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Megakaryoblastic Leukemia 1, a Potent Transcriptional Coactivator for Serum Response Factor (SRF), Is Required for Serum Induction of SRF Target Genes

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Megakaryoblastic leukemia 1 (MKL1) is a myocardin-related transcription factor that we found strongly activated serum response element (SRE)-dependent reporter genes through its direct binding to serum response factor (SRF). The c-*fos* SRE is regulated by mitogen-activated protein kinase phosphorylation of ternary complex factor (TCF) but is also regulated by a RhoA-dependent pathway. The mechanism of this pathway is unclear. Since MKL1 (also known as MAL, BSAC, and MRTF-A) is broadly expressed, we assessed its role in serum induction of c-*fos* and other SRE-regulated genes with a dominant negative MKL1 mutant (DN-MKL1) and RNA interference (RNAi). We found that DN-MKL1 and RNAi specifically blocked SRE-dependent reporter gene activation by serum and RhoA. Complete inhibition by RNAi required the additional inhibition of the related factor MKL2 (MRTF-B), showing the redundancy of these factors. DN-MKL1 reduced the late stage of serum induction of endogenous c-*fos* expression, suggesting that the TCF- and RhoA-dependent pathways contribute to temporally distinct phases of c-*fos* expression. Furthermore, serum induction of two TCF-independent SRE target genes, SRF and vinculin, was nearly completely blocked by DN-MKL1. Finally, the RBM15-MKL1 fusion protein formed by the t(1;22) translocation of acute megakaryoblastic leukemia had a markedly increased ability to activate SRE reporter genes, suggesting that its activation of SRF target genes may contribute to leukemogenesis.

The transcription of cellular immediate-early genes such as c-*fos* is activated rapidly by mitogenic signals (13, 23, 35). Regulation of many of the immediate-early genes is mediated by serum response elements (SREs) that bind serum response factor (SRF) (reviewed in references 30 and 57). Some SRE-containing genes contain a ternary complex factor (TCF) binding site adjacent to the SRF binding site. TCF contributes to serum regulation, is encoded by three Ets-related genes, Elk-1, SAP1, and SAP2, and can be activated by mitogen-activated protein (MAP) kinase phosphorylation (reviewed in reference 67). However, extracellular signals can also control SRF activity in the absence of TCF binding (22, 27, 31). This second, TCF-independent pathway can be blocked by inhibitors of the RhoA GTPase and other agents that affect actin treadmilling (28, 58). These results suggest that a serum-to-RhoA-to-actin pathway regulates SREs, yet the pathway from RhoA to SRF has not been determined.

SRF also plays an important role in the expression of muscle-specific genes (reviewed in reference 57). Many promoters of muscle-specific genes contain SREs, often termed CArG boxes, but lack obvious associated TCF binding sites. Muscle-specific expression of these genes has in some cases been shown to involve SRF-complexing factors (reviewed in references 5 and 57). The cardiac-restricted transcription factor Nkx2.5, a homeodomain protein, and GATA4 interact with SRF to facilitate the expression of cardiac genes (55). Wang and colleagues found that myocardin, a heart-specific gene product, binds to SRF and is a potent coactivator of SRF transcriptional activity (62). Recently, HOP was identified as an antagonist of SRF, inhibiting SRF-dependent cardiac-specific gene expression (7, 56). In addition, SRF binds and functionally cooperates with several other transcriptional factors, including Sp1, C/EBPβ, and TFII-I (24, 33, 52, 54). There is limited evidence, however, that any of these interactions are critical for serum regulation of SREs.

Megakaryoblastic leukemia 1 (MKL1), also termed megakaryocytic acute leukemia (MAL), BSAC, and MRTF-A, was initially identified due to its involvement as the chromosome 22-encoded protein altered by the t(1;22) translocation of acute megakaryoblastic leukemias in infants and young children (39, 44, 53, 63). As a result of this translocation, MKL1 is fused with the RBM15 protein (RNA-binding motif protein 15), also known as OTT, which is encoded on chromosome 1, to form an RBM15-MKL1 fusion protein that is believed to possess oncogenic properties (39, 44). Sequence comparison shows that MKL1 is weakly similar to myocardin throughout its full length of 931 amino acids, with higher similarity in several domains, including an SAP (SAF-A/B, acinus, PIAS) domain thought to be involved in nuclear scaffold attachment (37, 50).

During the course of this work, a mouse homologue of MKL1, MRTF-A/BSAC, was also identified and found to ac-

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tivate SRE-containing reporters (53, 63). We also recently identified a human MKL1-related gene, MKL2, that was also found in mouse and termed MRTF-B (63; A. Selvaraj and R. Prywes, submitted for publication). In contrast to myocardin, which is specifically expressed in the heart, MKL1 is broadly expressed, so that it has the potential to be a common regulator of growth factor-induced immediate-early genes (39, 44, 62). We therefore tested whether MKL1 is involved in serum induction of SRF target genes. We also tested the effect of the RBM15-MKL1 fusion on MKL1's ability to activate SRF as a possible explanation for aspects of the transforming potential of this chimeric protein.

MATERIALS AND METHODS

Cloning and plasmids. The cDNA sequence of a myocardin-related protein (AB037859) was used to identify human expressed sequence tag (EST) clones. EST BG716386, which encodes a shorter form of MKL1, and EST AA721352, which contains a partial sequence of MKL1, were used to generate the full-length MKL1 cDNA by PCR. Full-length MKL1 cDNA was subcloned into the p3×Flag-CMV-7.1 expression vector (Sigma) with three copies of the Flag tag at the N terminus to make p3×Flag-MKL1. Site-directed mutagenesis was performed with PCR-based methods. The N- and C-terminal deletion mutants lack the region up to and including the amino acid in their names, e.g., N100 lacks amino acids 1 to 100. The internal deletion mutants lack the following amino acids of MKL1: ΔB, 222 to 237; ΔQ, 264 to 281; ΔSAP, 343 to 378; and ΔCC, 527 to 555.

The expression plasmids for 1×Flag-RBM15 (single N-terminal Flag tag), 1×Flag-MKL1 (single N-terminal Flag tag) and 1×Flag-RBM15-MKL1 (single C-terminal Flag tag) containing the indicated human cDNAs (39) were cloned into pcDNA3neo (Invitrogen). The retroviral vector for MKL1 C630, pBabe-puro-C630, was constructed by placing the C630 coding region in pBabe-puro (45).

Luciferase reporter plasmids with the promoter sequences of cardiac α-actin (α-CA), SM22, atrial natriuretic factor, Egr-1 (Egr-1[1.2]Luc), c-*jun* (JC6), and 5×GAL4-E1b-luc were as described (6, 10, 12, 14, 40, 60). The human smooth muscle α-actin (α-SA) luciferase reporter was a gift of C. Chandra Kumar. The cytomegalovirus and Rous sarcoma virus luciferase reporters were constructed by cloning the promoters upstream of the luciferase gene in pBasicGL3 (Promega). The 4×MEF2 reporter contains four MEF2 sites upstream of the c-*fos* minimal promoter and luciferase gene similar to the pMEF2Fluc reporter (26). The 4×CRE and 5×SRE reporters were from Stratagene. The 1×SRE reporter contains the c-*fos* SRE sequence 5′-AGGATGTCCATATTAGGACATCT-3′ upstream of the c-*fos* minimal promoter and the luciferase gene in pOFlucGL3 (12). Luciferase reporter plasmids for the mouse c-*fos* promoter (−355 to +109), c-*fos* enhancer (−356 to −297) fused to the minimal c-*fos* promoter, c-*fos* enhancer with TCF site mutation (pm18), and c-*fos* enhancer with the c-*fos* AP1 site (FAP1) (−355 to −285) were as described (64, 65). The simian virus 40 promoter internal control plasmid pRL-SV40P and expression plasmids for activated *raf* (RafBXB), ERK-2, RhoA-Val14, and hemagglutinin (HA)-tagged human SRF (pCGNSRF) were as described (42, 65).

Cell culture, transfections, and luciferase assays. HeLa and NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% newborn calf serum (NCS; Sigma) at 37°C in a 5% CO_2 incubator. NIH 3T3 cells stably expressing Flag-tagged C630 were prepared by retroviral infection and selection with 2 μg of puromycin per ml. The virus expressing C630 was generated by transfection of 293 cells with pBabe-puro-C630 and a packaging site-defective Moloney murine leukemia virus construct.

HeLa cells were transiently transfected on six-well plates with the standard calcium phosphate DNA precipitation method. The cells were incubated with the transfection cocktail for 16 h and then grown in DMEM with 0.2% NCS for 24 h for serum starvation. The cells were then stimulated with DMEM containing 20% NCS, 10 μM L-α-lysophosphatidic acid (LPA; Sigma), or 100 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) per ml for 3 h. Luciferase assays were performed as described (65). The firefly luciferase activities were normalized to the *Renilla* luciferase activities to compensate for variability in transfection efficiencies. All experiments were performed with duplicate plates of cells for each time point.

For RNase protection assays, HeLa cells were transiently transfected with the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 5 h, cells were serum starved for another 24 h before being stimulated with 20% NCS or 10 μM LPA. The transfection of greater than 90% of the cells was confirmed by cotransfection with a green fluorescent protein (GFP) expression plasmid (pEGFP-N1).

Immunoprecipitations and immunoblots. For immunoprecipitation, cells from 6-cm plates were rinsed once in ice-cold phosphate-buffered saline and then lysed in 300 μl of immunoprecipitation buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA, and a protease inhibitor cocktail [Sigma no. P8340]). The lysates were rotated for 2 h at 4°C and cleared by centrifugation at 13,000 × *g* for 10 min at 4°C. MKL1 (3xFlag-MKL1) and its mutants were immunoprecipitated with 1 μl of anti-Flag antibody (M2; Sigma) overnight. Protein A-Sepharose (Amersham Biosciences) (120 μl of a 10% slurry in immunoprecipitation buffer) was then added for 2 h. The protein A beads were washed three times with immunoprecipitation buffer, and the proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to nitrocellulose membranes and immunoblotted with anti-Flag or anti-HA (HA.11; Covance) antibodies, as indicated, at a 1:1,500 dilution and horseradish peroxidase-conjugated secondary antibodies at a 1:5,000 dilution. The signals were visualized with ECL Plus reagents (Amersham Biosciences).

For immunoprecipitation of endogenous SRF and MKL1, 1.5 μl of rabbit anti-SRF serum (42) was used for the immunoprecipitation, followed by immunoblotting with a 1:500 dilution of anti-MKL1 serum. The rabbit anti-MKL1 serum was generated by injecting rabbits with glutathione *S*-transferase (GST)-MKL1 (amino acids 601 to 931) purified from *Escherichia coli*. Antiserum to MKL2 was generated by injecting rabbits with GST-MKL2 (amino acids 703 to 1049) purified from *E. coli.*

Gel mobility shift assays. MKL1 was in vitro transcribed and translated from pcDNA3.1-MKL1-HA, which contains a T7 promoter upstream of MKL1 and a C-terminal HA tag, with an STP3 kit (Novagen). In vitro-translated MKL1 (5 to 8 μl) and bacterially expressed SRF (amino acids 114 to 508) (10 ng) were used with a high-affinity SRF binding site, XGL, as a probe (47). SRF (amino acids 114 to 508) with an N-terminal polyhistidine tag was expressed in pET28b in *E. coli* and purified with a nickel-chelate resin (Novagen). The double-stranded oligonucleotide probe XGL was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The samples were incubated with 1 ng of probe in 20 μl of binding buffer (40 mM KCl, 10 mM Tris [pH 7.5], 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 60 mM spermidine, 1 μg of sheared herring sperm DNA) for 1 h at room temperature and then separated on a 4% polyacrylamide gel in 0.25× TBE (25 mM Tris base, 25 mM boric acid, 1 mM EDTA), dried, and exposed to film.

RNA interference. For RNA interference (RNAi) analysis of MKL1, the 19-nucleotide MKL1-specific sequence 5′-AGTAGCAGACAGCTCTTCC-3′ was cloned into the pSUPER expression vector as described (4). The pSUPER plasmid was used as a control. A 21-nucleotide small interfering RNA (siRNA) duplex with 3′dTdT overhangs corresponding to the MKL2 sequence 5′-AAGA GCTCGACTAGCAGATGA-3′ was synthesized (Qiagen). A GFP-22 siRNA duplex, 5′-AACGGCAAGCTGACCCTGAAGTTCAT-3′, was synthesized and used as a nonspecific control. RNAi transfections were performed with Lipofectamine 2000 reagent (Invitrogen). HeLa cells (1.5×10^5) were plated on 24-well dishes, and starting the next day, the cells were transfected as described by the manufacturer (Invitrogen) three times at 24-h intervals. For the third transfection, the c-*fos* enhancer luciferase reporter plasmid with the TCF site mutation (pm18GL3), the simian virus 40 promoter internal control plasmid pRL-SV40P, and/or an expression plasmid for RhoAV14 were cotransfected. The cells were then serum starved and induced as described for the luciferase assays above.

[^{35}S]methionine labeling. The RNAi-mediated reduction of endogenous MKL1 and MKL2 expression was tested by [^{35}S]methionine labeling of endogenous proteins. The transfected HeLa cells were washed twice with methionine-free DMEM (ICN). The cells were then incubated for 30 min at 37°C to deplete the intracellular pools of methionine and metabolically labeled for 4 h with [^{35}S]methionine (100 μCi/ml; ICN). The immunoprecipitations were performed as described above with anti-MKL1 and anti-MKL2 sera (1:200). Proteins were resolved on an SDS–8% polyacrylamide gel, and the gel was then dried and exposed to film.

RNase protection assays. RNase protection assays were performed essentially as described (9). Briefly, total RNA was prepared from serum-starved or -induced HeLa or NIH 3T3 cells with Trizol reagent (Invitrogen). An antisense human c-*fos* RNA probe, pGH, was as described (17). The plasmid was digested with *Bss*HII and transcribed with SP6 RNA polymerase, yielding a 394-nucleotide probe and a 286-nucleotide protected fragment. A human cyclophilin probe (Ambion) was used as an internal control. A mouse c-*fos* RNA probe, p149, was as described (20). The plasmid was digested with *Hin*dIII and transcribed with

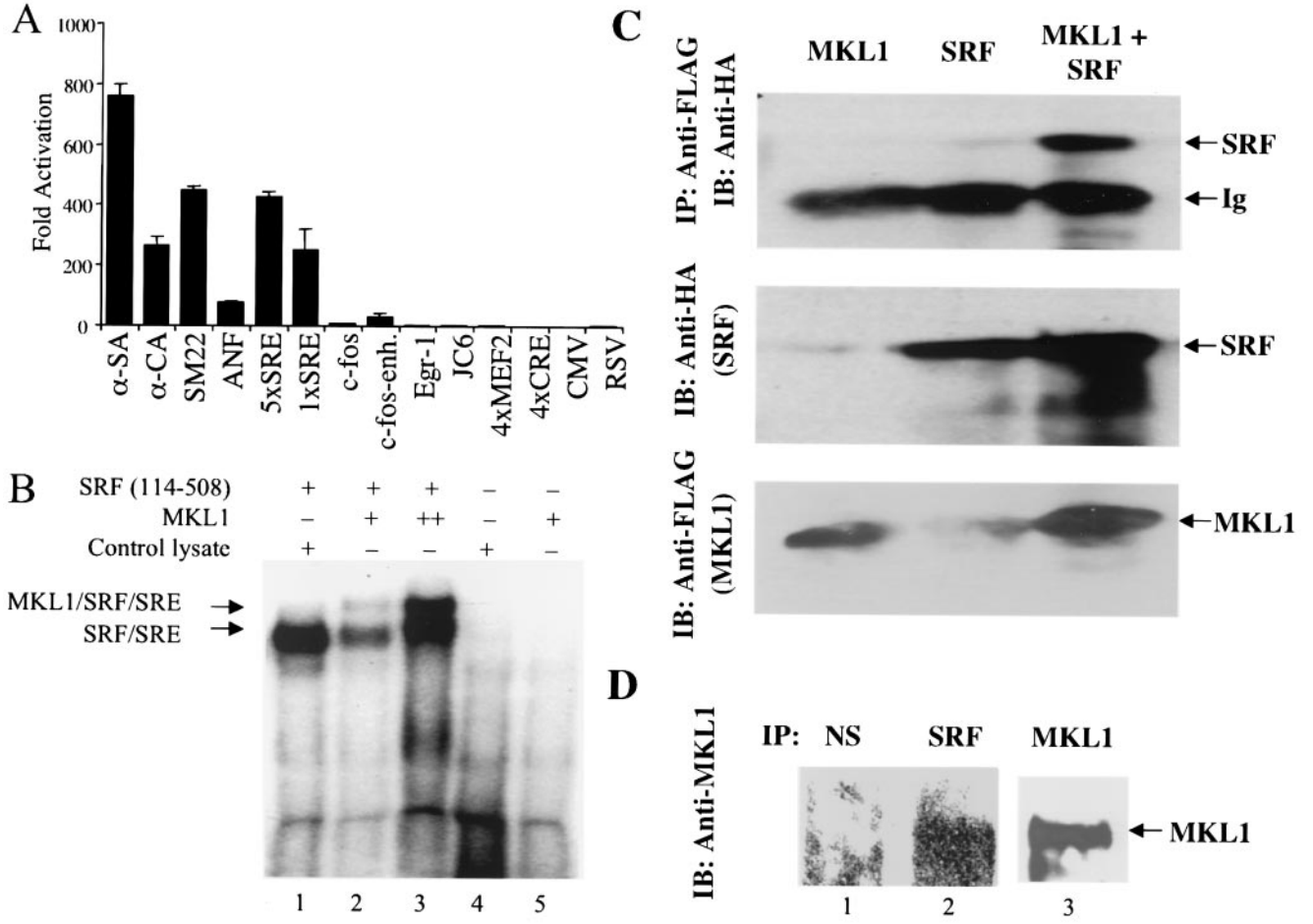

FIG. 1. Transcriptional activation of SRE-dependent promoters by MKL1 and binding of SRF to MKL1. (A) SRE-dependent or -independent luciferase reporter genes (0.5 μg), together with pRL-SV40P (50 ng) as an internal control, were transiently transfected into HeLa cells with vector (pcDNA3) or an MKL1 expression plasmid, p3×Flag-MKL1 (1 μg). Two days after transfection, luciferase assays were performed and normalized to the *Renilla* luciferase control. The values are expressed as the increase with MKL1 compared to the vector. Data are represented as the mean ± standard deviation of three independent experiments. α-SA, smooth muscle α-actin; α-CA, cardiac α-actin; ANF, atrial natriuretic factor. (B) Binding of MKL1 to SRF in gel mobility shift assays. Gel mobility shift assays were performed with ^{32}P-labeled oligonucleotide probe for a high-affinity SRF binding site (XGL) with bacterially expressed SRF(114-508) and/or in vitro translation products of MKL1 (5 and 8 μl in lanes 2 and 3, respectively) or control translation lysates (5 μl) as indicated. (C) Coimmunoprecipitation of MKL1 and SRF. HA-tagged SRF and 3×Flag-tagged MKL1 (2 μg each) were transfected into HeLa cells alone (with 2 μg of control plasmid pEGFP-N1) or together as indicated. Immunoprecipitates (IP) with anti-Flag antibodies were immunoblotted (IB) for SRF with anti-HA antibodies (top) and reprobed with anti-Flag antibodies to determine the presence of Flag-tagged MKL1 (bottom). One-thirtieth of the cell lysates was directly immunoblotted with anti-HA antibodies to detect HA-tagged SRF (middle). The positions of HA-SRF, Flag-MKL1, and the immunoglobulin heavy chain (Ig) are indicated. (D) Coimmunoprecipitation of endogenous SRF and MKL1. HeLa cell lysates were immunoprecipitated with nonspecific rabbit serum (NS) or rabbit antiserum to SRF or MKL1 and immunoblotted with anti-MKL1 serum. For lane 3, 1/10 as much lysate was used. MKL1 migrated at 160 kDa relative to markers.

SP6 RNA polymerase, yielding a 225-nucleotide probe and a 110-nucleotide protected fragment. Fragments of coding sequences from mouse SRF (nucleotides 1060 to 1780 in GenBank accession no. NM_020493) and vinculin (nucleotides 2710 to 3210 in GenBank accession no. NM_009502) from EST clones 8920535 and 6600824, respectively, were subcloned into pcDNA3. The plasmids were digested with *Pvu*II and *Eco*RV, respectively, and transcribed with T7 RNA polymerase, yielding a 459-nucleotide probe with a 445-nucleotide protected fragment for SRF and a 349-nucleotide probe with a 335-nucleotide protected fragment for vinculin.

Acidic ribosomal phosphoprotein P0 (ARPP-P0) RNA was measured as an internal control with a full-length mouse cDNA in pBS-SK (9). This plasmid was digested with *Stu*I and transcribed with T3 RNA polymerase to yield a 230-nucleotide probe and a 150-nucleotide protected fragment. Total RNA (15 μg) from each time point was hybridized with 2×10^5 cpm of probe at 48°C overnight. The samples were then digested with RNases A and T_1, followed by proteinase K digestion. The products were analyzed on 5% polyacrylamide–7 M urea gels. The gels were dried, exposed to X-ray film, and then quantitated with a Phosphorimager (Molecular Dynamics).

RESULTS

Activation of SRE-dependent reporters by MKL1. In order to test the SRF coactivator activity of MKL1, we expressed MKL1 with a series of SRE-containing promoters linked to luciferase reporters in HeLa cells. MKL1 very strongly activated smooth muscle α-actin (α-SA), cardiac α-actin (α-CA), and SM22 promoters by over 200-fold (Fig. 1A). MKL1 also strongly activated the atrial natriuretic factor (ANF) promoter

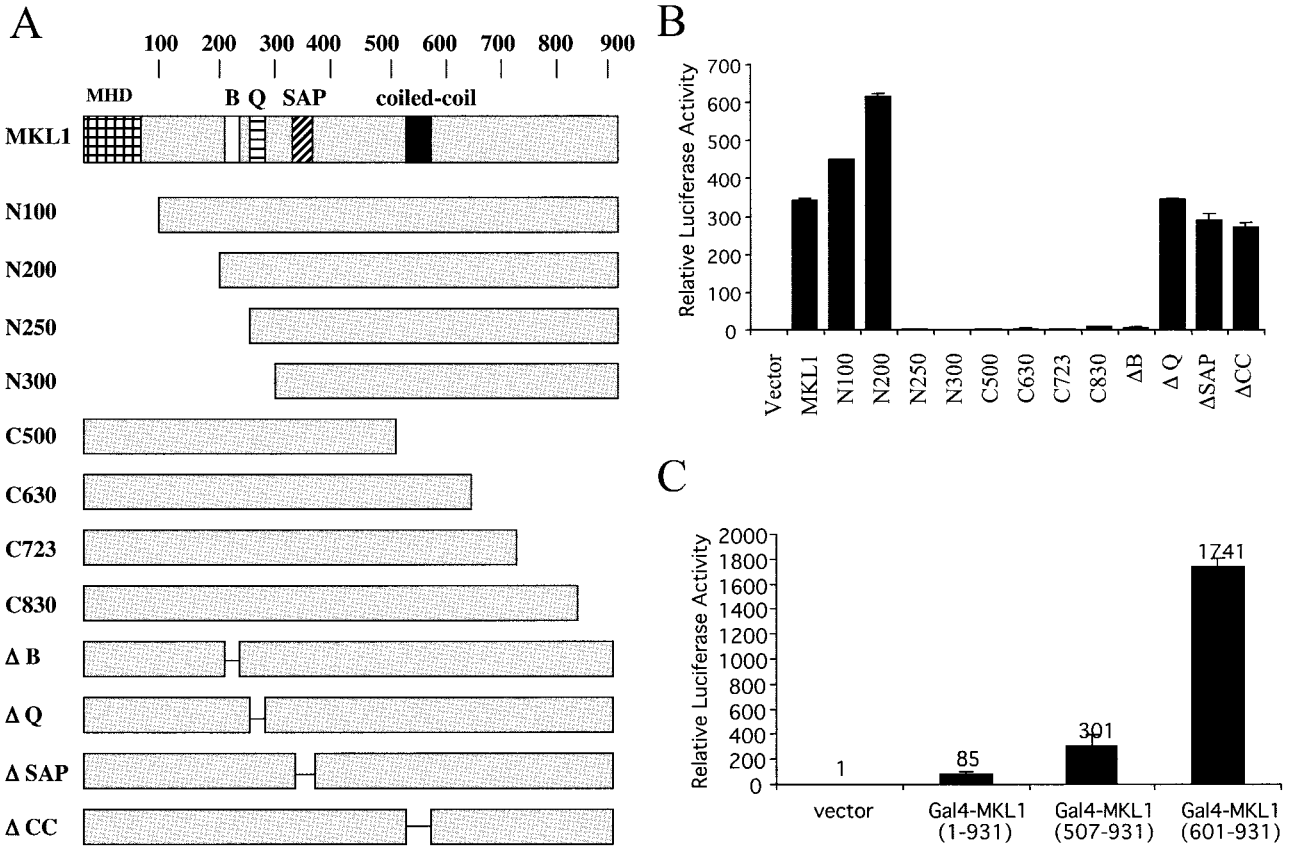

FIG. 2. Domains of MKL1 required for SRE promoter activation. (A) Diagram of MKL1 mutants. All MKL1 mutants contain a 3×Flag epitope at the amino terminus. MHD, MKL homology domain; B, basic domain; Q, glutamine-rich domain. (B) The indicated MKL1 constructs were tested for SRF activation with the 1×SRE luciferase reporter as in Fig. 1A. (C) HeLa cells were transiently transfected with expression vectors (0.2 μg) encoding the indicated regions of MKL1 fused to the GAL4 DNA binding domain (amino acids 1 to 147) and the 5×GAL4-E1b-luciferase reporter (0.5 μg), which contains binding sites for the GAL4 DNA binding domain. Luciferase activity is expressed as the increase above that observed with the GAL4 DNA binding domain alone and is the mean ± standard deviation of two independent experiments.

reporter and reporters containing one or five copies of the c-*fos* SRE. MKL1 activated the c-*fos* promoter less well than the other SRE-containing promoters but still activated it ninefold. A c-*fos* promoter construct with just the c-*fos* enhancer (containing the SRE) and the minimal c-*fos* promoter was more strongly activated (20-fold). Surprisingly, the Egr-1 promoter, an immediate-early gene with five SREs, was only activated twofold. In contrast, MKL1 did not activate several non-SRE promoters, including the c-*jun* promoter (JC6), promoters with four copies of myocyte enhancer factor 2 sites (4×MEF2), or cyclic AMP-responsive element sites (4×CRE), and the cytomegalovirus and Rous sarcoma virus promoters (Fig. 1A).

MKL1 forms a complex with SRF. To determine whether MKL1 binds to SRF, we used two assays. First, in a gel mobility shift assay, we used a high-affinity SRE probe (XGL) and bacterially expressed SRF (amino acids 114 to 508), which contains the MADS box (MCM1, AG, DEFA, and SRF) DNA binding domain and a C-terminal transcriptional activation domain. Full-length MKL1 was in vitro translated. MKL1 did not bind the SRE alone but caused a supershift of the SRF-SRE complex, suggesting that it forms a complex with SRF (Fig. 1B). Second, we assayed for the SRF-MKL1 interaction by coimmunoprecipitation of transfected epitope-tagged proteins in HeLa cells. We found that immunoprecipitated MKL1 specifically precipitated SRF, further demonstrating the SRF-MKL1 interaction (Fig. 1C). We also tested for the interaction of endogenous SRF and MKL1 with antiserum to SRF and MKL1. Immunoprecipitation with anti-SRF serum was able to coprecipitate MKL1 (at about 160 kDa) while MKL1 was not detected with immunoprecipitation by nonspecific serum (Fig. 1D, lanes 1 and 2). The level of coimmunoprecipitation was low compared to the amount of MKL1 in the cell, as can be seen by a comparison with lane 3, where 1/10 of the lysate was immunoprecipitated with anti-MKL1 and immunoblotted with the same serum (Fig. 1D).

Mapping domains of MKL1. To investigate the properties of MKL1, we generated a series of amino- and carboxyl-terminal deletion mutants (Fig. 2A). Deletion of the first 200 residues (mutants N100 and N200) did not impair MKL1 transcriptional activity on a 1×SRE promoter (Fig. 2B). This region contains a segment highly homologous to myocardin; however, its removal caused a slight increase in activity. Further amino-terminal deletion to residue 250 (N250), which eliminates the basic region, totally abolished MKL1 transcriptional activity. This deletion also resulted in the relocalization of MKL1 from the nucleus to the cytoplasm, while all the other deletion mu-

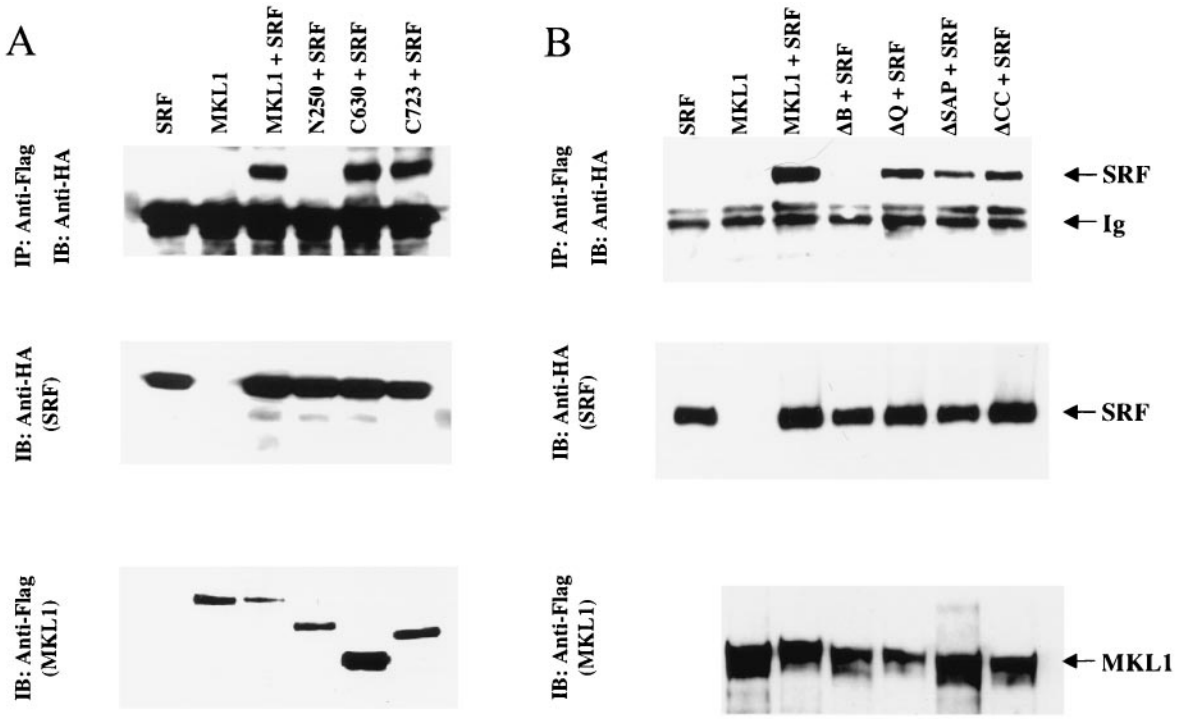

FIG. 3. Coimmunoprecipitation of SRF with MKL1 mutants. HA-tagged SRF and the indicated Flag-tagged MKL1 constructs were transfected into HeLa cells and immunoprecipitated (IP) with anti-Flag antibodies as described for Fig. 1C. Two separate sets of MKL1 mutant proteins that were examined for their ability to associate with SRF in independent experiments are shown in panels A and B. IB, immunoblot.

tants retained MKL1's nuclear localization (data not shown). All of the carboxyl-terminal deletion mutants (C500, C630, C723, and C830) completely lost MKL1 activity (Fig. 2B). This is likely due to the loss of a transcriptional activation domain, since fusion of the C terminus of MKL1 to the GAL4 DNA binding domain caused strong activation of a GAL4 site reporter gene (Fig. 2C). Deletion of amino-terminal regions of MKL1 in the GAL4 fusions led to higher activation. Since we found that each of the GAL4-MKL1 fusions was similarly expressed (data not shown), this suggests that MKL1's transcriptional activation domain is regulated by other regions of the molecule (Fig. 2C). In addition, the expression of each of the MKL1 deletion mutants was similar, as measured in immunoblots; thus, variations in protein levels cannot explain the changes in activity (Fig. 3 and data not shown).

To more specifically investigate the roles of the domains of MKL1 that are conserved with myocardin, we made internal deletions of the basic region (B), the glutamine-rich region (Q), the SAP domain, and a leucine zipper-like region (coiled-coil region) (Fig. 2A). Deletion of the basic region totally abolished MKL1 activity, although this mutant remained in the nucleus (Fig. 2B and data not shown). Somewhat surprisingly, deletion of all of the other domains had no effect on SRE activation. This contrasts with myocardin, in which deletion of the glutamine-rich region abolishes activation (62). We found that binding of the MKL1 mutants to SRF correlated well with SRE activation, since only the N250 and basic region deletion mutants abolished SRF binding in coimmunoprecipitation experiments (Fig. 3).

Dominant negative MKL1 blocks serum and RhoA induction of SRE reporter genes. We sought to use the defective MKL1 mutants as dominant negative inhibitors of endogenous MKL1 activity. We tested the ability of a given mutant to act as a dominant negative by cotransfection with wild-type MKL1 and found that the C630 and C723 C-terminal deletion mutants strongly inhibited MKL1 activation of the cardiac α-actin, smooth muscle α-actin and 1×SRE promoters (Fig. 4A and data not shown). We subsequently used C630 as a dominant negative mutant since C723 caused a weak activation of three fold.

To investigate the requirement of MKL1 in serum induction of the c-*fos* SRE, we transfected into HeLa cells the dominant negative MKL1 mutant C630 (DN-MKL1) with the c-*fos* enhancer reporter gene and induced expression by addition of serum. DN-MKL1 only modestly inhibited serum induction of the c-*fos* enhancer reporter (Fig. 4B); however, the c-*fos* enhancer SRE contains a TCF site that can be activated by MAP kinase pathways. We therefore tested a TCF site mutation in the c-*fos* enhancer reporter gene pm18, which strongly reduces MAP kinase and TCF activation of the promoter (22, 65). DN-MKL1 more strongly reduced serum induction of the pm18 reporter by 70% (Fig. 4B). We obtained similar results with induction by lysophosphatidic acid (LPA), a major mitogenic component of serum (Fig. 4C), and with serum induction of mouse NIH 3T3 cells (data not shown). As controls, DN-MKL1 did not inhibit TPA induction of a c-*fos* enhancer reporter (Fig. 4D). TPA induction is primarily mediated by the ERK1/2 MAP kinases, which phosphorylate TCF factors and

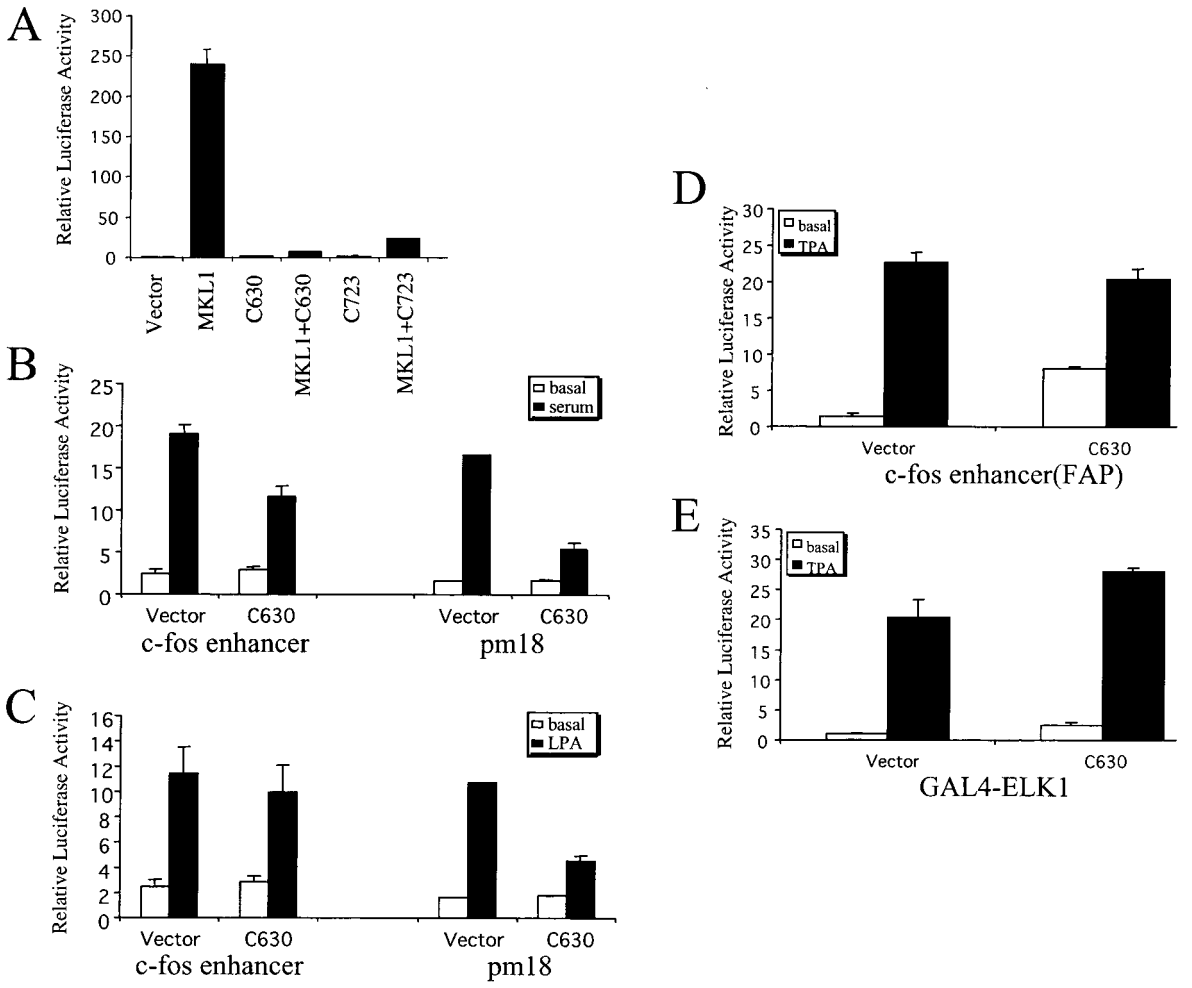

FIG. 4. Dominant negative MKL1 inhibits serum and LPA induction of the SRE. (A) Expression plasmids for MKL1 and MKL1 C-terminal deletion mutants (1 μg each) were transfected with the cardiac α-actin promoter luciferase reporter plasmid and pRL-SV40P. (B and C) HeLa cells were transfected with the c-*fos* enhancer reporter gene or the c-*fos* enhancer with a mutated TCF site (pm18) in the presence or absence of the MKL1 dominant negative mutant C630 (1 μg). The transfected cells were serum starved and induced with serum (B) or LPA (C) for 3 h. (D) Cells were transfected with a c-*fos* enhancer reporter gene that includes the c-*fos* AP1 site (FAP) in addition to the SRE and TCF sites. All three sites are required for TPA induction (65). Cells were transfected with or without the DN-MKL1 C630 mutant and stimulated with TPA. (E) Cells were transfected with GAL4-ELK1 and the 5×GAL4 site reporter gene with or without DN-MKL1. Cell lysates were assayed for luciferase activity and normalized for *Renilla* luciferase activity as described for Fig. 1A.

activate ATF1/CREB factors binding adjacent to the SRE at the c-*fos* AP1 (FAP) site (22, 64, 65, 67). Activation of the TCF factor Elk-1 can also be seen with a GAL4-ELK1 fusion and a GAL4 site reporter gene. DN-MKL1 also had no significant effect on TPA induction of GAL4-ELK1 (Fig. 4E).

The TCF-independent pathway for serum activation of the SRE largely utilizes a Rho-dependent pathway (21, 28, 64). We tested whether Rho activation of the SRE requires MKL1 by transfection of activated RhoA, RhoA-Val14, with SRE reporter genes with and without DN-MKL1. DN-MKL1 strongly inhibited RhoAV14 activation of both the c-*fos* enhancer pm18 (TCF binding site mutant) and 1×SRE reporter genes (Fig. 5A and B). The c-*fos* enhancer with the TCF and FAP sites adjacent to the SRE can also be induced by the combination of activated Raf (RafBXB) and ERK2 (65). In control experiments, DN-MKL1 did not inhibit Raf activation of the c-*fos* enhancer reporter or its activation of GAL4-ELK1 (Fig. 5C and D).

Inhibition of serum and RhoA activation by RNA interference. To clarify that the effect of dominant negative MKL1 was due to inhibition of endogenous MKL factors rather than blocking the action of another factor, we used RNA interference to reduce endogenous levels of MKL1. Since an MKL1-related factor, MKL2 (MRTF-B), is expressed in HeLa cells along with MKL1 (63; A. Selvaraj and R. Prywes, submitted for publication), we also used RNAi to inhibit MKL2 levels.

To generate small interfering RNAs, we used two systems. For MKL1, we had best success with a plasmid/RNA polymerase III expression system, where a short hairpin double-stranded RNA was generated in pSUPER (pSUPER-MKL1) (4). For MKL2, we used synthetic 21-bp double-stranded RNA oligonucleotides. These reagents or control plasmid or oligo-

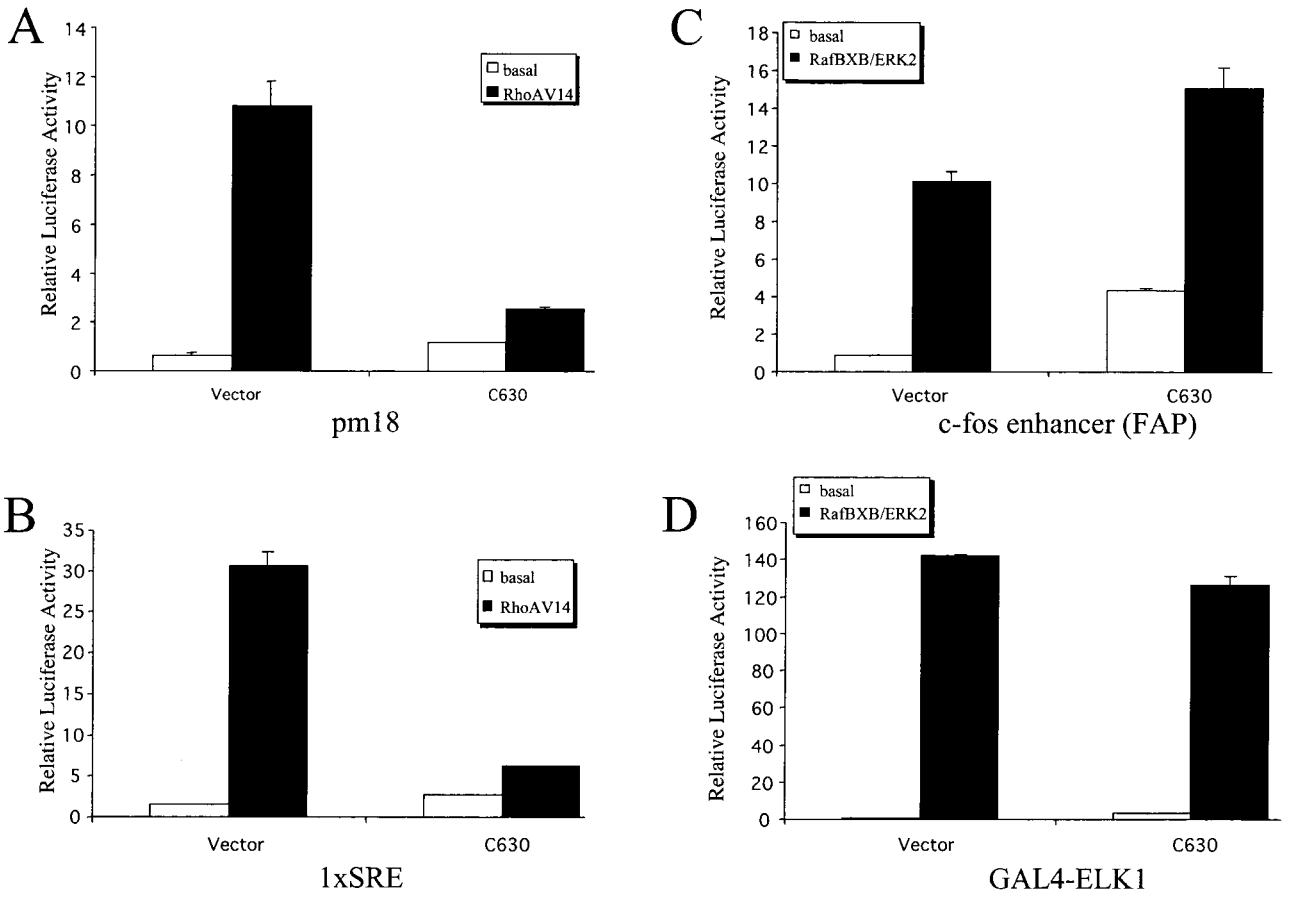

FIG. 5. Inhibition of RhoA induction of SRE reporter genes by dominant negative MKL1. HeLa cells were transfected with or without DN-MKL1 C630 and with an activated form of RhoA (RhoA-Val14) (A and B) or the combination of activated Raf (RafBXB) and wild-type ERK2 (1 μg each) (C) with the indicated reporter genes as described for Fig. 4. (D) GAL4-ELK1 and the 5×GAL4 site reporter were transfected with activated Raf, ERK2, and DN-MKL1 as indicated.

nucleotides were transfected into HeLa cells under conditions where greater than 90% of the cells were transfected, as determined by cotransfection with a GFP expression vector.

To determine whether the levels of endogenous MKL1/2 were affected, we generated antiserum in rabbits to the C-terminal domains of MKL1 and MKL2. These sera were most effective in immunoprecipitations and did not cross-react. We therefore metabolically labeled the transfected cells with [^{35}S]methionine and immunoprecipitated with anti-MKL1 or anti-MKL2 serum (Fig. 6A, top and bottom, respectively). MKL1 and MKL2 were specifically detected as bands migrating at about 160 kDa (Fig. 6A and data not shown). The pSUPER-MKL1 plasmid specifically reduced MKL1 levels without affecting MKL2. Similarly, MKL2 siRNA reduced MKL2 levels without affecting MKL1 (Fig. 6A).

We next used these RNAi reagents singly and together in cotransfections with the c-*fos* enhancer reporter plasmid pm18GL3. RNA interference of MKL1 or MKL2 reduced serum induction of the reporter gene; however, strikingly, the combination of MKL1 and MKL2 RNAi completely abolished serum induction (Fig. 6B). Similar inhibition of induction was observed for RhoAV14 activation of the reporter gene (Fig. 6C). The control points contained both the pSUPER plasmid and GFP-RNAi oligonucleotides. We also did not observe inhibition with other specific pSUPER-RNAi plasmids (data not shown). While a greater inhibition was observed with MKL1 RNAi than MKL2, it is difficult to determine the relative contribution of each because the effectiveness of RNAi inhibition on their target genes was not identical. Nevertheless, the combined inhibition strongly suggests that these factors can act redundantly in the serum and RhoA pathways for activation of the SRE.

Dominant negative MKL1 blocks induction of endogenous SRF target genes. The induction of the endogenous c-*fos* gene has not always mimicked that of c-*fos* reporter genes (1, 21). Hence, we sought to determine whether DN-MKL1 affected endogenous SRF target genes. We transfected HeLa cells with conditions under which greater than 90% of the cells were transfected, as shown by cotransfection with a GFP expression plasmid (data not shown). The expression of endogenous c-*fos* was then monitored by an RNase protection assay. Interestingly, DN-MKL1 had only a modest effect on c-*fos* expression at 30 min of serum induction but strongly decreased expression at 60 min (Fig. 7A and B). Similar results were obtained with LPA induction (data not shown). In contrast, there was no effect on serum induction of c-*jun* expression, which is not

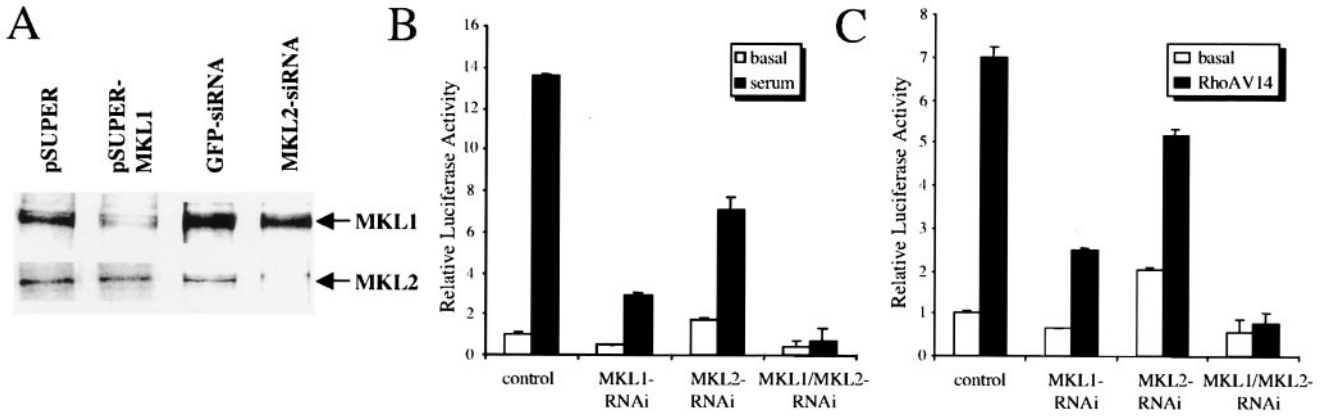

FIG. 6. RNA interference of MKL1 and MKL2 inhibits serum and RhoA activation. (A) HeLa cells were transfected with control or MKL1-specific pSUPER plasmids or GFP- or MKL2-specific double-stranded RNA oligonucleotides. The cells were metabolically labeled with [^{35}S]methionine and immunoprecipitated with anti-MKL1 or MKL2 serum (A, top and bottom, respectively). (B) HeLa cells were transfected with a c-*fos* enhancer reporter gene (pm18GL3) with control (pSUPER and GFP-siRNA), pSUPER-MKL1 (MKL1-RNAi), MKL2 siRNA, or pSUPER-MKL1 and MKL2-RNAi. The cells were serum starved and induced with serum, as indicated, and luciferase activities were measured as for Fig. 4. (C) As in B, except that the indicated samples were cotransfected with a RhoAV14 expression vector as in Fig. 5A.

regulated by SREs (25), or on expression of the constitutively expressed cyclophilin gene (Fig. 7A). Since endogenous c-*fos* would still be activated by the MAP kinase/TCF pathway, this pathway may account for the expression of c-*fos* that was not inhibited by DN-MKL1.

We also tested for inhibition by DN-MKL1 in mouse NIH 3T3 fibroblasts. We could not attain sufficiently high transfection efficiencies in these cells; therefore, we made a stably transfected cell line with a retroviral vector expressing C630 (pBabe-C630). This cell line also had reduced c-*fos* expression at the later 60-min time point, though there was little effect at 30 min (Fig. 7C and D). This result is seen more clearly in the quantitation in Fig. 7D, which represents the averages of three experiments. To confirm that the greater decrease in c-*fos* expression levels caused by DN-MKL1 at 60 min compared to 30 min was significant, we performed a *t* test. In HeLa cells (Fig. 7B), the difference of 59% versus 14% reduction was significant at $P = 0.014$, while in NIH 3T3 cells (Fig. 7D), the difference of 56% versus 7% reduction was significant at $P = 0.005$.

To more clearly determine the effect of DN-MKL1 on serum induction of SRE target genes, we examined two genes, vinculin and SRF, that have SREs but do not appear to have TCF binding sites and whose induction is more dependent upon the Rho signaling pathway (21, 58, 59). While we were not able to detect serum induction of these genes in HeLa cells, they were clearly induced in NIH 3T3 cells, though with slower kinetics than c-*fos* (Fig. 7E and data not shown). The effect of DN-MKL1 could clearly be seen in the stably transfected NIH 3T3 cells, in which serum induction of vinculin and SRF expression was almost completely blocked (Fig. 7E, F, and G). In contrast, there was no effect on an internal control gene, acidic ribosomal phosphoprotein P0 (ARRP-P0). These results suggest that MKL1 or closely related factors such as MKL2 are required for the serum induction of TCF-independent SRF target genes and that MKL1 and/or MKL1-like proteins affect the late phase of induction of TCF-dependent genes such as c-*fos*.

RBM15-MKL1 fusion protein has far greater SRE transcriptional activation capabilities than MKL1. The t(1;22) translocation in acute megakaryoblastic leukemia results in the fusion of MKL1 with RBM15 (39, 44). The RBM15-MKL1 fusion protein is thus a candidate oncoprotein. We compared the transcriptional activity of RBM15-MKL1 and MKL1 on a series of SRE-dependent reporters to test whether the translocation activates the SRF coactivator activity of MKL1. RBM15-MKL1 had activity comparable to that of MKL1 for the cardiac and smooth muscle α-actin promoters, but exhibited a greatly increased activity for the c-*fos* and Egr-1 promoters (Fig. 8A). RBM15 alone had no effect on expression of any of the reporter genes. Titration of MKL1 and RBM15-MKL1 with the c-*fos* promoter showed that RBM15-MKL1 was very poorly expressed relative to MKL1 yet activated the c-*fos* promoter much more strongly even at its lowest expression levels (Fig. 8B and C). Since RBM15-MKL1 was expressed at lower levels than MKL1, this suggests that it is also more active for the other reporters used in Fig. 7A. These results show that the SRF coactivator activity of MKL1 is greatly activated by its fusion with RBM15 and suggest a possible mechanism for the leukemogenic effects of the RBM15-MKL1 chimera.

DISCUSSION

Serum and growth factors can induce c-*fos* expression by at least two mechanisms, one dependent upon phosphorylation of TCF and the second independent of TCF. This second pathway can be blocked by Rho inhibitors, but the mechanism of regulating SRF has been unclear. We find here that a dominant negative MKL1 specifically blocked the TCF-independent pathway for serum induction and RhoA activation of SRE reporter genes. These pathways were also blocked by RNAi-mediated inhibition of MKL1 and MKL2 levels. The late phase of serum induction of endogenous c-*fos* expression was specifically blocked, suggesting that the TCF signaling pathway contributes more to the early induction, while a RhoA/MKL path-

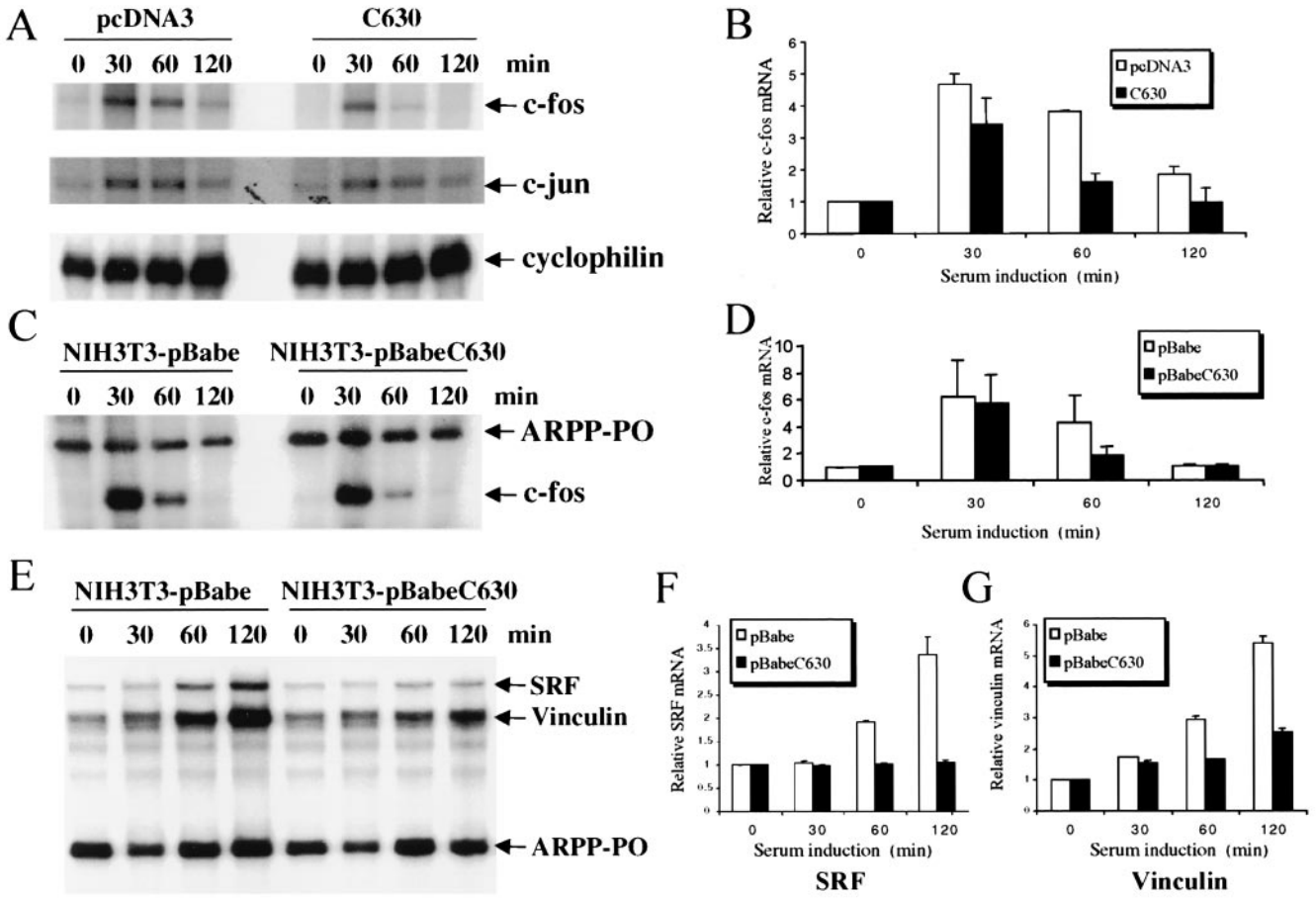

FIG. 7. Inhibition of endogenous SRF target genes by dominant negative MKL1. (A) HeLa cells were transfected with vector pcDNA3 or MKL1 dominant negative mutant C630 (4 μg) under conditions yielding greater than 90% transfected cells as confirmed by cotransfection with 0.5 μg of the GFP expression vector pEGFP-N1 (data not shown). Cells were serum starved for 24 h before stimulation with 20% newborn calf serum for the times indicated. Total RNA was prepared, and mRNAs for c-*fos*, c-*jun*, and cyclophilin detected by RNase protection assays. (B) The c-*fos* RNase protection bands in A were quantitated with a Phosphorimager and normalized to the cyclophilin expression levels. (C to G) Cell lines were generated with an empty retroviral vector (pBabe-puro) or the vector driving expression of C630 in NIH 3T3 mouse fibroblasts. The cells were serum starved and treated with 20% serum for the indicated times, and c-*fos* (C) or SRF and vinculin (E) and control acidic ribosomal phosphoprotein-P0 (ARPP-P0) mRNAs were detected by RNase protection. The signals were quantitated with a Phosphorimager, with c-*fos* (D), SRF (F), and vinculin (G) expression levels normalized to that of ARRP-P0. The quantitated results are the averages of three determinations ± standard deviation.

way is involved in later induction. The effect of DN-MKL1 could be more clearly seen on SRF target genes that do not contain TCF sites, vinculin and SRF. These genes had slower kinetics of induction than c-*fos* and were nearly completely blocked by DN-MKL1. These results suggest that MKL1 is a key component for the TCF-independent pathway of serum activation of SRF target genes. Since MKL1 was found to complex with SRF and to possess a transcriptional activation domain, recruitment of MKL1 or activation of the MKL1/SRF complex likely directly contribute to transcription from SRF-bound promoters.

Myocardin/MKL gene family. MKL1 and myocardin constitute a gene family together with a third member that we have identified, termed MKL2, which is broadly expressed in human tissues, binds SRF, and activates SRE reporter genes (A. Selvaraj and R. Prywes, submitted for publication). During the course of this work, mouse homologues of MKL1 (BSAC and MRTF-A) and MKL2 (MRTF-B) were also identified and found to activate SRE reporter genes (53, 63). Interestingly, BSAC was identified in a screen for genes that protect cells from tumor necrosis factor alpha-induced cell death. We found that MKL1 and MKL2 but not myocardin are expressed in HeLa and NIH 3T3 cells and that DN-MKL1 blocks activation of SRE reporter genes by MKL2 (A. Selvaraj and R. Prywes, submitted for publication). Our results with RNA interference show that inhibition of both MKL1 and MKL2 is required to completely block serum and RhoA induction of reporter genes. These results strongly suggest that MKL1 and MKL2 can act redundantly in these pathways.

The members of the myocardin/MKL gene family contain a number of conserved domains, including a highly conserved N-terminal domain, a basic domain, a glutamine-rich region, a SAP domain, and a leucine zipper-like region. Despite its high degree of conservation, deletion of the N-terminal domain did not abolish activation of SRE reporter genes. Similarly, removal of the leucine zipper-like region had little effect. As with

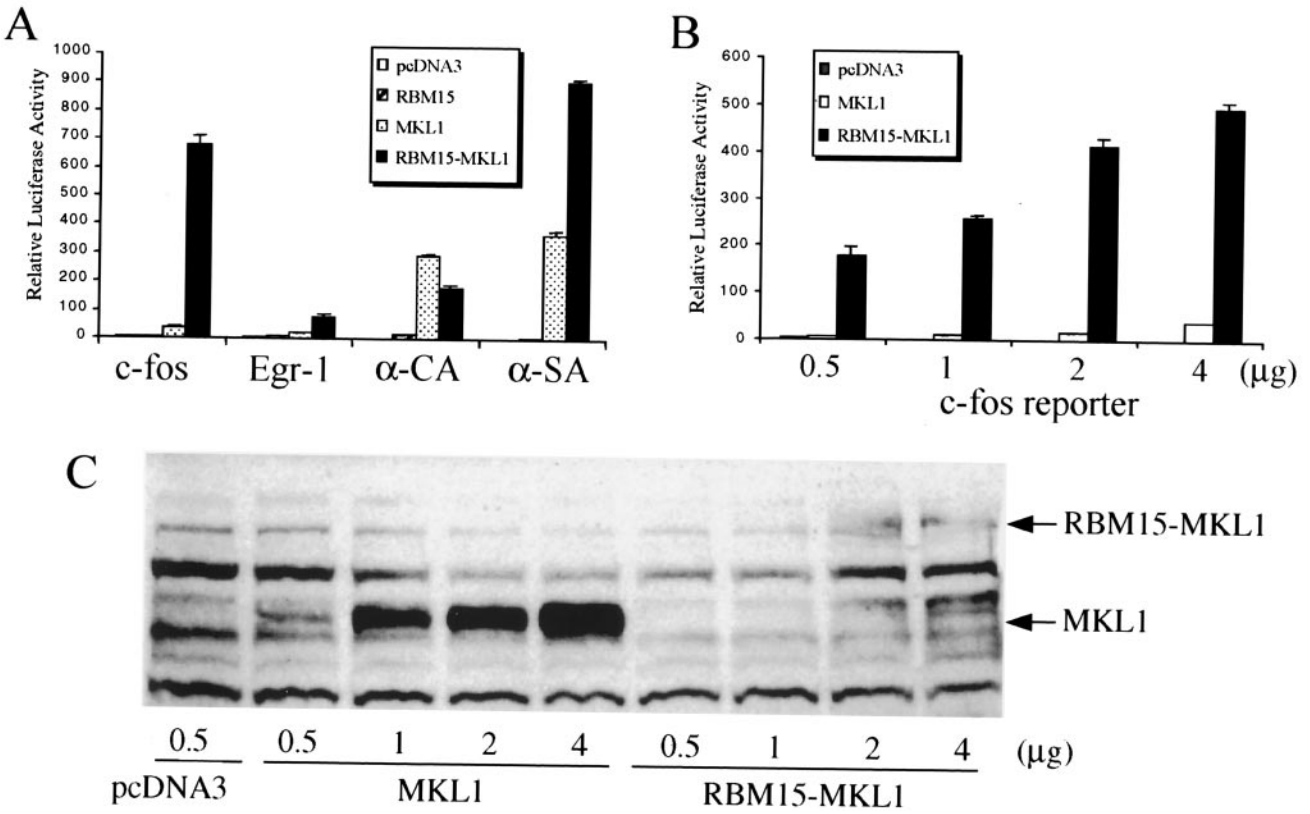

FIG. 8. RBM15-MKL1 fusion protein has a markedly increased ability to activate SRE reporter genes. (A) HeLa cells were transfected with the indicated reporter genes (0.5 μg), together with pRL-SV40P (50 ng) as an internal control, and vector pcDNA3, 1×Flag-RBM15, 1×Flag-MKL1 or 1×Flag-RBM15-MKL1 (1 μg). Cell lysates were assayed for firefly luciferase activity and normalized for *Renilla* luciferase activity. The averages of three determinations ± the standard deviation are shown. (B and C) HeLa cells were transfected with the c-*fos* promoter-luciferase gene with or without increasing amounts of 1×Flag-MKL1 or 1×Flag-RBM15-MKL1 expression plasmids, as indicated. Cell lysates were assayed for luciferase activity (B) and for protein expression by immunoblotting with anti-Flag antibodies (C).

myocardin, however, deletion of the basic region abolished SRE activation and SRF binding. In contrast to myocardin, deletion of the glutamine-rich domain had no effect on SRE activation by MKL1, suggesting that this domain is not critical for this function. The SAP domain is thought to be involved in attachment to nuclear scaffolds (3, 37, 50). Deletion of this domain did not affect activation by MKL1, while mutations in the SAP domain of myocardin affected activation of some SRE reporters but not others (62). It is possible that some of the regions of MKL1 are required for growth factor regulation but that this requirement is not observed in the SRE reporter assays performed here that involve MKL1 overexpression.

Rho pathway signaling to the SRE. The mechanism of TCF-independent activation of SRF by serum has been elusive. While SRF is phosphorylated at a number of residues, none of these modifications have been definitively linked to serum induction of SREs (29, 38, 41, 43, 49). Activated Rho family members increase expression of SRE reporter genes, and Rho inhibitors block their serum induction (28). Since RhoA can be activated by LPA receptors (34), these observations suggested that this pathway mediates TCF-independent activation of SREs. Downstream of RhoA, the formin-related protein mDia and Rho kinase can control SRE activation (11, 15, 19, 61). Besides activating the SRE, RhoA also causes the formation of cytoskeletal stress fibers, and the RhoA effectors mDia and Rho kinase are required for stress fiber formation (reviewed in references 18 and 32). Together with the effects of actin overexpression and actin polymerization mutants and inhibitors, these results suggested that the formation of stress fibers or the reduction of free actin results in activation of SREs (46, 58). However, mutants of RhoA have been found that distinguish between the formation of stress fibers and SRE activation; i.e., these mutants can activate stress fibers but not SREs or vice versa (51, 68). This leaves open the possibility that RhoA uses unique effectors and pathways to activate the SRE from those employed to regulate stress fibers, although there must be some degree of cross talk between the cytoskeleton and SRE regulation. Resolution of this issue will require linking the pathway from RhoA to SRE.

DN-MKL1 and MKL-1 and -2 RNAi blocked RhoA-mediated activation of SREs, suggesting that MKL-1 and -2 are distal targets of this pathway since they bind SRF. Elucidation of the mechanisms by which MKL-1 and -2 are regulated by serum and/or RhoA should reveal whether this process is mediated by changes in the cytoskeleton or by other pathways. As a step in examining these mechanisms, we detected a serum-induced increase in MKL1 phosphorylation (unpublished re-

sults) and are investigating whether this change affects its activity.

SRF target gene activation by MKL1. We found that some SRE-containing promoters, similar to myocardin, were activated better than others. Several muscle-specific promoters were highly activated, while two immediate-early promoters, c-*fos* and Egr-1, were relatively poorly activated. This variation in activation does not appear to be due to differences in the precise SRE sequences, since a single c-*fos* SRE was strongly activated by MKL1. Thus, the context of the SREs in the promoters appears to control their level of activation. In addition, although we detected low activation for the c-*fos* promoter, there was still a significant increase of ninefold. It is possible that the immediate-early gene promoters are under tighter control than the muscle-specific promoters because the former need to be regulated rapidly by growth factors. This does not preclude MKL1 from contributing to transcriptional activation after stimulation of growth factor signaling pathways. Further work with DN-MKL1 will be useful to determine which endogenous genes in addition to vinculin and SRF depend upon MKL-1 or -2 for serum induction.

Activation of MKL1 in acute megakaryoblastic leukemia. The t(1;22) chromosomal rearrangement in acute megakaryoblastic leukemias results in the fusion of the RBM15 and MKL1 genes (also known as OTT and MAL, respectively), analogous to the aberrant chimeric transcriptional regulatory factors found in other forms of acute myeloid leukemia, such as PML-retinoic acid receptor alpha in acute promyelocytic leukemia and AML1-ETO in acute myelogenous leukemia (2, 16, 39, 44). RBM15 contains three RNA recognition motifs as well as a C-terminal domain with homology to *Drosophila spen* previously described as a Spen paralog and ortholog C-terminal domain (66). Spen affects *hox* function and *ras* signaling in *Drosphila melanogaster*, though its precise mechanism of action is not known (8, 36, 48, 66).

In our reporter assays, the RBM15-MKL1 fusion strongly enhanced MKL1 function, particularly activating the c-*fos* reporter gene despite significantly lower levels of expression of the chimeric protein compared to normal MKL1. RBM15 alone had no effect in these assays, suggesting that it does not regulate SREs but rather affects the activity of MKL1 when the two are linked due to the t(1;22) translocation. These results suggest that these leukemias may be caused in part by the inappropriate activation of MKL1 and SRF target genes. It will be interesting to determine how the fusion of RBM15 to MKL1 causes its activation. We have not had sufficient material to determine whether SRF target genes are activated in acute megakaryoblastic leukemia cells. However, we tested a myeloid cell line (32Dcl3) engineered to overexpress RBM15-MKL1 and found that neither the SRF, vinculin, nor c-*fos* gene was more highly expressed compared to parental cells (unpublished results). Future work will require more comprehensive gene expression profiling to determine whether SRF target genes are activated in cells containing the RBM15-MKL1 fusion.

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