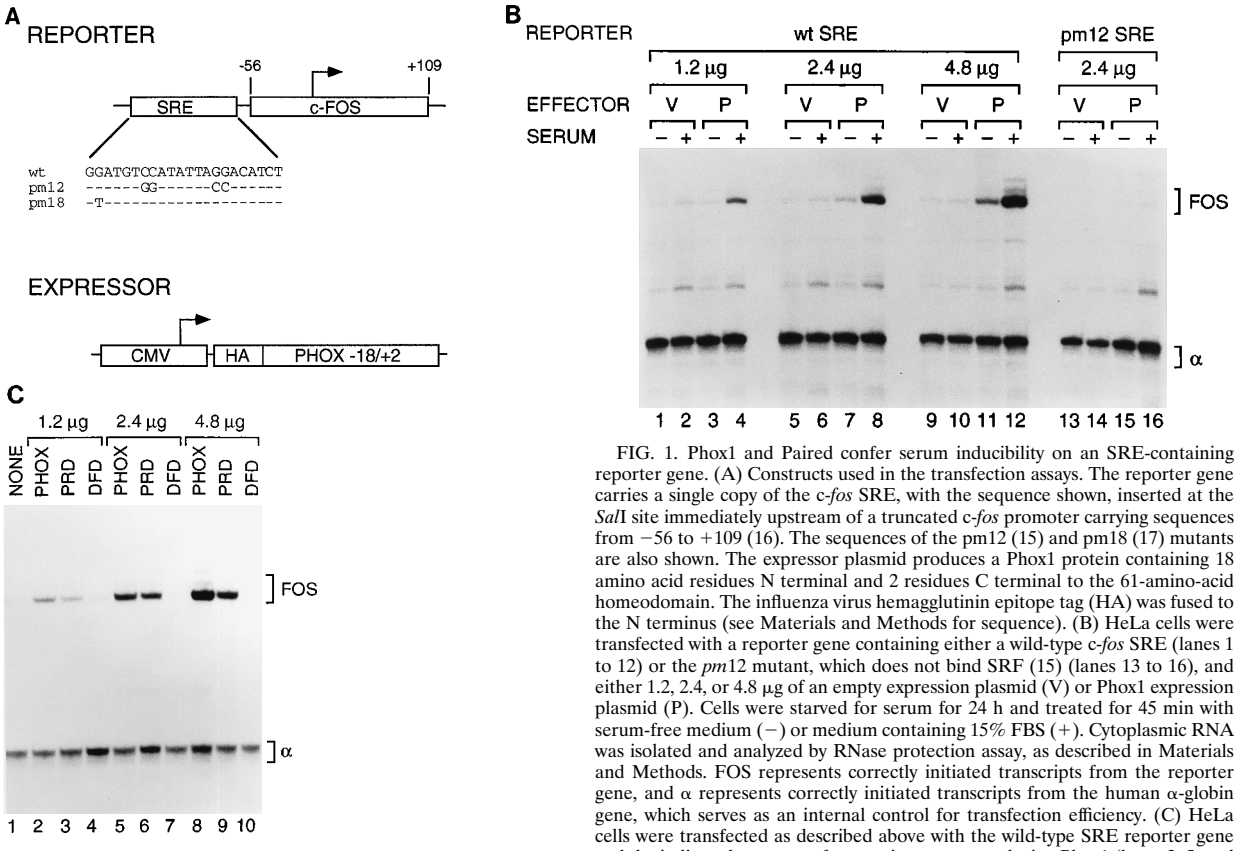

FIG. 1. Phox1 and Paired confer serum inducibility on an SRE-containing reporter gene. (A) Constructs used in the transfection assays. The reporter gene carries a single copy of the c-*fos* SRE, with the sequence shown, inserted at the *Sal*I site immediately upstream of a truncated c-*fos* promoter carrying sequences from −56 to +109 (16). The sequences of the pm12 (15) and pm18 (17) mutants are also shown. The expressor plasmid produces a Phox1 protein containing 18 amino acid residues N terminal and 2 residues C terminal to the 61-amino-acid homeodomain. The influenza virus hemagglutinin epitope tag (HA) was fused to the N terminus (see Materials and Methods for sequence). (B) HeLa cells were transfected with a reporter gene containing either a wild-type c-*fos* SRE (lanes 1 to 12) or the *pm*12 mutant, which does not bind SRF (15) (lanes 13 to 16), and either 1.2, 2.4, or 4.8 μg of an empty expression plasmid (V) or Phox1 expression plasmid (P). Cells were starved for serum for 24 h and treated for 45 min with serum-free medium (−) or medium containing 15% FBS (+). Cytoplasmic RNA was isolated and analyzed by RNase protection assay, as described in Materials and Methods. FOS represents correctly initiated transcripts from the reporter gene, and α represents correctly initiated transcripts from the human α-globin gene, which serves as an internal control for transfection efficiency. (C) HeLa cells were transfected as described above with the wild-type SRE reporter gene and the indicated amounts of expression vector producing Phox1 (lanes 2, 5, and 8), Paired (lanes 3, 6, and 9), or Deformed (lanes 4, 7, and 10) homeodomains.

D. melanogaster homeodomains, including those encoded by the *paired* and *orthodenticle* genes. These results led us to propose that one aspect of the identity function of homeodomains may be the ability to route signals to cell-type-specific genes and that homeodomains accomplish this by recruiting MADS box proteins to these sites, thereby connecting cell-type-specific genes to signal transduction pathways. Here we have tested several predictions of this model. We find that human and *D. melanogaster* homeodomain proteins can indeed impart growth factor responsiveness to a reporter gene that is unresponsive to signals in their absence. They apparently do this by promoting the assembly of a multiprotein complex that includes SRF and its signal-responsive accessory proteins.

MATERIALS AND METHODS

Plasmid constructions. For transient transfection assays in HeLa cells, the reporter plasmid carried a single copy of the c-*fos* SRE positioned upstream of a c-*fos*–chloramphenicol acetyltransferase fusion gene containing mouse c-*fos* sequence from position −56 to +109 (4, 16) (see Fig. 1A). A fragment of Phox1 encompassing amino acids 58 to 138 was expressed from the cytomegalovirus-based vector pCGN (56). This fragment includes the Phox1 homeodomain, 18 N-terminal amino acids, and 2 C-terminal amino acids. In addition, the vector incorporates an N-terminal extension with the sequence MASSYPYDVPDYASLGGPSRM, which includes the influenza virus hemagglutinin epitope tag (underlined). Analogous 81-amino-acid fragments of the *Drosophila* Paired and Deformed proteins were produced from the same vector.

The Elk1-VP16 expression vector was constructed by inserting the entire Elk1 cDNA (45) into a pCGN-based expression vector. This vector fuses to the N terminus of Elk1, the influenza virus hemagglutinin epitope, the nuclear localization signal of simian virus 40 large T antigen, and amino acids 411 to 490 of the herpes simplex virus VP16 protein.

The SRF in vitro translation vector was pT7-ATG (40). The Phox1 cDNA was inserted into the plasmid pT7βXBL (1), which expresses cDNAs under the control of the T7 promoter and the human β-globin 5′ untranslated region.

Site-directed mutagenesis of the Phox1 cDNA (31) was performed with single-stranded DNA derived from the pCGN expression plasmids. The mutagenic oligonucleotides were as follows (substitutions are underlined): *pm1*, 5′-CTG CAGGCTTTGGCGGCTGTCTTTGAG-3′; *pm2*, 5′-CGAGAAGACCTTGCC GCGGCTGTGAACCTC-3′; and *pm3*, 5′-GATGCTTTTGTGCGAGCAG CGCTTGCCCGC-3′. The sequence encoding the N51Q mutant of Phox1 (i.e., with an N-to-Q change at residue 51) was excised from the previously described GST fusion plasmid (19) as an *Xba*I-*Bam*HI fragment and inserted into pCGN in a fashion identical to that used for other pCGN constructs.

The variant SRE reporter genes were constructed by annealing the following oligonucleotides [in which $(A/T)_6$ refers to six positions that were synthesized with an equimolar ratio of adenosine and thymidine precursors]: 5′-AGCTTG CATGCCTGCAGGTCGTCGGATGTCC$(A/T)_6$GGACATCTG-3′ and 5′-TC GACAGATGTCC$(A/T)_6$GGACATCCGACGACCTGCAGGCATGCA-3′. The oligonucleotides were phosphorylated, annealed, and ligated into the c-*fos*–chloramphenicol acetyltransferase reporter plasmid that had been cleaved with *Hin*dIII and *Sal*I to remove the wild-type SRE. The ligation products were transformed into *Escherichia coli*, and transformants were grown as a pooled population. Plasmid DNA was extracted from the pool and retransformed into *E. coli*. Individual colonies were picked at random, and their plasmids were sequenced.

Transient-transfection assay. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). All transfections were performed with calcium phosphate coprecipitation as described previously (19). Cells were seeded on 10-cm-diameter plates and transfected at 30% confluence. Transfection cocktails contained 3 μg of reporter plasmid, 4.8 μg of Phox1 expression plasmid, unless otherwise indicated, and 0.1 μg of human α-globin plasmid as an internal control for transfection efficiency. In all cases, the total DNA concentration was adjusted to 20 μg with pUC119 DNA. The cells were incubated with the transfection precipitates for 16 h, washed three times with phosphate-buffered saline, and incubated for 24 h in Dulbecco's modified Eagle's medium containing 0.5% FBS to achieve serum starvation. The serum-starved cells were treated with serum-free Dulbecco's modified Eagle's medium containing either no inducer, 15% FBS, 100 ng of phorbol myristate acetate (PMA) per ml, 100 ng of epidermal growth factor per ml, 3 μM ionomycin, or a

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mixture of 10 μM forskolin and 0.5 mM isobutylmethylxanthine. After 45 min of treatment, cells were harvested and resuspended in 200 μl of 10 mM Tris (pH 8.0)–150 mM NaCl. A 180-μl portion of this suspension was used to prepare total cytoplasmic RNA (55), and a 20-μl portion was used to prepare whole-cell extracts for DNA-binding assays and immunoblots.

RNase protection assays were performed as previously described (15, 19). Aliquots of the final RNase protection products representing equal numbers of transfected cells were analyzed on a first gel. Protected products on this gel were quantitated with a Fuji BioImage analyzer. The remaining aliquots of the RNase protection reaction mixtures were adjusted to normalize the signal from the α-globin internal control and run on a second gel.

DNA-binding assays. For mobility shift assays of transfected cells, the 20-μl aliquot of cell suspension was centrifuged, resuspended in 70 μl of immunoprecipitation (IP) buffer (0.1% Nonidet P-40, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride), and sonicated twice with 2-s pulses. The extracts were centrifuged for 20 min to remove cell debris, and 2 μl of the supernatant was used in DNA-binding assays. Assays contained 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 5% glycerol, and 50 μg of poly(dI-dC) · (dI-dC) per ml. Assays of in vitro-translated proteins contained 1 μl of lysate in a 20-μl reaction mixture identical to the one described above, except that it contained 5 μg of poly(dI-dC) · (dI-dC) per ml and 5 μg of bovine serum albumin. The samples were further processed as described in reference 19.

RESULTS

The Phox1 and paired homeodomains confer response to cellular signal transduction pathways on an SRE-containing reporter gene in mammalian cells. In yeast cells, Phox1 activates a reporter gene under the control of three tandemly repeated copies of the α-cell-specific, pheromone-inducible upstream activation sequence (UAS) of the *STE3* gene (19). This UAS is normally bound cooperatively by a complex containing the yeast MADS box protein Mcm1 and the product of the mating type gene *MATα1* (3, 26, 44). In the absence of α1, Mcm1 does not bind to this site and reporter gene expression is extinguished. Phox1 restored reporter gene expression, presumably by substituting for α1 and recruiting Mcm1 to the reporter gene via a cooperative interaction. Biochemical analysis of extracts of yeast cells expressing Phox1 supports this hypothesis (18).

To detect a similar interaction between Phox1 and SRF in mammalian cells, we constructed a reporter gene carrying a single copy of the c-*fos* SRE positioned upstream of a basal c-*fos* promoter containing sequences from −56 to +109 (Fig. 1A). Although this site confers serum responsiveness in its natural promoter context, it imparts only weak constitutive activity to the reporter gene in HeLa cells and in other cell lines we have tested (Fig. 1B and data not shown). We speculate that SRF function in the natural c-*fos* promoter may require cooperation with other elements missing from this simplified promoter. If Phox1 were able to interact with SRF in vivo and enhance its binding to the SRE as we observed in our biochemical assays, then expression of Phox1 should impart serum-inducible transcription to the reporter gene. To test this prediction, we cotransfected the reporter gene into HeLa cells with an expression vector that produced an 81-amino-acid fragment of the Phox1 protein (Fig. 1A). This fragment encompassed the Phox1 homeodomain, 18 amino-terminal amino acid residues, and 2 carboxy-terminal residues. In all experiments, protein expression was verified by immunoblot or indirect immunofluorescence with a monoclonal antibody directed against an N-terminal epitope tag incorporated into the protein or by mobility shift assay of extracts (data not shown). Following transfection, cells were starved for serum for 24 h and then treated with 15% serum for 45 min. RNA was isolated from the cells and analyzed by RNase protection for correctly initiated transcripts arising from the reporter gene and from a human α-globin plasmid used as an internal control.

Figure 1B shows that when the SRE-containing reporter gene was cotransfected with an empty expression vector, it directed the expression of low levels of mRNA that were not significantly elevated by serum stimulation. In contrast, when cotransfected with an expression vector producing the Phox1 homeodomain, the reporter became inducible by serum (Fig. 1B, lanes 4, 8, and 12). The degree of serum-induced transcription of the reporter gene increased with the dose of Phox1 expression plasmid, suggesting that this assay responds to the concentration and activity of Phox1 protein. We conclude that the Phox1 homeodomain can impart serum responsiveness to an SRE-containing reporter gene.

Phox1 is capable of binding directly to the c-*fos* SRE, albeit with low affinity (19). To test whether Phox1 acted alone to activate the reporter gene or required the cooperation of endogenous cellular proteins such as SRF, we constructed a reporter that carries four nucleotide substitutions in the SRE (pm12) (Fig. 1A). This mutation reduces the affinity of this sequence for SRF by at least 2 orders of magnitude without significantly affecting the binding of Phox1 (reference 15 and data not shown). This mutant reporter was not activated by Phox1 in vivo (Fig. 1B, lanes 15 and 16). Thus, binding of Phox1 to DNA is not sufficient to impart serum inducibility (see also Fig. 5). Rather, Phox1 requires the cooperation of cellular proteins with DNA-binding specificity related to that of SRF. These data, together with our previous observation that Phox1 enhances the activation of this reporter gene by an SRF-VP16 fusion protein (19), suggest that Phox1 interacts with endogenous SRF in HeLa cells and recruits it to the reporter gene in a functional form that enables the gene to respond to serum growth factors.

We originally observed that the ability of the Phox1 homeodomain to enhance the binding of SRF to the SRE in in vitro assays was shared with the *D. melanogaster* Paired (Prd) protein but not with more distantly related homeodomains (19). To determine if this specificity was preserved in this in vivo assay, we tested the Prd homeodomain and the distantly related homeodomain encoded by the *Deformed* (*Dfd*) gene. Figure 1C shows that, like Phox1, Prd was able to impart serum responsiveness to the SRE reporter gene in a dose-dependent fashion. In contrast, Dfd was inactive. All three proteins were expressed at equivalent levels in this experiment (data not shown). Thus, the ability to impart serum responsiveness to this gene is specific for certain homeodomains and does not reflect a more general activity shared by all homeodomain proteins. Moreover, this activity is conserved in a *D. melanogaster* segmentation gene product.

Phox1 enables the recruitment of SRF-dependent accessory factors. As a further test of the hypothesis that Phox1 recruits functional SRF to the reporter gene, we asked whether the reporter gene adopted a pattern of signal responsiveness consistent with the known functions of SRF in the c-*fos* promoter. The c-*fos* SRE is sufficient for response to whole serum, purified polypeptide growth factors, and phorbol esters but not for response to cyclic AMP (cAMP) or calcium ionophores (57). Figure 2A (lanes 1 to 6) shows that the reporter gene exhibited an identical pattern of responsiveness to these signals in the presence of Phox1. It was induced by serum, epidermal growth factor, and PMA but not by agents that elevate intracellular cAMP or calcium. The reporter gene responded to none of these signals in the absence of Phox1 (data not shown). Thus, Phox1 imparts responsiveness to a subset of cellular signal transduction pathways previously associated with SRF.

The ability of SRF to mediate a transcriptional response to certain signal transduction pathways requires the recruitment of signal-responsive ternary complex factors (TCFs), including

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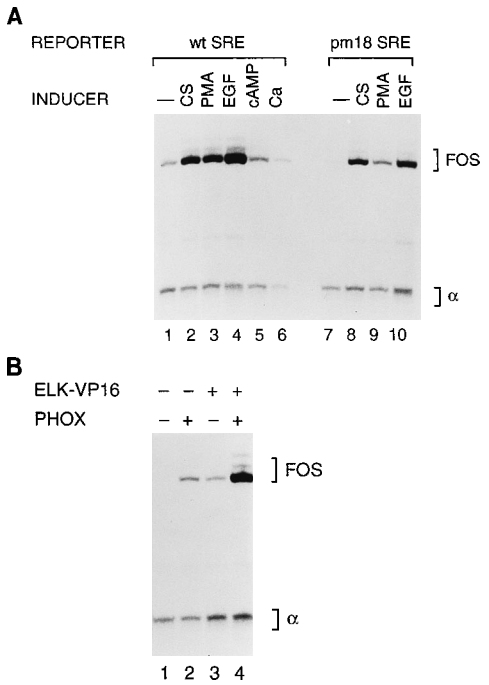

FIG. 2. Phox1 confers response to growth factors and phorbol ester and promotes recruitment of SRF-dependent ternary complex factors. (A) HeLa cells were transfected with wild-type Phox1 expression plasmid and the wild-type SRE reporter (lanes 1 to 6) or the pm18 mutant (lanes 7 to 10), which binds SRF but does not recruit ternary complex factors (17). After serum starvation, the transfected cells were treated for 45 min with serum-free medium supplemented with nothing (lanes 1 and 7), 15% FBS (lanes 2 and 8), 100 ng of PMA per ml (lanes 3 and 9), 100 ng of epidermal growth factor per ml (lanes 4 and 10), 10 μM forskolin and 0.5 mM isobutylmethylxanthine (lane 5), and 3 μM ionomycin (lane 6). RNA was isolated and analyzed by RNase protection assays. (B) HeLa cells were cotransfected with the wild-type SRE reporter alone (lane 1), with 2.4 μg of Phox1 expression vector (lane 2), with 50 ng of expression vector producing an Elk1-VP16 fusion protein (lane 3), or with both (lane 4). Cells were deprived of serum for 24 h with no further treatment. Cytoplasmic RNA was isolated and analyzed by RNase protection.

the ets domain protein Elk1 (11, 17, 23, 24, 52). The TCFs do not bind to the SRE on their own; instead, they recognize the SRF-SRE complex, making additional DNA contacts to the 5′ side of the SRF recognition site. Substitutions in these nucleotides prevent the recruitment of TCFs and abolish the response of the SRE to some signal transduction pathways, particularly protein kinase C-dependent signals (17). If Phox1 imparts response to the protein kinase C activator PMA by recruiting SRF and SRF in turn recruits TCFs, then activation by PMA in the presence of Phox1 should depend on sequences recognized by TCFs. To test this prediction, we assayed Phox1 on a reporter gene carrying an SRE mutant (pm18) (Fig. 1A) that binds SRF but cannot recruit TCFs (17). Figure 2A (lanes 7 to 10) shows that Phox1 imparts to this reporter only a subset of the responses observed with the wild-type sequence: response to PMA was significantly reduced, whereas responses to serum and epidermal growth factor were largely unaffected. This pattern is consistent with the activity of the SRE in the natural c-*fos* promoter and suggests that Phox1 recruits SRF to the reporter in a configuration that allows SRF in turn to recruit TCFs.

To measure directly the ability of Phox1 to effect the recruitment of SRF-dependent accessory proteins, we constructed an expression vector producing the Elk1 protein fused to the transcriptional activation domain of VP16. Recruitment of Elk1-VP16 to the SRE should activate the reporter gene even in the absence of signals because of the constitutive activity of the VP16 domain. Figure 2B shows that Elk1-VP16 elicited weak but detectable activation of the reporter gene in serum-starved cells in the absence of Phox1 (lane 3), suggesting that the reporter retains a limited ability to bind endogenous SRF in the absence of Phox1 and perhaps reflecting the ability of Elk1 to interact cooperatively with SRF (49). In the presence of Phox1, however, Elk1-VP16 strongly activated the reporter gene, which is consistent with enhanced occupancy of the SRE by SRF under these conditions. Thus, we conclude that Phox1 interacts cooperatively with SRF in vivo to enhance the binding of SRF to the SRE. In the resulting complex, SRF retains the ability to recruit at least one of its signal-responsive accessory proteins.

Phox1 homeodomain function requires solvent-exposed amino acid side chains. We used the serum response assay to determine what portions of the Phox1 homeodomain were required for its ability to recruit SRF to the SRE in vivo. We originally found in our biochemical analysis that the ability of Phox1 to enhance the binding of SRF to the SRE requires a direct interaction between Phox1 and SRF (19). This observation predicts that the activity of Phox1 in vivo should require amino acid side chains on the solvent-exposed surfaces of the homeodomain (30). To test this prediction, we generated three mutant Phox1 homeodomains, each carrying two amino acid substitutions at positions predicted to lie on the solvent-exposed surfaces of homeodomain helices 1 and 2, where they could potentially contact other proteins. In all cases, the substitutions changed charged amino acid residues to alanines. The mutants were tested for their ability to impart serum responsiveness to the SRE reporter gene in HeLa cells. The expression and activity of the mutant proteins were verified by immunoblotting and mobility shift assays of transfected cell extracts.

Figure 3 shows that the three mutants had significantly reduced activity in the reporter gene assay, suggesting that the substituted amino acid residues were required for full activity of the homeodomain in this assay. Mutant proteins 1 and 2 were expressed and bound DNA at levels equivalent to those of wild-type Phox1 (data not shown). This observation suggests that these proteins were properly folded in vivo and therefore that the defect observed in this assay is likely due to a specific requirement for the altered amino acid side chains. Mutant 3 was expressed at similar levels but had reduced activity on a wild-type SRE probe, which carries a low-affinity Phox1-binding site, although it had wild-type activity on an SRE variant that carries a high-affinity binding site. In view of the possible reduction in the DNA-binding activity of mutant 3, we are uncertain of the significance of the reduced activity of this mutant in the reporter gene assay. Nevertheless, we conclude that the amino acid side chains on helices 1 and 2 defined by mutants 1 and 2, respectively, are required for the full activity of the Phox1 homeodomain in imparting serum responsiveness to the SRE reporter gene. On the basis of their predicted positions on the homeodomain, we infer that these residues are engaged in direct contact with SRF or another protein in the complex that forms on the SRE.

Phox1 homeodomain function in vivo requires DNA-binding activity. We found previously that the ability of the Phox1 homeodomain to interact with SRF in vitro and enhance the kinetics of its binding to the SRE was not affected by an amino acid substitution in helix 3 that abolished the DNA-binding activity of the homeodomain. Although this observation supported the argument that protein-protein contacts must play a predominant role in the SRF-Phox1 interaction under our in

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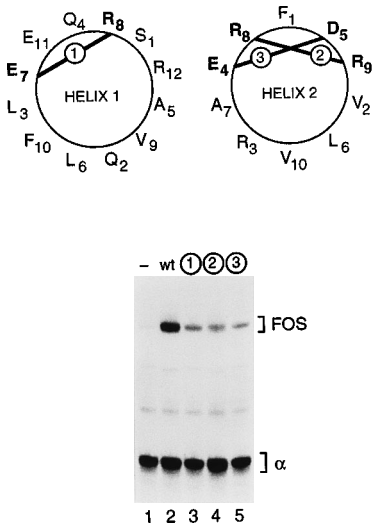

FIG. 3. Amino acid residues on the solvent-exposed surfaces of homeodomain helices 1 and 2 are required for maximal activity. The diagrams at the top represent homeodomain helices 1 and 2 oriented so that homeodomain helix 3 and the DNA are down and the solvent is up. The amino acid residues in boldface and joined by numbered bars are the residues replaced with alanines in the indicated mutants. HeLa cells were transfected with the wild-type SRE reporter with an empty expression vector (lane 1), vector expressing wild-type (wt) Phox1 (lane 2), or vectors expressing the three Phox1 mutants (lanes 3 to 5). Cells were starved for serum for 24 h and treated for 45 min with medium containing 15% FBS. Cytoplasmic RNA was isolated and analyzed by RNase protection assays. Protein expression was verified by immunoblotting and mobility shift assays of aliquots of each transfection. All proteins were expressed at equivalent levels. As discussed in the text, mutant 3 may have a mild defect in DNA binding. Fos and α are as described for Fig. 1.

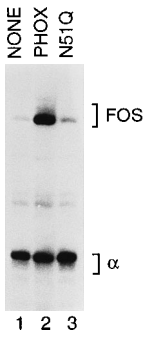

FIG. 4. Phox1 DNA-binding activity is required for activity in vivo. HeLa cells were transfected with wild-type SRE reporter and empty expression vector (lane 1), vector expressing wild-type Phox1 (lane 2), or vector expressing a mutant Phox1 protein carrying an asparagine-to-glutamine substitution at position 51 of the homeodomain. This mutation abolishes Phox1 DNA-binding activity (19). Cells were starved for serum for 24 h and treated for 45 min with medium containing 15% FBS. FOS and α are as described for Fig. 1.

vitro assay conditions, the lack of a requirement for Phox1 DNA-binding activity was surprising, since DNA binding is a highly conserved activity of all homeodomains (51). It seemed likely to us that DNA-binding activity would be important for homeodomain function in vivo. Therefore, we tested the same Phox1 DNA-binding mutant in our in vivo assay. This mutant carries a substitution of glutamine for a conserved asparagine common to nearly all homeodomains. In the engrailed homeodomain, this asparagine side chain makes two hydrogen bond contacts with an adenine base (28). Substitution of this residue in the Phox1, Prd, and Bicoid homeodomains effectively abolishes DNA-binding activity (11a, 19, 20).

Figure 4 shows that this mutant, N51Q, was unable to impart significant serum responsiveness to the SRE reporter gene (compare lanes 2 and 3). Western blotting (immunoblotting) indicated that this protein was expressed at levels equivalent to those of the wild type, and indirect immunofluorescence revealed no difference in its subcellular localization (data not shown). Thus, we conclude that under in vivo conditions the DNA-binding activity of the Phox1 homeodomain is required for the formation of active complexes at the SRE, even though this activity is dispensable in vitro.

The activity of Phox1 in vivo is DNA sequence specific. If homeodomain proteins impart specificity to incoming signals by recruiting SRF, or related proteins, to cell-type-specific genes, then Phox1's activity in the serum response assay should display DNA sequence specificity; that is, Phox1 should impart serum responsiveness to only a subset of potential SRF-binding sites. To test this hypothesis, we made a series of mutations in the c-*fos* SRE sequence. The SRE is a partially palindromic sequence, containing at its center the sequence CCATATTA GG. The flanking pairs of G · C base pairs are critical contact sites for SRF at the SRE; they are found in essentially all known SRF-binding sites (42, 58). We have focused our attention here on the AT-rich core of the SRE for two reasons. First, this is the site of the Phox1 footprint in vitro (19). Second, among many known and synthetic SRF-binding sites, these six positions show a nearly absolute requirement for an adenine or thymine base with relatively weak preferences for either (42, 58). This observation suggests either that SRF does not discriminate well between A · T and T · A base pairs in this region or that the sequence of the AT core varies because it also functions as a recognition site for other proteins.

Figure 5 shows assay results for a number of these variant SREs. Figure 5A shows in vivo activities of variant SREs following serum stimulation in the absence and presence of Phox1. All were essentially inactive in the absence of Phox1. In the presence of Phox1, there was a dramatic range in the degree of serum responsiveness, ranging from sites as strong as or stronger than those of the wild type (e.g., pm102 [Fig. 5A, lane 8] and pm104 [lane 12]) to sites nearly as weak as the pm12 mutant, which does not bind SRF at all (e.g., pm106 [lane 14] and pm116 [lane 24]). Thus, all potential SRF-binding sites are not equally responsive to Phox1.

Figure 5B shows results of mobility shift assays of each of these sites, with SRF produced by in vitro translation. These assays indicated that the ability of Phox1 to impart serum responsiveness to these sites in vivo correlated partially with the affinity of the sites for SRF. For example, the three strongest SRF-binding sites—wild-type, pm102, and pm104—were all strongly serum responsive in the presence of Phox1. In contrast, very poor SRF-binding sites exhibited weak serum responsiveness in the presence of Phox1. This result is consistent with the observation that the pm12 mutant, which fails to bind SRF, is not activated by Phox1 (Fig. 1B). However, several sites exhibited activities in vivo inconsistent with their binding affinities in vitro. For example, pm58 and pm101 bound SRF with similar affinities (Fig. 5B, lanes 2 and 3), but pm101 was a much more efficient SRE in the presence of Phox1 (Fig. 5A, lanes 4 and 6). The pm113 site bound SRF with lower affinity than pm116 (Fig. 5B, lanes 11 and 12) but was a much stronger Phox1-dependent SRE in vivo (Fig. 5A, lanes 22 and 24). pm106 bound SRF more strongly than either pm103, pm107, or pm109, but it was a weaker Phox1-dependent SRE than these sites. pm58, another weakly Phox1-dependent site in vivo, bound SRF more strongly in vitro but functioned less well in vivo than pm107, pm109, pm112, or

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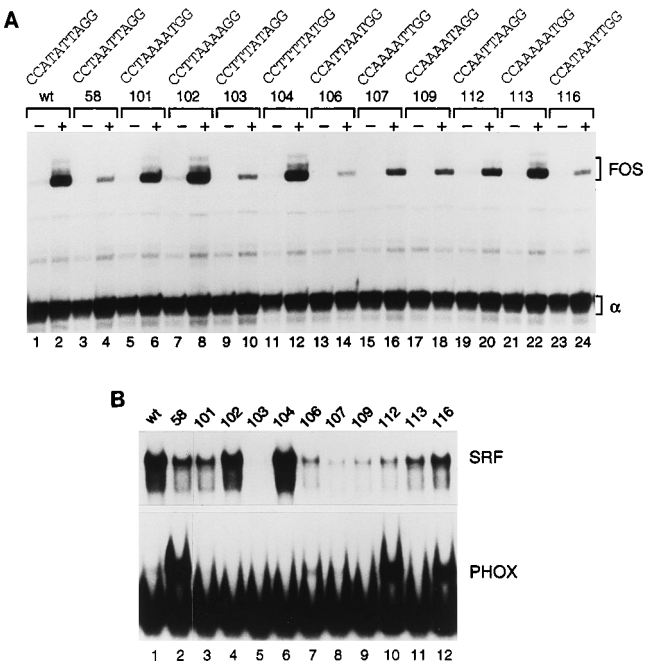

FIG. 5. Phox1 imparts serum responsiveness in a sequence-specific fashion. (A) Variant SRE reporter genes with the core sequences shown were transfected into HeLa cells with empty expression vector (−) or Phox1 expression vector (+). All cells were serum starved for 24 h and treated for 45 min with medium containing 15% FBS. (B) Human SRF produced by in vitro translation was assayed by mobility shift on probes derived from each of the variant SREs (upper gel). The probes were prepared by PCR amplification of the corresponding reporter gene plasmids with a common pair of end-labeled primers, generating a set of probes with identical specific activity. Only a portion of the gel is shown. The Phox1 homeodomain fragment corresponding to the protein expressed in the transfection experiments was produced by in vitro translation and assayed as described for SRF (lower gel).

pm113. We conclude that the ability of an SRF-binding site to respond to serum in the presence of Phox1 does not correlate with its simple affinity for SRF in vitro. This observation suggests that Phox1 modifies the binding specificity of SRF in vivo and is consistent with the hypothesis that Phox1 can interact cooperatively with SRF to recruit SRF to novel sites in vivo.

Results of binding assays for Phox1 are also shown in Fig. 5B. There was a marked lack of correlation between Phox1 binding affinity in vitro and Phox1-induced serum responsiveness in vivo. Two of the three high-affinity Phox1-binding sites, pm58 and pm116, were poor SREs in the presence of Phox1. The third high-affinity site, pm112, had moderate activity in vivo. In contrast, the two strongest Phox1-dependent SREs in vivo, pm102 and pm104, had little detectable affinity for Phox1 in vitro. Thus, the specificity of action of Phox1 in vivo differs strikingly from its DNA-binding specificity in vitro, consistent with the hypothesis that Phox1 acts in conjunction with other cellular proteins to impart serum responsiveness to these reporter genes.

Taken together, these data support the hypothesis that SRF and Phox1 interact cooperatively in vivo to impart to target genes the ability to respond to extracellular signals. They do this with a sequence specificity that is distinct from the simple DNA-binding specificities of either protein alone. The data support the idea that Phox1 and other Paired class homeodomain proteins have the ability to impart specificity to signal transduction information by recruiting SRF and its signal-responsive accessory proteins to novel cell-specific DNA sites.

DISCUSSION

We originally isolated the Phox1 cDNA in a yeast genetic screen for human cDNAs encoding proteins that could activate a cell-type-specific, pheromone-inducible reporter gene in cooperation with the yeast MADS box protein Mcm1 (19). Biochemical studies using recombinant proteins showed that Phox1 interacts with both Mcm1 and the closely related human MADS box protein SRF. Here, we have shown that Phox1 has a similar activity in human cells and can functionally recruit SRF to a reporter gene carrying an SRF-binding site. The effect of Phox1 in this assay is to enable this gene to respond to growth factors.

Several lines of evidence support the interpretation that Phox1 acts by recruiting SRF to the SRE-containing reporter gene. First, the activity of Phox1 requires a site that can be bound by SRF (Fig. 1B). Second, Phox1 confers on the reporter a spectrum of responses to extracellular signals characteristic of natural SRF-binding sites (Fig. 2A). Third, Phox1 enables the recruitment of Elk1, a protein whose interaction at the SRE is dependent on the presence of DNA-bound SRF (Fig. 3B). Thus, we conclude that the Phox1 homeodomain and the related *D. melanogaster* Prd homeodomain have the ability to interact with SRF in vivo and recruit it to DNA sites that SRF cannot activate on its own. Bringing SRF to the DNA in turn allows the recruitment of signal-responsive accessory proteins such as Elk1. Thus, the function of the homeodomains in this assay is to connect these sites to cellular signal transduction pathways. The ability to specify the transcriptional response to extracellular signals is a novel activity for the homeodomain. We speculate that this activity may account for a portion of the cell identity function displayed by many homeodomains in animal development.

What is the nature of the SRF-homeodomain complex? The effect of Phox1 on the binding of SRF to the SRE appears to be principally kinetic in biochemical assays. Although this effect requires a direct interaction between the proteins, we do not detect stable complexes containing both proteins under standard mobility shift assay conditions, nor do we detect stable SRF-Phox1 complexes in solution in the absence of DNA. How then does Phox1 enable the SRF-dependent activation of our reporter genes? We envision three different models.

First, the effect of Phox1 on SRF binding in vivo may be purely kinetic. Although genomic footprinting studies indicate that SRF is constitutively bound at the SRE (22, 29), activation might require the exchange of a new SRF molecule onto the SRE in place of the molecule previously occupying the site. Indeed, there is strong evidence for a growth factor-induced phosphorylation of SRF that alters its kinetics of DNA binding (47). Because the signals that act on the SRE are short-lived, enhancing the rate of SRF exchange would increase the probability that a new SRF molecule arrives at the SRE while signals are still active. Thus, SRF exchange could be the rate-limiting step for activation of the SRE, and homeodomains could act simply to accelerate this exchange reaction.

Second, Phox1 could act by eliciting a conformational change in SRF molecules already bound at the SRE. This conformational change would either permit the association of signal-responsive accessory factors with SRF or enhance the ability of SRF to interact with the basal transcription machinery. Eliciting this conformational change would presumably require a direct interaction between Phox1 and SRF as well as at least transient association of Phox1 with DNA.

Third, SRF and Phox1 could form stable cooperative complexes on DNA. This is the model we favor for several reasons. In yeast cells, Phox1 functionally replaces the α1 protein at the

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STE3 UAS, where α1 clearly interacts cooperatively with Mcm1 (3, 26, 44). Presumably, therefore, Phox1 is also able to interact cooperatively with Mcm1. Indeed, in extracts of yeast cells expressing Phox1, we detect a stable complex on an *STE3* probe that contains both Phox1 and Mcm1 (18). Formation of this complex is required for reporter gene activation. In contrast, we do not observe the formation of this stable complex using purified Phox1 and Mcm1 produced in bacteria. Instead, under these conditions, Phox1 elicits a purely kinetic effect on Mcm1 binding, as we had observed with SRF. Thus, the formation of stable Phox1-Mcm1 complexes must require a component missing from our purified preparations. We have identified an activity in yeast extracts that permits the formation of stable complexes of recombinant Phox1 and Mcm1 (18), which is consistent with this hypothesis. A similar activity may exist in HeLa cells.

A second reason we favor the hypothesis that stable SRF-Phox1 complexes are required to activate reporter genes in vivo is that the sequence specificity of reporter gene activation in vivo is distinct from the binding specificity displayed by SRF in vitro. If Phox1 were simply accelerating the kinetics of SRF-DNA association or inducing a conformational change in SRF already bound to DNA, there would be a strict correlation between reporter gene activity in vivo and SRF affinity in vitro. Instead, by altering the apparent sequence specificity of SRF in vivo, Phox1 must be differentially altering the equilibrium binding constant of SRF at each sequence. The most likely way it could do so is by interacting cooperatively with SRF at selected sites. The degree of cooperativity presumably depends on stereospecific contacts between Phox1 and SRF and between Phox1 and DNA.

What is the organization of these putative SRF-Phox1 complexes? On the basis of the results presented here, we can make several inferences about their physical organization. First, complex formation requires SRF-DNA contact, because sites that are not bound by SRF cannot be activated by Phox1 (Fig. 1B and 5). Second, complex formation requires homeodomain-DNA contact, because a mutant homeodomain that cannot bind DNA cannot activate the SRE (Fig. 4). Third, complex formation requires contact between the Phox1 homeodomain and another cellular protein, possibly SRF, because full activity of the homeodomain requires amino acid side chains predicted to be oriented away from the DNA (Fig. 3). Fourth, the sites on SRF contacted by Phox1 are likely to be distinct from the sites contacted by Elk1, since a complex containing all three proteins can apparently assemble on the SRE (Fig. 2). However, confirmation of these inferences will require biochemical analysis of these complexes.

Implications for the biochemical function of the homeodomain. In *D. melanogaster*, the specificity of action of homeotic genes resides within the homeodomain of the encoded proteins (14, 32, 36). But these proteins are closely related in sequence, and their DNA-binding specificities in vitro are nearly identical. Furthermore, the amino acid residues responsible for the different biological activities of two homeodomains that have been identified are largely residues that do not contact DNA in the binary complex (7, 34, 65). For the pair-rule gene *fushi tarazu*, the DNA-binding specificity of the homeodomain is also insufficient to explain all of the gene's activities in vivo (48). Therefore, the simple DNA-binding specificity of most homeodomains observed under in vitro conditions is not sufficient to explain the biological activity of the corresponding genes.

The relatively degenerate DNA recognition specificity displayed by homeodomains has led many investigators to propose that homeodomains must act in conjunction with other proteins in vivo (21). Several examples of protein-protein interactions involving homeodomains have now been reported. One common theme is direct interaction between two homeodomains, including the yeast homeodomain proteins **a**1 and α2 (25, 35), the **a**1-related *D. melanogaster* Extradenticle protein and the homeotic gene products (6, 46, 60), the *Caenorhabditis elegans* homeodomain proteins mec-3 and unc-86 (63), and the human HOXD8 and HOXD9 proteins (64). A distinct example of homeodomain-homeodomain interaction is the cooperative homodimer formation observed with *paired* class homeodomains (62).

Homeodomains also interact with other proteins. The yeast α2 homeodomain interacts with Mcm1, forming cooperative protein-DNA complexes on highly specific operator sites (27, 53). This interaction contributes to the determination of cell identity in yeast cells. Nevertheless, although Mcm1 and SRF are closely related proteins, the biochemical details of the Mcm1-α2 interaction differ significantly from those of the SRF-Phox1 interaction, showing a dependence on sequences that are N terminal to the homeodomain rather than on the homeodomain itself (61). The SRF-Phox1 interaction is more similar to the interaction of the POU homeodomain protein Oct-1 with the viral transactivator VP16. The interaction of Oct-1 and VP16, like that of Phox1 and SRF, depends on amino acid residues on the exposed surfaces of helices 1 and 2 of the homeodomain, although the precise positions of apparent contact differ (33, 43). Second, the effect of the Oct-1-VP16 interaction is the recruitment of both proteins to sites that neither protein binds efficiently without its partner (8); that is, the complex binds DNA with specificity which is distinct from that of the individual components, as we observe with SRF and Phox1.

SRF-homeodomain interactions and the specificity of signal transduction to the nucleus. The ability of homeodomains to interact with SRF could account for some of the biological activities of homeodomain genes in development. These genes have a variety of roles in imparting positional identity and ultimately cellular identity in organisms ranging from yeasts to vertebrates (37). The determination of cellular identity must be intimately related to the ability of a cell to respond to extracellular signals. We propose that homeodomains may impart identity to a cell by directly programming the transcriptional response of the cell to inductive signals. Our molecular data suggest that homeodomains can do this by recruiting proteins of the MADS box family, such as SRF, to novel DNA sites. Because SRF in turn recruits a set of signal-responsive accessory proteins, homeodomains may physically link these new genes to the cell's signal transduction machinery. Specifying the primary transcriptional response to an inductive signal may be the critical step in defining the developmental fate of a cell.

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