Combinatorial Expression of GATA4, Nkx2-5, and Serum Response Factor Directs Early Cardiac Gene Activity*

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Herein, the restricted expression of serum response factors (SRF) closely overlapped with Nkx2-5 and GATA4 transcripts in early chick embryos coinciding with the earliest appearance of cardiac α-actin (αCA) transcripts and nascent myocardial cells. The combinatorial expression of SRF, a MADS box factor Nkx2-5 (a NK4 homeodomain), and/or GATA4, a dual C4 zinc finger protein, in heterologous CV1 fibroblasts and Schneider 2 insect cells demonstrated synergistic induction of a CA promoter activity. These three factors induced endogenous aCA mRNA over a 100-fold in murine embryonic stem cells. In addition, the DNA-binding defective mutant Nkx2-5pm efficiently coactivated the αCA promoter in the presence of SRF and GATA4 in the presence of all four SREs and was substantially weakened when individual SREs were mutated and or serially deleted. In contrast, the introduction of SRFpm, a SRF DNA-binding mutant, blocked the activation with all of the αCA promoter constructions. These assays indicated a dependence upon cooperative SRF binding for facilitating the recruitment of Nkx2-5 and GATA4 to the αCA promoter. Furthermore, the recruitment of Nkx2-5 and GATA4 by SRF was observed to strongly enhance SRF DNA binding affinity. This mechanism allowed for the formation of higher ordered αCA promoter DNA binding complexes, led to a model of SRF physical association with Nkx2-5 and GATA4.

A theme is emerging in which the appearance and diversification of nascent embryonic cardiac and smooth muscle cells requires the combinatorial interactions of serum response factor (SRF)† with other transcription factors enriched in early progenitor cells. A feature of a large number of cardiac and virtually all of the smooth muscle-expressed genes to date is their dependence upon a consensus sequence CC(A/T)₆GG, a high affinity binding site for SRF. As a member of an ancient DNA-binding protein family, SRF shares a highly conserved DNA-binding/dimerization domain of 90 amino acids termed the MADS box. SRF serves as a versatile protein that binds to its cognate sites in a multitude of promoters to integrate intracellular signals and assists as a docking surface for the binding of accessory factors that may confer the regulation of specific gene programs (1–6). SRF is a key regulator of immediate early gene expression, which frequently results in mitogenesis, and also a key regulator of myogenic terminal differentiation. Most SRF accessory proteins identified as coregulators of c-fos induction also act as endpoints of growth factor-induced signal transduction cascades. In contrast to the c-fos gene, which contains a single high affinity SRE, many muscle-specific genes including skeletal, cardiac, and smooth muscle α-actins contain combinations of at least three or more strong and weak affinity SREs that bind SRF in a highly cooperative manner and do not contain an adjacent ETS sequence (6–8). Collateral accessory factors may then play essential roles in either facilitating or impeding SRF binding on multi-SRE muscle gene promoters, thus stimulating or repressing the transcription of SRF-dependent gene targets.

The recent homologous recombinant knock-out of the murine SRF gene locus supports the observation that SRF is absolutely required for the appearance of cardiac mesoderm during mouse embryogenesis (9). One of the SRF cofactors is Nkx2-5, a homeobox vertebrate homologue of Drosophila tinman (10, 11) that is one of the earliest markers of vertebrate heart development and is important for the regulation of cardiac-restricted gene activity required for cardiac morphogenesis. We determined that the Nkx2-5 target sequences resemble the AT-rich central core of the serum response element and subsequently demonstrated that Nkx2-5 binds weakly to the consensus SREs of the αCA promoter. Nkx2-5 is also recruited by SRF to SREs, resulting in modest activation of endogenous αCA gene transcription (2, 3, 12, 13). In addition, Nkx2-5 can associate with the dual C4 zinc finger transcription factor GATA4 to activate a variety of cardiac specified genes (14–16). Likewise, SRF recruits GATA factors to activate SRE-containing myogenic and non-myogenic promoters (1, 17, 18). Recently, Nishida et al. (19) showed that the triad of SRF, Nkx-3.2, and GATA-6 was coexpressed in the medial smooth muscle layer of arteries. These factors transactivated the promoters of smooth muscle genes including β₁ integrin, SM22, and caldesmon genes. This triad of factors provides transcriptional potency similar to the recently identified myocardin, a SAP factor, enriched during cardiogenesis, which also serves as a potent SRF corepressor factor (20). Herein, we also provide strong evidence for SRF in playing a leading role in the commitment of cardiac progenitors by virtue of its obligatory requirement for acting as a myogenic restricted platform to interact with other early cardiac en-
riched transcription factors, Nkx2.5 and GATA4. However, in this study, we show that Nkx2.5 and GATA4 facilitate potent activation of the cardiac α-actin promoter via the recruitment of these factors to multiple serum response elements, coincident with the earliest activation of the cardiac α-actin gene in promyocardial and embryonic stem cells.

MATERIALS AND METHODS

Recombinant Plasmids—The luciferase reporter vectors described previously (2, 3, 14) consist of the following plasmids: αCA-luciferase containing the StuI-BstBI genomic fragment of the avian αCA promoter (−315 to −15 relative to transcription start site) ligated to the αCA-luciferase gene; the deletion derivatives (−200del, −150del, and −100del) and the site-directed mutations of SRE1 (SRE1M), SRE2 (SRE2M), SRE3 (SRE3M), SRE4 (SRE4M), and −100del + SRE1M (−100del +1M). The expression of Nkx2-5, SRF, and GATA4 was controlled by CMV promoter expression vectors described previously (14). Insect cell expression vector pαX-SRF was kindly provided by Dr. Michael Gilman (Ariad Pharmaceuticals) (for review see Ref. 21). pαX-GATA4 was constructed by excising a 726-bp fragment from pCG-GATA4 with XbaI and XcmI and a 600-bp band from pBS-GATA4 (1–126) with XcmI and BamHI. The two fragments were ligated into the pαX vector after the removal of SRF from pαX SRF with XbaI and BamHI. pαX-CAG-NNKX2.5 was constructed by exciting Nkx2-5 from pCG-Nnkx2-5 and EcoRI, blunting the sticky ends, and cloning into the pαX vector after the removal of SRF from pαX SRF with XbaI and BamHI and blunting. pαCTp1 (22) was a kind gift from Dr. Thomas Shenk (Howard Hughes Medical Institute, Princeton University, Princeton, NJ). The plasmid pCMV-PA-I expressing a tetramer of the IgG-binding domain of Staphylococcus aureus α-chain protein A was a gift from Dr. Peter Uetz (European Molecular Biology Laboratory, Heidelberg, Germany) (for review see Ref. 23). SRF was excised from pCGN-SRF with XbaI and ligated into the same site of pCMVPA-II, resulting in protein A fused to the N terminus of SRF.

SRF, GATA4, Nkx2-5, and Cardiac α-Actin Expression by in Situ Hybridization Analysis—Chick embryos at Hamburger-Hamilton (HH) stage 8 (4 somites) and HH10 (10 somites) were fixed in MEMFA (0.1 mM MOPS, 2 mM EDTA, 1 mM MgSO\textsubscript{4} 3% paraformaldehyde) and stored in 90% methanol at −20 °C until use for hybridization (24). The synthesis of digoxigenin-labeled RNA probes corresponding to cNkx2-5, cGATA4, and cαCA and whole mount in situ hybridization were performed as described previously (24). All of the probes were alkaline hydrolyzed to the size of −300 bp to enhance probe penetration. Cross section of 10 μm was cut from paraffin-embedded whole mounts.

Cell Culture, Transfection, and Luciferase Assays—CV1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium containing 10% newborn calf serum. Cells were grown to 50% confluence in 100 mm tissue culture dishes and were transfected with either pCGN (200 ng of expression vectors and 200 ng of reporter DNA) in 100 μl serum-free medium (Invitrogen) at 26 °C until use for hybridization (24).

Nuclear Extract Preparation—Nuclear extracts of transfected cells were prepared by using a mini-extract procedure (25). The protein concentration was determined by the method of Bradford using a Bio-Rad DC protein assay.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA were performed with 20-μl reaction mixtures at room temperature as previously described by Chen and Schwartz (2) in which 0.5 μg of poly(dIdC) was used as a nonspecific competitor. For EMSA antibody interference assays, proteins were incubated with cold antisem for 5 min before the addition of the probe.

Affinity Purification of Protein Complexes with Protein A Fusion Proteins—Protein A pull-down assays were performed as described previously (14). Whole cell extracts from CV1 cells transfected with pCGN-Nnkx2-5 and pCG-GATA4 together with plasmids expressing either protein A or protein A-SRF were lysed in EBC buffer, incubated with IgG-Sepharose beads (Amersham Biosciences) for 15 min at 4 °C, and washed four times with 500 μl of EBC buffer. Proteins were solubilized in Laemmli’s SDS-loading buffer, separated by SDS-PAGE, and visualized by immunoblotting with anti-HA and anti-GATA4 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

DNA Affinity Chromatography Assay—Biotinylated αCA promoter DNA was reacted with translated αCA-luciferase and GATA4. Casein kinase II was used to label Nnkx2-5pm with [α-32P]ATP. DNA binding solution (50 mM NaCl, 15 mM HEPES, pH 7.6, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol) contained 5 mg/ml bovine serum albumin, 5 μg of poly(dIdC) as detailed by Cheng and Schwartz (2) with the addition of 5% fat-free milk. Avidin magnetic beads (Promega) at 100 μg/assay point were concentrated with a magnetic rack (Promega) and repeatedly washed in binding buffer. After the complete removal of binding buffer, the beads were resuspended in SDS-loading buffer, split into two equal fractions, and analyzed by SDS-10% PAGE.

RESULTS

Overlapping SRF, GATA4, Nkx2-5 Expression Patterns Coincided with αCA Gene Activity in Early Heart Development—SRF, GATA4, and Nkx2-5 gene expression patterns were mapped to determine whether they coincided with that of cardiac restricted αCA gene activity, a marker for committed cardiac myocytes. Whole mount-sectioned in situ hybridized chick embryos (Fig. 1) revealed that by HH stage 8 (4 somites), Nnkx2-5, cSRF, cGATA4, and cαCA have overlapping patterns of expression with the anterior intestinal portal (AIP) marking the apex of expression (Fig. 1, A–D). However, SRF and cαCA are not expressed in the medial AIP (Fig. 1, B and D, d and j). At the level of the AIP, Nnkx2-5 was expressed throughout the mesoderm and endoderm (Fig. 1, e, α), whereas GATA4 was expressed only in the endoderm (Fig. 1G, g). In the paired heart tubes, both genes were expressed in the mesoderm (Fig. 1C, b). The first somite marks the posterior border of SRF and cGATA4 expression (Fig. 1, F and G), but Nnkx2-5 expression barely extends to this level (Fig. 1, E, c). Thus, SRF expression appears to mark the anterior border of αCA expression, whereas Nnkx2-5 expression appears to coincide with the posterior border. The approximate overlap of SRF mRNA with Nnkx2-5 and GATA4 transscripts was coincident with αCA gene activity and the appearance of the myocardial cells (as shown in Fig. 1b, e, h, and k). At HH stage 10 (~10 somites), the heart tube is fused at the midline, and Nnkx2-5, cSRF, and cαCA were expressed throughout the straight heart tube (Fig. 1, E, F, and H), whereas cGATA4 expression is primarily in the sinus venosus. By HH stage 10, all four genes were expressed in the myocardium.

SRF Cooperated with GATA4 and Nnkx2-5 to Activate the αCA Promoter—The reporter plasmid αCA-luciferase contains the
avian oCA promoter from −315 to +15 relative to the transcription start site. When cotransfected with SRF alone, oCA-luciferase activity varied between 6- and 10-fold, whereas GATA4 or Nkx2-5 alone resulted in only 1-4-fold transcriptional activation in CV1 fibroblasts. The pairings of SRF and GATA4 resulted in the coactivation of −20-30-fold above the basal level. The transfection of all of the three factors resulted in robust synergistic activation of the oCA promoter to levels up to 160-fold (Fig. 2A). In contrast, no coactivation was seen with the SV40 promoter (data not shown). Fig. 2B shows robust expression of plasmid DNA expression vectors driving wild-type gene activity of transfected CV1 fibroblasts as detected by antibody protein blots. These results suggest that most of the synergistic coactivation requires the efficient binding of SRF to the SRE. Consistent with this idea, the cotransfection of SRFpm1, a triple point mutant of SRF that weakens DNA binding with GATA4 and Nkx2-5, resulted in a 75% reduction in coactivation (Fig. 2, A and C). To investigate whether triad factor coactivation requires the binding of Nkx2-5 to the oCA promoter, the non-DNA-binding mutant Nkx2-5pm, a point mutation (Asn to Gln) at position 10 of the DNA recognition helix, was substituted for wild-type Nkx2-5 in the cotransfection assay with SRF and GATA4 (Fig. 2, A and C). No reduction in activity was noted. In fact, a small increase of −15% reporter activity was seen. These findings suggest that as shown for the interaction of Nkx2-5 with SRF (2), the binding of Nkx2-5 to the oCA promoter is not required and may actually decrease the efficiency of the coactivation with SRF and GATA4.

It is conceivable that the interaction of Nkx2-5 with SRF and GATA4 allows the activation domain of Nkx2-5 to contribute significantly to the synergistic effect as demonstrated in cotransfection assays of Nkx2-5 plus GATA4 (14, 15, 26). To investigate whether other members of the Nkx2 family are also able to cooperate with SRF and GATA4 in a triple interaction, cotransfections were performed as described above, substituting Nkx2-5 for Nkx2-1 (TTF1), Nkx2-8, and D. tinman (Fig. 2C). The results showed that although Nkx2-1 and D. tinman were able to replace Nkx2-5 albeit at a lower efficiency, Nkx2-8 was unable to cooperate with SRF and GATA4 in activating the oCA promoter, indicating non-equivalent roles for the Nkx2 factors.

Drosophila S2 cells, which are devoid of significant SRF expression (21), allowed us to study the activation of the oCA promoter in the absence of endogenous SRF. The transfection of SRF into S2 cells resulted in minimal (2-fold) activation of the oCA promoter (Fig. 2D). Transfection of VP16-SRF resulted in 190-fold activation of the same promoter (results not shown), indicating that this promoter binds SRF and is functional in S2 cells, whereas the natural activation domain of SRF is relatively weak in S2 cells. Similarly, GATA4 and Nkx2-5 by themselves or in combination did not significantly activate the oCA promoter in these cells. A double transfection of SRF with GATA4 or with Nkx2-5 resulted in activation below the additive level (<3 times). However, triple cotransfection of SRF, GATA4, and Nkx2-5 resulted in synergistic (13-fold) stimulation of the oCA promoter. We also observed that the removal of the strong inhibitory C-terminal domain in the Nkx2-5 mutant Nkx2-5-(1–203) was by itself a weak activator of oCA (3 times) but a much stronger coactivator with SRF (18-fold) and with GATA4 and SRF (40-fold). These results together with the cotransfection experiments in CV1 fibroblasts (Fig. 2, A and C) (for review see Ref. 14) suggest that GATA4 activated Nkx2-5 by the removal of the repressive effect of the C-terminal domain of Nkx2-5. Moreover, the interaction of Nkx2-5 and GATA4 on the oCA promoter was mediated by exogenous SRF in insect cells, because the level of activation in the absence of SRF is minimal.

Sp1 has been reported to physically interact with GATA1 (27–29), Nkx2-1 (30), and MEF2C (31) and to functionally interact with SRF to regulate the oCA promoter (32–34). Because S2 cells have been shown to lack Sp1 (22), we then asked whether Sp1 interacts with these factors. When Sp1 was cotransfected into S2 cells with a minimal promoter containing multimerized Sp1 sites, luciferase activity was 160-fold compared with the same promoter without Sp1 sites (results not shown). However, Sp1 alone or with SRF had minimal effect on oCA-luciferase (Fig. 2D). Sp1 had only an additive effect on the
GATA4, and Nkx2-5 expression vectors were cotransfected with 1 µg of the indicated luciferase reporter construct. pCGN-SRF and pCGN-SRFpm (150 ng), pCGN-Nkx2-5 and pCGN-Nkx2-5pm (400 ng), or pCG-GATA4 (400 ng) were added in various combinations with an appropriate amount of pCG empty vector to make the total mass of pCG-derived vectors equal to 1 µg. CV1 cells were transfected at ~50% confluence, and extracts collected after 48 h culture in Dulbecco’s modified Eagle’s medium were transfected with 3% horse serum and 15 µg/ml of insulin. The luciferase activity of the empty vector alone was set as 1. Error bars represent the mean ± S.D. of at least two separate assays. B, Western blot of transfected CV1 fibroblasts with 2 µg of pCGN-SRF, pCGN-Nkx2-5, or pCG-GATA4. After 72 h, extracts were prepared in EBC buffer, and proteins were separated by SDS-PAGE. Immunoblotting was done with anti-HA (lanes 1–5) or GATA4 (lanes 6–10). HA antibody nonspecifically labels three cellular proteins (asterisk) that indicate equal loading of the gel and specifically show SRF and Nkx2-5 expression. The indicated expression plasmids were cotransfected with an aCA-SRE1 into CV1 cells and assayed as in A. The full-length wild-type sequences are represented as wt, whereas pm represents point mutants that are unable to bind DNA. C, other members of the Nk2 family cotransfected with SRF and GATA4 in a triple interaction were performed as above, substituting Nkx2-5 for Nkx2-1 (TTF1), Nkx2-8, and D. tinman. D, Drosophila Schneider 2 embryonic cells were transfected in 12-well plates with 200 ng of aCA-luciferase and 50 ng of each of the indicated insect cell expression vectors or empty vector to a total of 200 ng of plasmid DNA. The full-length wild-type sequences are represented. 1–203 represents Nkx2-5 with amino acids C-terminal to residue 203 removed. After 48 h post-transfection, luciferase activity was assayed and compared with the activity obtained by transfection with the empty expression vector alone. Error bars represent the mean ± S.D. of at least two assays.

coactivation of the aCA promoter by SRF plus GATA4 and reduced coactivation by SRF plus GATA4 and Nkx2-5 (Fig. 2D). These results indicate that Sp1 does not stimulate aCA transcription via interactions through this triad of factors. SRF, Nkx2-5, and GATA4 Activated aCA Gene Activity in Murine ES Cells—In the coexpression of Nkx2-5, GATA4, and SRF capable of activating the endogenous aCA gene? The expression vectors encoding these transcription factors were transiently transfected into pluripotent murine AB2-129 embryonic stem cells. aCA mRNA was not detected in the parental 6-day post-aggregated ES cells transfected with the empty pCGN plasmid as shown in Fig. 3. Increased expression of any one of these factors in ES cells reproducibly elicited significant transcription of the aCA gene. SRF and GATA4 alone were sufficient to increase aCA transcripts by 40-fold over control ES cells. The paired groupings of SRF with GATA4 or with Nkx2-5 stimulated aCA activity ~30% more than the combination of Nkx2-5 and GATA4 (Fig. 3). The combination of SRF, Nkx2-5, and GATA4 induced aCA mRNA levels over 100-fold versus control levels, indicating a central role for SRF in directing embryonic cardiac gene expression.
Expression of endogenous cardiac restricted genes were analyzed by RT-PCR according to recommendations by the manufacturer (Invitrogen) using gene-specific primers as described under “Materials and Methods.” After 72 h post-transfection, the relative amount of aCA transcripts was measured by RT-PCR with ³²P-labeled 3' gene-specific primers following 24, 26, and 28 cycles and analyzed on 8% polyacrylamide gels and quantified by Betagen autoradiography. Error boxes represent the mean ± S.D. of three assays, and the standard difference of the means shows a significance of p < 0.02 for accumulation of aCA transcripts in ES cells transfected with all three expression vectors versus the control ES cells transfected with empty vectors.

The luciferase activity of full-length aCA promoter SRE mutations and serial deletion constructs were previously described in Chen and Schwartz (2, 12). SREs were indicated by closed boxes. A, transcription analysis of various aCA promoter mutant reporter constructs in CV1 fibroblasts is shown. The luciferase activity of full-length aCA promoter in these cells was set at 100, transfection of a SRE1M promoter constructions. Thus, these assays indicate the importance for cooperative SRF binding in facilitating the recruitment of nonbinding Nkx2-5pm mutant and GATA4.

Indicating the recruitment of nonbinding Nkx2-5pm mutant and GATA4 to biotinylated aCA DNA. Also, the DNA-binding defective mutant Nkx2-5pm was pulled out only by SRF wild type, not by mutant SRFpm. Thus, Nkx2-5 and GATA4 brought about robust synergy with SRF by strongly enhancing the formation of promoter DNA binding complexes.

**DISCUSSION**

In vertebrates, SRF expression is restricted to tissues of mesoderm and neuroectoderm origins (3, 37). During chicken embryogenesis and the progression of gastrulation, strongly localized SRF mRNA expression was observed in the lateral plate mesoderm, the precardiac splanchnic mesoderm, the myo-
cardium, and the myotomal portion of the somites (37). We have shown that SRF is expressed at high levels in a symmetrically split cusp forming the extreme anterior and lateral parts of the embryo appearing like the split cardiac progenitor cell populations described from fate mapping experiments (37). The initial expression pattern resolves into a complete crescent and undergoes change consistent with morphogenesis of the linear and S-shaped heart tube. In the mouse, we showed that the highest SRF mRNA levels were seen in adult skeletal and cardiac muscle. During mouse embryonic development, SRF transcripts were found to be enriched in smooth muscle media of the vessels, the myocardium of the heart, and myotome portion of somites (1). Recently, the homologous recombinant knock-out of the murine SRF gene demonstrated a severe block for mesoderm formation during mouse embryogenesis (9). The very early lethal SRF-deficient embryos, which appear to have normal cell replication, also have a severe gastrulation defect and do not develop to term. They consist of misfolded ectoderm and endoderm cell layers that do not form primitive streak or any detectable mesoderm and fail to express the very early developmental marker genes Bra(T), Bmp2/4, and Shh (9).

We observed that the overlap of SRF transcripts with Nkx2-5 and GATA4 transcripts coincided with αCA gene activity and the appearance of the early committed myocardial cells of the heart (Fig. 1). The consequence of expression of any one of these transcription factors in ES cells was adequate to stimulate significant endogenous αCA gene activity, whereas the combination of SRF, Nkx2-5, and GATA4 induced αCA RNA levels over 100-fold versus control levels (Fig. 3). The coactivation was dependent on intact SRF as shown by the dominant inhibition of αCA expression with mutant SRFpm (Figs. 2 and 4). In addition, Nishida et al. (19) showed that the cardiac muscle-specific triad of Nkx2-5, serum response factor, and GATA-4 transactivated the cardiac atrial natriuretic factor gene, which contains three separate elements, a CArlG-like box, a GATA binding box, and a NK binding element. These observations demonstrate that transcripts emanating from the endogenous αCA and ANF genes can be increased by elevated levels of SRF, GATA4, and Nkx2-5 and lend further support to the notion that the combination of these three factors are among the earliest endogenous activators of cardiac gene transcription.

**A Model of GATA4 and Nkx2-5 Interaction with SRF—SRF** serves as a versatile protein that binds to its cognate sites in a
multitude of promoters and serves as a docking surface for the binding of many different accessory proteins that confer specific functional abilities to the promoter (2, 4, 38). The elucidation of the x-ray crystal structure of the SRF core bound to DNA (39, 40) provides an explanation for mutually inclusive binding of coaccessory factors to a single SRE. As shown schematically in Fig. 6, the coiled coil formed by the MADS box α helices (amino acids 153–179) lies parallel and on top of a narrow DNA major groove making contacts with the phosphate backbone on a SRE half-site. In addition, the unstructured N-terminal extension from the α helix (amino acids 132–152) makes critical base contacts in the minor groove. The dimerization of the MADS box occurs above the α helix by a structure composed of two β-sheets in the monomer that interact with the same unit in its partner. A second αII helix in the C-terminal portion of the MADS box stacked above these β-sheets completes this stratified structure. B, the second zinc finger of GATA4 binding to the N-terminal extension and α-coil I of the MADS box. Removal of the first zinc finger of GATA4 actually stimulated GATA4 synergy with SRF by the removal of interfering multi-zinc finger coaccessory inhibitory proteins such as FOG-1 and FOG-2. C, the binding of Nkx2-5 homeodomain through its helix II/I to the other monomer of SRF and the interaction with GATA4 through helix III. Note that helix III may be positioned to bind the exposed major groove at the center of the SRE. Nkx2-5 and SRF interact directly in the absence of the SRE. A short 30-amino acid peptide (amino acids 142–171), which encompasses the basic region of SRF in the α1 coil of the MADS box is sufficient to mediate protein-protein contacts with the Nkx2-5. The N terminus/helix I and helix II regions of the Nkx2-5 homeodomain interact with the MADS box. The fact that Phox-1-SRF (45, 46) and Nkx2-5-SRF (2) interactions required N-terminal arm/helix I/helix 2 region of the homeodomain lead us to propose an earlier model that helixI/II is responsible for homeodomain-SRF interactions (2). As shown above in C, this model shows GATA factors such as GATA4 binding to a monomer of SRF and Nkx2-5 binding to the other SRF monomeric subunit. Cross-linking assays from our laboratory indicated that a monomer of Nkx2-5 was bound to a SRF dimer (C. Y. Chen and R. J. Schwartz, unpublished observation). Thus, this model represents our data presented here and elsewhere that demonstrated Nkx2-5 and GATA4 coassociation with SRF in solution and on SRE DNA binding targets.

FIG. 6. A structural model for SRF, GATA4, and Nkx2-5 interactions with the αCA SRE. A, SRF dimer binding to SRE through helix I in the major groove and N-terminal extension wrapping around to the minor groove causing bending of the DNA. This figure is adapted from Pellegrini et al. (40) who described the x-ray crystallographic structure of structure of serum response factor core bound to DNA. The paired monomers form a coiled coil by the MADS box α helices (amino acids 153–179) and lies parallel and on top of a narrow DNA major groove making contacts with the phosphate backbone on a SRE half-site, whereas an unstructured N-terminal extension from the α helix (amino acids 132–152) makes critical base contacts on paired Gs in the minor groove. Dimerization of the MADS box occurs above the α helix by a structure composed of two β-sheets in the monomer that interact with the same unit in its partner. A second αII helix in the C-terminal portion of the MADS box stacked above these β-sheets completes this stratified structure. B, the second zinc finger of GATA4 binding to the N-terminal extension and α-coil I of the MADS box. Removal of the first zinc finger of GATA4 actually stimulated GATA4 synergy with SRF by the removal of interfering multi-zinc finger coaccessory inhibitory proteins such as FOG-1 and FOG-2. C, the binding of Nkx2-5 homeodomain through its helix II/I to the other monomer of SRF and the interaction with GATA4 through helix III. Note that helix III may be positioned to bind the exposed major groove at the center of the SRE. Nkx2-5 and SRF interact directly in the absence of the SRE. A short 30-amino acid peptide (amino acids 142–171), which encompasses the basic region of SRF in the α1 coil of the MADS box is sufficient to mediate protein-protein contacts with the Nkx2-5. The N terminus/helix I and helix II regions of the Nkx2-5 homeodomain interact with the MADS box. The fact that Phox-1-SRF (45, 46) and Nkx2-5-SRF (2) interactions required N-terminal arm/helix I/helix 2 region of the homeodomain lead us to propose an earlier model that helixI/II is responsible for homeodomain-SRF interactions (2). As shown above in C, this model shows GATA factors such as GATA4 binding to a monomer of SRF and Nkx2-5 binding to the other SRF monomeric subunit. Cross-linking assays from our laboratory indicated that a monomer of Nkx2-5 was bound to a SRF dimer (C. Y. Chen and R. J. Schwartz, unpublished observation). Thus, this model represents our data presented here and elsewhere that demonstrated Nkx2-5 and GATA4 coassociation with SRF in solution and on SRE DNA binding targets.

The recruitment of Nkx2-5 to a SRE is dependent upon SRF DNA binding activity. The dominant negative SRFpm1 mutant, which dimerizes with wild-type SRF, blocked the recruitment of Nkx2-5. Even though Nkx2-5 can bind weakly to some SREs, we found that the activation of a minimal promoter consisting of a single SRF binding site was dependent upon increasing the cellular levels of SRF (3, 37). When Nkx2-5 binding activity was blocked by a point mutation in the third helix of the homeodomain, SRF was still capable of recruiting mutated Nkx2-5 to the αCA promoter (2). An investigation of protein-protein interactions demonstrated that Nkx2-5 could bind to SRF in the absence of DNA as soluble protein complexes isolated from cardiac myocyte nuclei. In addition, Nkx2-5 and SRF complexes could be detected as coassociated binding complexes on the proximal SRE1 (2). The recruitment of Nkx2-5 to a SRE was dependent upon SRF DNA binding activity and could be blocked by the dominant negative SRFpm1 mutant, which dimerizes with wild-type SRF monomers but cannot itself bind to DNA. In addition, Nkx2-5 protein and SRF interact directly in the absence of the SRE. A short 30-amino acid peptide (amino acids 142–171), which encompasses the basic region of SRF in the α1 coil of the MADS box, is sufficient to mediate protein-protein contacts with the Nkx2-5. The N terminus/helix I and helix II regions of the Nkx2-5 homeodomain interacts with the MADS box as modeled in Fig. 6.

Nkx2-5 can also cooperate with GATA4 to activate the αCA (14), ANF (15, 16, 26), and minimal A20 (14) promoters containing multimerized Nkx2-5 DNA binding sites. Transcriptional activity requires the N-terminal activation domain of Nkx2-5 and binding activity through its homeodomain, but it does not require the activation domain of GATA4. Minimal interactive regions were mapped to the homeodomain of Nkx2-5 and the second zinc finger of GATA4. The removal of Nkx2-5 C-terminal inhibitory domain stimulates robust transcriptional activity compared with the effects of GATA4 on wild-type Nkx2-5, which in part facilitates Nkx2-5 DNA binding activity. We postulated a simple model that GATA4 induces a conformational change in Nkx2-5 that displaces the C-terminal inhibitory domain; thus, eliciting transcriptional activation of promoters containing Nkx2-5 DNA binding targets. Also, coexpression of both GATA4 and SRF in fibroblasts resulted in robust activation of both muscle-restricted and ubiquitous SRE-dependent promoters. GATA4 second zinc finger binds avidly to the α1 coil of the MADS box. Interestingly, deletion of the N-terminal activation domain and the first zinc finger of GATA4 increased the ability of GATA4 to synergize with SRF, suggesting that the domain surrounding the first zinc finger may interfere with interaction of SRF and GATA4. This notion is supported by recent reports describing interaction the multizinc finger coaccessory proteins such as FOG-1 and FOG-2 which inhibit the transcriptional activity of GATA-1 and GATA4 by interacting with the first zinc finger (41–43) which in Drosophila is also similar to the interaction of pannier with U-shaped (44). The coexpression of all three proteins resulted in robust synergistic activation of the αCA promoter two orders of magnitude above base line. The combination of Nkx2-5 homeodomain and GATA4 enhanced the formation of SRF dependent DNA binding complexes. Possibly, conformational changes in SRF structure facilitated by Nkx2-5 and GATA4 made SRF a more efficient DNA binding factor; allowing it to bind to weaker nonconsensus SREs and, stimulating αCA activity under limiting amounts of SRF. This level of activation required intact SRF-SRE binding, but was even higher when Nkx2-5pm was...
substituted for wild-type Nkx2-5. The lack of requirement for Nkx2-5 binding to DNA is also observed with Nkx2-5/SRF coactivation of the cA-SRE1 promoter (2, 3). This may indicate that Nkx2-5 acts by providing a strong transcriptional activation domain, whereas the role of SRF is to attract Nkx2-5 to the cA promoter and facilitate the recruitment of GATA4. We did not observe significant synergistic activity of SRF and Nkx2-5 in insect cells. However, the deletion of the C-terminal repressor domain of Nkx2-5 resulted in increased cooperative activation with SRF similar to the levels obtained with coexpressed GATA4, SRF, and Nkx2-5. These results suggest that the interaction with SRF is not sufficient to remove the repressive effect of the C-terminal domain and that interaction with GATA4 (recruited by SRF or Nkx2-5) is required for coactivation of the repressor domain of Nkx2-5. This effect of GATA4 on the repressor domain of Nkx2-5 may thus explain why triple coexpression resulted in much greater activation of the cA promoter.

Recently, Wang et al. (20) identified myocardin, a novel and highly potent transcription factor that is expressed in cardiac and smooth muscle cells. Myocardin belongs to the SAP domain family of nuclear proteins and activates cardiac muscle promoters by recruitment to SRF. By virtue of the ability of myocardin to restructure chromatin, it is probable that myocardin could further stimulate the ability of this triad to transactivate target genes. In conclusion, the reciprocal recruitment among Nkx2-5, GATA4, and SRF may expand the spectrum of genes regulated by either one of these factors while conferring additional level of specificity. Our study underscores the ability of these proteins to interact in a combinatorial manner to drive cardiogenic gene expression programs.

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