Megakaryoblastic Leukemia-1/2, a Transcriptional Co-activator of Serum Response Factor, Is Required for Skeletal Myogenic Differentiation*

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Serum response factor (SRF) is required for the expression of a wide variety of muscle-specific genes that are expressed upon differentiation and is thus required for both striated and smooth muscle differentiation in addition to its role in regulating growth factor-inducible genes. A heart and smooth muscle-specific SRF co-activator, myocardin, has been shown to be required for cardiac development and smooth muscle differentiation. However, no such co-factors of SRF have been identified in the skeletal myogenic differentiation program. Myocardin and the related transcription factor megakaryoblastic leukemia-1 (MKL1/MAL/MRTF-A) can strongly potentiate the activity of SRF. Here we report the cloning of the third member of the myocardin/MKL family in humans, *MKL2*. MKL2 binds to and activates SRF similar to myocardin and MKL1. To determine the role of these factors in skeletal myogenic differentiation we used a dominant negative MKL2 to show that the MKL family of proteins is required for skeletal myogenic differentiation. Expression of the dominant negative protein in C2C12 skeletal myoblasts blocked the differentiation-induced expression of the SRF target genes skeletal α-actin and α-myosin heavy chain and blocked differentiation of the myoblasts to myotubes *in vitro*. C2C12 cells express both MKL1 and MKL2, but not myocardin, implicating MKL1 and/or MKL2 in the requirement for skeletal myogenic differentiation. MKL1 was predominantly cytoplasmic in C2C12 cells, with a small amount in the nucleus, however, no movement of MKL1 to the nucleus was observed upon differentiation.

Serum response factor (SRF)[1] is a transcription factor that belongs to the MADS box family and binds as a dimer to a conserved CArG box sequence $(CC(A/T)_6GG)$ (1–3). CArG boxes, also known as serum response elements (SREs), are found in the promoters of a diverse set of genes such as the immediate early genes c-*fos*, *egr-1*, and *nur77*, and the muscle-specific genes α-actin, α-myosin heavy chain, and muscle creatine kinase (4–8). SRF is required for both the induction of immediate early genes by growth factors and the induction of muscle-specific genes during differentiation. Microinjection of anti-SRF antibodies blocked the serum induction of the c-*fos* gene in NIH3T3 cells as well as the expression of muscle-specific genes and the terminal differentiation of C2C12 skeletal myoblasts (9, 10).

Skeletal muscle differentiation involves a series of events, first the determination of paraxial mesodermal cells as skeletal myoblast precursors by muscle regulatory factors such as MyoD and Myf5, then the withdrawal of proliferating myoblasts from the cell cycle in response to specific differentiation signals, the expression of a battery of muscle-specific genes and the fusion of myoblasts to form myotubes and subsequently myofibers (11, 12). Many of the muscle-specific genes that are expressed during differentiation, such as α-actin, α-myosin heavy chain, and muscle creatine kinase have CArG box(es) in their promoters and thus are dependent on SRF for their expression. Consistent with the requirement of SRF for gene expression, inhibition of SRF expression or activity prevented skeletal muscle differentiation (10, 13). Unlike the c-*fos* SRE that has a ternary complex factor binding site adjacent to the CArG box, muscle-specific promoters do not have obvious ternary complex factor binding sites. Hence it appears that activation of these promoters occurs through the complexing of SRF with other factors. SRF physically interacts with numerous cardiac-specific factors including GATA-4, Nkx2.5, TEF-1, myocardin, CRP1/2, Barx1b, and Hop and thus regulates the cardiac differentiation program (14–19). In smooth muscle differentiation, the SRF interacting proteins CRP1/2 and myocardin have been shown to be necessary (15, 16). For the case of skeletal muscle differentiation fewer SRF complexing factors have been identified. Notably, however, the homeodomain factor Barx2b binds to SRF and Barx2b antisense oligonucleotides inhibited the differentiation of skeletal muscle cells (20, 21).

SRF can be activated by at least two pathways, one involving the phosphorylation of ternary complex factor and the second being activation by a RhoA-induced pathway (22, 23). The RhoA GTPase can activate SRF though actin treadmilling, connecting cytoskeleton changes to nuclear gene activation (24). Because the muscle-specific promoter CArG boxes do not have obvious ternary complex factor binding sites, it is possible that they are regulated by the Rho pathway. Consistent with this notion is the finding that dominant negative RhoA can reduce myogenic differentiation (25). Furthermore, STARS, an actin-binding protein is expressed specifically in striated muscles and has been shown to activate RhoA and SRF activity (26). The Rho effector p160 rho kinase and actin treadmilling have also been shown to be necessary for smooth muscle cell

[1] The abbreviations used are: SRF, serum response factor; SRE, serum response element; MKL, megakaryoblastic leukemia; RT, reverse transcription; RACE, rapid amplification of cDNA ends; UTR, untranslated region; aa, amino acid(s); CMV, cytomegalovirus; HA, hemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CPE, cytoplasmic polyadenylation elements; DN, dominant negative.

differentiation (27). The connection of Rho signaling to SRF activation, however, in skeletal muscle cell differentiation has not been previously identified.

Myocardin was identified as a heart-specific protein that binds to SRF and is a potent activator of SRF transcriptional activation (15). Subsequently, myocardin has also been shown to be expressed in smooth muscle cell lineages (28–30). Use of dominant negative myocardin suggests that it is required for cardiac and smooth muscle cell differentiation (15, 29). Because of the tissue specificity, however, myocardin was not a candidate for regulation of skeletal muscle-specific genes or growth factor-inducible immediate early genes. We recently identified megakaryoblastic leukemia-1 (MKL1), a protein with a similarity to myocardin, as a potent transcriptional activator of SRF that is broadly expressed in many tissues (31). MKL1/MAL was originally identified as part of a fusion protein with RBM15/OTT at a t(1;22) translocation in acute megakaryoblastic leukemia (32, 33). Others have also recently identified MKL1/MAL/BSAC/MRTF-A as an activator of SRF (34–36). We and others have also recently found that MKL1, or related factors, are required for RhoA activation of SRE reporter genes in HeLa and NIH3T3 cells suggesting that MKL family members could be mediating the requirement of Rho for muscle cell differentiation (31, 34). Here we report the cloning of a third member of the myocardin/MKL family, MKL2, in humans. A mouse homologue of this gene, *MRTF-B*, was also recently identified and a fragment of the gene was previously identified as *MAL16* (33, 36). We find that MKL2 is broadly expressed, with strong expression in skeletal muscle, and can interact with SRF and activate many CArG box-containing promoters. We have used the C2C12 skeletal myoblast cell line as a model to test for the role of MKL proteins in skeletal muscle differentiation.

EXPERIMENTAL PROCEDURES

Cloning of Human MKL2—Human genomic sequences were searched using BLAST for sequences that were similar to MKL1 and myocardin. A sequence identified on chromosome 16 was then used to search for human ESTs. Two EST clones (GenBank™ accession numbers BG831958 and AB033069) were obtained encoding partial cDNAs. The EST sequences were used to design primers to the 5′ and 3′ end of the coding sequence (primer sequences available upon request). The full coding sequence was then isolated by RT-PCR using these primers and HeLa cell RNA. 5′ Rapid amplification of cDNA ends (5′ RACE) was performed using the Clontech 5′ RACE kit to determine the 5′ end of the cDNA. A single major amplified band was identified and sequenced suggesting that the major 5′ end has been identified. The conserved motifs were identified with the SMART program (37).[2] 5′ and 3′-UTR scans for regulatory sequence elements were performed using the UTRScan program (38).[3]

Plasmid Constructs—MKL2 cDNA encoding the full-length protein or deletion mutants were subcloned into the expression vector p3×-FLAG-CMV-7.1 (Sigma) in-frame with an N-terminal 3× FLAG epitope tag. The deletion mutants lack the following amino acids: ΔN, 1–71; ΔC700, 700–1049; ΔL, 574–604. The MKL1 coding region was also expressed in p3×-FLAG-CMV-7.1 as described (31).

For the Gal4 fusion protein constructs, full-length MKL2 or amino acids 703–1049 were subcloned into an HA-Gal4 (aa 1–147) vector driven by a CMV promoter (39). The retroviral vector for MKL2ΔNΔC700 was constructed by placing amino acids 71–700 of MKL2 with a 3× FLAG epitope tag at the N terminus in the pBabepuro vector (40). For *in vitro* translation products, the indicated MKL2 coding regions were subcloned into the pcDNA3.1(−) vector (Invitrogen).

Luciferase reporter plasmids with the promoter sequences of cardiac α-actin, SM22, atrial natriuretic factor, c-*jun* (JC6), and 5×GAL4-E1B-luc were as described (41–44). The human smooth muscle α-actin luciferase reporter was a gift of C. Chandra Kumar. The CMV luciferase reporter was constructed by cloning the promoter for the cytomegalo-

virus immediate early gene 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 (45). The 4× CRE reporter with four CRE sites upstream of a TATA box was from Stratagene. The 1× SRE reporter contains the c-*fos* SRE sequence 5′-AGGATGTCCATATTAGGACATC-T-3′ upstream of the c-*fos* minimal promoter (−53 to +45) and the luciferase gene in pOFluc-GL3 (42). The internal control plasmid pRLSV40P (containing the SV40 promoter driving the Renilla luciferase gene) and expression plasmids for RhoA-V14 and HA-tagged human SRF (pCGNSRF) were as described (41, 46, 47).

Cell Culture, Transfection, and Luciferase Assays—HeLa cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% newborn calf serum (Sigma) at 37 °C in a 5% CO$_2$ incubator. The cells were transiently transfected on six-well plates using the calcium phosphate DNA precipitation method. The cells were incubated with the transfection mixture for 16 h and then changed to fresh media for another 24 h. Luciferase assays were performed as described (48). 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.

The C2C12 skeletal myoblast line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For differentiation studies, C2C12 cells were plated at 8×10^5 cells per 6-cm plate in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The next day (day 0), differentiation was induced by replacing the media with Dulbecco's modified Eagle's medium with 2% horse serum for 3 days. For proliferation studies, C2C12 cells and its stable derivatives were plated at a density of 2×10^5 cells per 6-cm plate and the cell numbers were counted every 24 h for 3 days.

Immunoprecipitations, Immunoblots, and Cellular Fractionation—For immunoprecipitations, cells from 6-cm plates were rinsed once in ice-cold phosphate-buffered saline and then lysed in 500 μl of IP buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture; Sigma number P8340). The lysates were rotated for 1 h at 4 °C and cleared by centrifugation at $13,000 \times g$ for 10 min at 4 °C. Transfected MKL2 (3×FLAG-MKL2), MKL1 (3×FLAG-MKL1), or endogenous MKL1 were immunoprecipitated with 2 μl of anti-FLAG (M2; Sigma) or anti-MKL1 antibodies overnight. Protein A-Sepharose (Amersham Biosciences) (25 μl of a 50% slurry in IP buffer) was then added for 1 h. The protein A beads were washed three times with IP buffer and the proteins were resolved on an SDS-polyacrylamide gel. The proteins were immunoblotted with anti-FLAG or anti-HA (HA.11; Covance) antibodies as indicated at a 1:2000 dilution and horseradish peroxidase-conjugated secondary antibodies at a 1:5000 dilution. The signals were visualized with ECL plus reagents (Amersham Biosciences). Rabbit anti-MKL1 sera was generated by injecting rabbits with glutathione *S*-transferase-MKL1 (aa 601–931) purified from *Escherichia coli*. For immunoblotting the immunoglobulin was partially purified with protein A-Sepharose.

For myoblast protein expression analysis, the differentiated and undifferentiated cells were lysed in IP buffer at the indicated time points and the protein concentrations of the lysates were determined by the Bradford method using the Bio-Rad protein assay dye reagent. Equal amounts of protein from each time point were immunoblotted with the indicated antibodies. For detecting dominant negative MKL2, α-actin, and β-tubulin, 20 μg of the cell lysates were immunoblotted with anti-FLAG, anti-α-actin (5C5; Sigma), or anti-β-tubulin (3F3 ascites fluid; a gift from Jim Lessard) antibodies at 1:2000, 1:1500, and 1:6000 dilutions, respectively. For skeletal α-myosin heavy chain, 80 μg of whole cell lysates were immunoblotted with undiluted MF-20 cell supernatant (Developmental Studies Hybridoma Bank).

For cellular fractionation of NIH3T3 and C2C12 cells, nuclear and cytoplasmic extracts were prepared essentially as described (49). Cells were incubated in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and fresh protease inhibitor mixture) for 15 min, Nonidet P-40 was added to 0.5% and the samples were centrifuged for 5 min at $2000 \times g$ and the supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in an equal volume of buffer A and used as the nuclear fraction. Equal volumes of cytoplasmic and nuclear fractions were immunoblotted with anti-MKL1, mouse monoclonal anti-HSP $90\alpha/\beta$ (sc-13119, Santa Cruz Biotechnology), goat polyclonal anti-topoisomerase I (sc-5342, Santa Cruz Biotechnology) antibodies at 1:500, 1:1000, and 1:500 dilutions, respectively.

Generation of Stable Cell Lines—We used the retroviral transduction method (40) to make C2C12 cell lines stably expressing the dominant negative MKL2 protein. 293 cells (plated at a density of 2.5×10^6

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[2] smart.embl-heidelberg.de.
[3] bighost.area.ba.cnr.it/BIG/UTRScan.

cells/100-mm tissue culture dish) were co-transfected with 10 μg of pBabe-puro-MKL2 (aa 71–700) and 10 μg of the defective helper virus pPSI⁻MLV. The supernatant containing the viral particles was collected after 2 days, filtered with a 0.45-μm filter and mixed with Polybrene to a final concentration of 8 μg/ml. The supernatant was then added to C2C12 cells (plated at a density of 4×10^5 cells/100-mm tissue culture dish) for overnight incubation. The C2C12 cells were allowed to recover for 1 day, split at 1:25 with fresh medium, and selected with puromycin (2.5 μg/ml) the next day. Individual colonies were picked ~7–9 days following the addition of selective media. Whole cell lysates from the colonies were checked for the expression of 3×FLAG-MKL2-(71–700) by immunoblotting with anti-FLAG antibodies.

RNA Analyses—A human multiple tissue blot (Clontech) was probed for MKL2 mRNA under high stringency conditions with a ^{32}P-labeled probe derived from the EST clone, accession number BG831958. A 567-bp region that matches 147 bases of the 5′-untranslated region and 420 bases of the coding region of the *MKL2* gene was amplified from the EST clone. This sequence was checked using BLAST for specificity to the *MKL2* gene. The BLAST results showed only one match to the human genome on chromosome 16. The probe was labeled using the Random Primers DNA labeling system (Invitrogen).

For RT-PCR experiments total RNA was made from differentiated and undifferentiated C2C12 cells using Trizol as described by the manufacturer (Invitrogen). Total RNA for mouse heart was obtained from Ambion. Total RNA (6 μg) was used for reverse transcription using the Powerscript reverse transcriptase kit from Clontech. For reverse transcription oligo(dT) primers were used for myocardin, MKL1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and a gene-specific primer was used for MKL2 (5′-TTAGTCCCATGGCAGCGGCAGGTC-CTGTGG-3′). For the PCR step, the number of cycles (20) was determined empirically to be in the linear phase of amplification. Primer pairs specific to mouse MKL1, MKL2, myocardin, and GAPDH were designed to give 500-bp products (MKL1, 5′-CAAAGAGCCACCATCC-CTACCAGGCAAG-3′ and 5′-CAAGCAGGAATCCCAGTGGAGCTGCA-AG-3′; MKL2, 5′-CTCCAATTTCCAAGATGAAACCAGTGACAGCCAG-C-3′ and 5′-TTAGTCCCATGGCAGCGGCAGGTCCTGTGG-3′; myocardin, 5′-GGTACTGAGGTGAGCCTCTCCAAGGCAGCAG-3′ and 5′-CC-ACTGCTGTAAGTGGAGATCCATAGGGG-3′; GAPDH, 5′-GGCATTG-TGGAAGGGCTCATGACCACAGTCC-3′ and 5′-CTCCTTGGAGGCCA-TGTAGGCCATGAGGTC-3′).

Gel Mobility Shift Assay—MKL2 and MKL2ΔN were *in vitro* transcribed and translated from pcDNA3.1-MKL2-HA, which contains a T7 promoter upstream of MKL2 and a C-terminal HA tag, using an STP3 kit (Novagen). The expression of the proteins was detected separately by translation with 50 μCi of [^{35}S]methionine and electrophoresis on an SDS-polyacrylamide gel. For the gel mobility shift assay, *in vitro* translated MKL2 or MKL2ΔN (5 μl) and bacterially expressed SRF (aa 114–508) (10 ng) were used with a high affinity SRF binding site, XGL, as a probe (50). SRF amino acids 114–508 in pET28b (Novagen) with an N-terminal polyhistidine tag were expressed in *E. coli* and purified with a nickel-chelate resin (ProBond, Invitrogen). 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.

RESULTS

Identification and Expression of Human MKL2—We searched human genomic and EST databases for sequence homology to myocardin and MKL1 and found a match on chromosome 16. The full-length gene was cloned by PCR methods based on available EST sequences. To confirm the 5′ end of the mRNA we performed 5′ RACE RT-PCR and found a predominant 5′ end of the mRNA. Based on its sequence similarity to MKL1, we have named the gene *MKL2*. The coding region of the gene encodes a 1049-amino acid long protein with 44% identity to MKL1 and 36% to myocardin. It has all the conserved domains seen in MKL1 and myocardin: N-terminal MKL homology domain with three RPEL motifs, basic region, glutamine-rich region, SAP domain, and leucine zipper-like region (Fig. 1*A*). The SAP domain has previously been implicated in attachment to the nuclear matrix while the function of

the RPEL motifs has recently been suggested to be actin binding (34, 51).

The *MKL2* gene is localized to the p13.1 region of human chromosome 16. It has 17 exons and spans greater than 126 kb on human chromosome 16 (Fig. 1*B*). Of the 17 exons, the two 5′ exons (in the 5′-untranslated region) have not as yet been localized to any contig on chromosome 16. However, we found that the two 5′ exons match a BAC clone from the 16p13 region, confirming that they are linked to the gene. The cDNA has a long 3′ non-coding region of over 3 kb in length. Sequence analysis of the 3′-UTR showed the presence of four cytoplasmic polyadenylation elements (CPE) that have been shown to activate translation of quiescent mRNAs in a temporal manner during development in *Xenopus*, *Drosophila*, and mouse (52, 53).

Northern hybridization of a human tissue blot with an MKL2 probe that hybridizes specifically to a 5′ 567-base pair region of the gene (5′-UTR and coding region) showed a broad pattern of expression of the MKL2 transcript with a size of about 9.5 kb (Fig. 1*C*). This size is significantly larger than the myocardin and MKL1 transcript sizes such that the signal cannot be because of cross-reactivity with these genes. Notably, there were elevated levels of expression in skeletal muscle, whereas no expression was detectable in lung.

MKL2 Is a Strong Transactivator of CArG Box-dependent Promoters—Because MKL1 and myocardin strongly activate CArG box containing promoters (15, 35, 36), we checked whether overexpression of MKL2 could also activate CArG box-dependent reporter genes and act as a transcriptional co-activator of SRF. To test this we co-expressed MKL1, MKL2, or an N-terminal deletion of MKL2 (MKL2ΔN) that lacks 71 aa such that it starts at the beginning of the MKL homology domain similar to MKL1 (Fig. 2*A*). Increasing amounts of each construct were transfected into HeLa cells with the 1× SRE reporter gene (Fig. 2*B*). In parallel the lysates were immunoblotted with anti-epitope antibodies to correlate activation with expression of the constructs (Fig. 2*C*). Expression of both MKL1 and MKL2 strongly activated the SRE reporter gene in a dose-dependent manner. However, MKL2 protein was made at lower levels than MKL1 or MKL2ΔN in part because a smaller form of about 55 kDa was also made (* in Fig. 2*C*). The significance of this fragment is unclear. Activation by MKL2 also had a threshold effect in that 0.5 μg caused little activation while 2.0 μg caused 40-fold activation (Fig. 2*B*). This activation was stronger than that by MKL2ΔN when normalized for protein expression levels (Fig. 2*C*) suggesting that the N terminus of MKL2 is involved in its regulation.
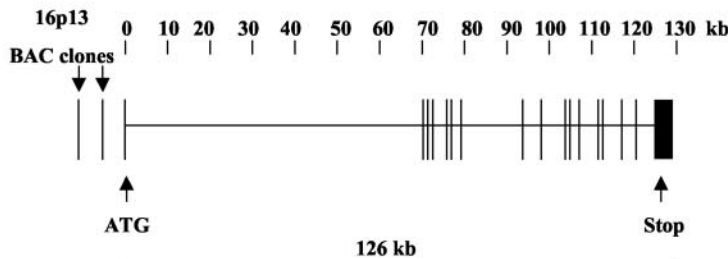
MKL2 overexpression strongly activated multiple cardiac and smooth muscle-specific promoters that contain CArG boxes while not activating a set on control promoters (Fig. 2*D*). In particular, the 4× MEF2 reporter contains the same minimal c-*fos* promoter as the 1× SRE construct, but was not activated by MKL2. Together these results show that MKL2, similar to MKL1, can activate a panel of CArG box containing promoters and suggest that activation is specific to the SRE site.

We tested for the transcriptional activation of MKL2 domain by fusing it with the DNA binding domain of GAL4 and transfecting it into HeLa cells with a GAL4 site reporter gene (Fig. 2*E*). Full-length MKL2 (Gal4-MKL2F) activated the reporter gene much more strongly than MKL1 (Gal4-MKL1F), however, activation by both was greatly increased by deletion of the N-terminal region (Gal4-MKL1/2C). The expression levels of all the mutants were similar as tested by immunoblots (data not shown). These results suggest that the C-terminal region of MKL2 (amino acids 701–1049) contains a strong transcrip-

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FIG. 1. **Protein sequence, gene structure, and tissue distribution of human MKL2.** *A*, alignment of human MKL2, MKL1, and myocardin protein sequences showing the conserved regions. The sequences used for human MKL1 and human myocardin are GenBank™ accession numbers AAK54721 and AAN33040. *B*, a schematic map of the human *MKL2* exon structure on chromosome 16. The exons are represented as *vertical lines*. The two 5′ exons in the 5′-UTR region map to a BAC clone on the 16p13 region of dif ference chromosome (GenBank™ accession number U91322), but have not been contiguously linked to the rest of the gene region. The ATG translation start site is in the third exon and the stop codon is in the 17th exon. *C*, human tissue RNA blot showing a 9.5-kb MKL2 transcript.
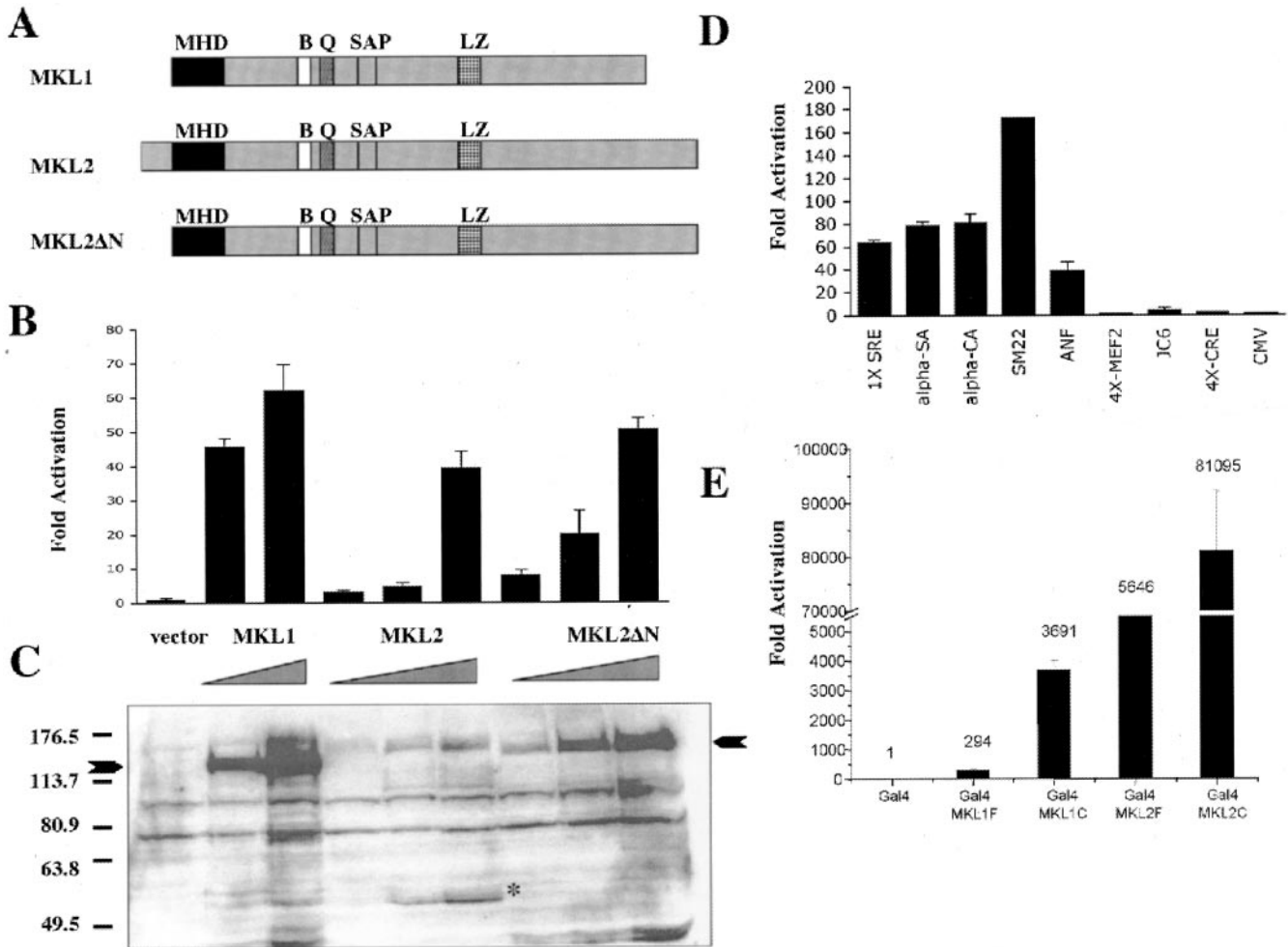
FIG. 2. **Transcriptional activation of CArG box-dependent promoters by MKL2.** *A*, diagram of MKL1, MKL2, and MKL2ΔN. MKL2ΔN lacks the N-terminal 71 aa. All expression constructs have a 3× FLAG epitope tag at the N terminus. *MHD*, MKL homology domain; *B*, basic domain; *Q*, glutamine-rich domain; *LZ*, leucine zipper-like domain. *B*, activation of the 1× SRE reporter gene by MKL1, MKL2, and MKL2ΔN. Increasing amounts of FLAG-tagged expression plasmids of MKL1 (0.5 and 1 μg), MKL2 and MKL2ΔN (0.5, 1 and 2 μg) were co-expressed with the 1× SRE luciferase reporter gene in HeLa cells. Two days after transfection, luciferase assays were performed and normalized to the Renilla luciferase activity of a cotransfected pRLSV40 plasmid. Data are represented as the mean -fold activation relative to vector alone ± S.D. of two experiments. *C*, expression levels of MKL1, MKL2, and MKL2ΔN. Whole cell lysates from the samples used for the luciferase assays in *B* (labeled on *top*) were immunoblotted with anti-FLAG antibodies. *Asterisk* (*) indicates an MKL2-specific product; *arrowheads* indicate MKL1 and MKL2 proteins. *D*, activation of CArG box-specific reporters by MKL2. Luciferase reporter genes (0.5 μg) were transfected together with MKL2 (2 μg) into HeLa cells and luciferase assays were performed as in *B*. 1× *SRE*, one SRE site upstream of a c-*fos* minimal promoter; α-*SA*, smooth muscle α-actin promoter; α-*CA*, cardiac α-actin promoter; *ANF*, atrial natriuretic factor promoter. 4×*MEF2*, four MEF2 sites upstream of a c-*fos* minimal promoter; *JC6*, c-*jun* promoter; 4×-*CRE*, four CRE sites upstream of a minimal promoter; *CMV*, cytomegalovirus immediate early promoter. *E*, transcriptional activation domain of MKL2. HeLa cells were transiently transfected with expression vectors (0.2 μg) encoding the indicated regions of MKL1 and MKL2 fused to the GAL4 DNA binding domain 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 -fold increase above that observed with the GAL4 DNA binding domain alone and is the mean ± S.D. of two experiments. MKL1F, aa 1–931; MKL1C, aa 601–931; MKL2F, aa 1–1049; MKL2C, aa 703–1049.

tional activation domain that can be regulated by its N-terminal domain(s).

Interaction of SRF and MKL2—To demonstrate that SRF and MKL2 can interact with each other and form a ternary complex on the CArG box we performed gel mobility shift assays using a high affinity SRE probe (XGL), *in vitro* translated MKL2, and bacterially expressed SRF (aa 114–508). Although MKL2 did not bind to the DNA by itself, it formed a complex with SRF on the DNA as seen by the supershifted SRF band (Fig. 3*A*). We also checked the binding properties of an N-terminal deletion mutant of MKL2 (MKL2ΔN). Although both MKL2 and MKL2ΔN were expressed at similar levels (Fig. 3*B*), MKL2ΔN formed a much stronger complex with SRF, suggesting that the N-terminal 71 aa of MKL2 has a repressive effect on SRF binding.

To further demonstrate the interaction, we tested for coimmunoprecipitation of epitope-tagged SRF and MKL2 trans-

fected into HeLa cells. SRF coprecipitated with immunoprecipitated MKL2 to a similar degree as with MKL1 (Fig. 3*C*). Although the association of SRF with MKL1/2 was weak, there was no precipitation of SRF without MKL1/2 transfection demonstrating the specificity.

MKL2 Can Interact with Itself and MKL1 Through the Leucine Zipper-like domain—MKL2 has a conserved leucine zipper-like region from aa 574 to 602 of the protein similar to MKL1 (Fig. 1*A*). Whereas the role of the basic, glutamine-rich and SAP domains have been shown for myocardin and MKL1 (15, 31, 34), the role of the leucine zipper region was not known. Because leucine zipper domains are implicated in protein dimerization, we checked whether MKL2 could associate with itself or MKL1. We made two differentially epitope-tagged MKL2ΔN constructs, one with a HA tag and the other with a FLAG tag. We chose to use the MKL2ΔN constructs, because MKL2ΔN was expressed at a higher level than MKL2 (Fig. 2*C*).
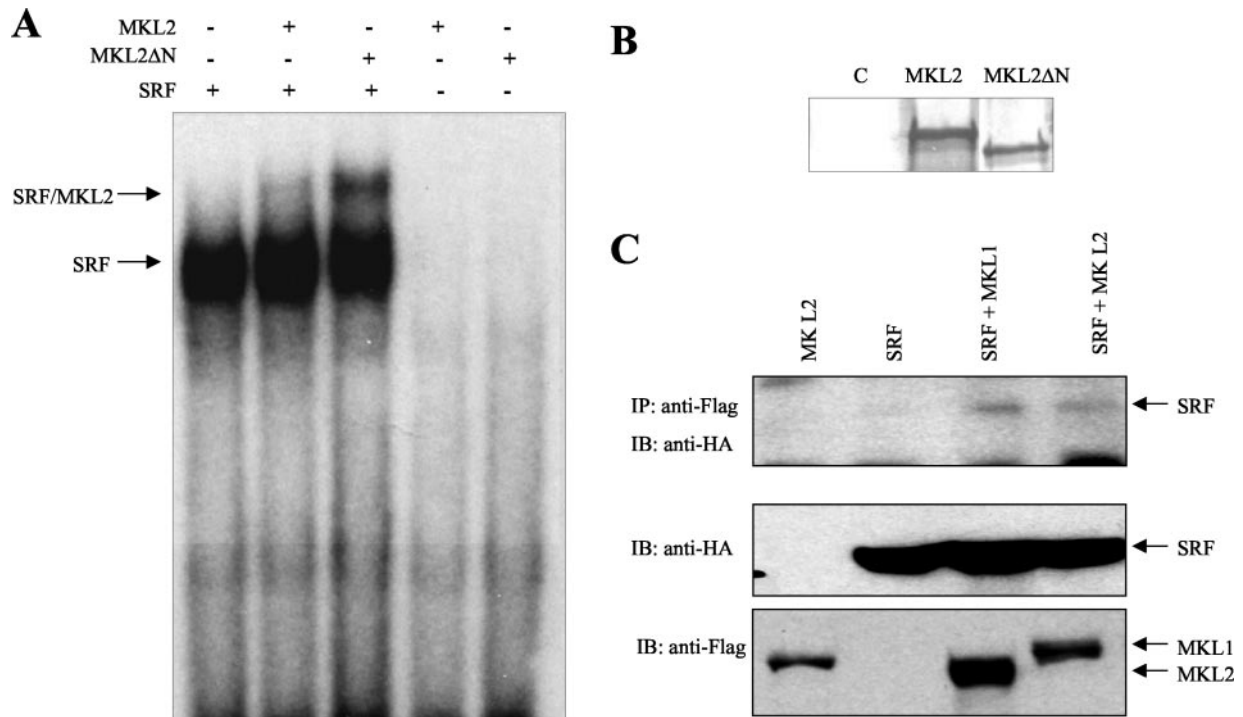
FIG. 3. **Interaction of SRF and MKL2.** *A*, binding of MKL2 to SRF in gel mobility shift assays. Gel mobility shift assays were performed with a ^{32}P-labeled oligonucleotide probe for a high affinity SRF binding site (XGL) with bacterially expressed SRF (aa 114–508) and/or *in vitro* translation products of MKL2, MKL2ΔN, or control translation lysates (*lane 1*). The positions of the SRF·DNA and SRF·MKL2·DNA complexes are indicated. *B*, [^{35}S]methionine-labeled *in vitro* translated products of MKL2 and MKL2ΔN were electrophoresed on an SDS-polyacrylamide gel and exposed to film. *C*, coimmunoprecipitation of MKL2 and SRF. HA-tagged SRF and 3× FLAG-tagged MKL1 or MKL2 were transfected into HeLa cells alone or together as indicated. Immunoprecipitates with anti-FLAG antibodies were immunoblotted (*IB*) for SRF with anti-HA antibodies (*top*). One-twentieth of the cell lysate was directly immunoblotted with anti-HA antibody to detect HA-tagged SRF (*middle*) and with anti-FLAG antibody to detect MKL1/2 (*bottom*). The positions of HA-SRF and FLAG-MKL1/2 are indicated.

HA-tagged MKL2ΔN specifically immunoprecipitated with FLAG-tagged MKL2ΔN and FLAG-tagged MKL1 (Fig. 4*A*). We also found that overexpressed MKL2ΔN associated with endogenous MKL1 in HeLa cells using MKL1-specific sera (Fig. 4*B*). We found that this association was dependent upon the leucine zipper region of MKL2 because deletion of this region greatly reduced, but did not abolish, the interaction (Fig. 4*B*).

To assess the role of the leucine zipper region and oligomerization of MKL factors, we assayed the ability of the leucine zipper deletion mutant, MKL2ΔNΔL, to activate the 1× SRE reporter gene. The deletion caused a 50% decrease in transactivation activity (Fig. 4*C*). However, MKL2ΔNΔL still strongly activated the reporter even though it was expressed at similar levels as MKL2ΔN (Fig. 4, *C* and *D*). These results suggest that the leucine zipper region and oligomerization promote activation by MKL2 but are not required.

Dominant Negative MKL2 Can Block the Activation of Both MKL1 and MKL2—To make a dominant negative mutant of MKL2 we tested C-terminal deletion mutants that lack the transcriptional activation domain. These mutants were made in the context of the N-terminal deletion of the first 71 amino acids because this allowed higher expression (Fig. 2*C*). C-terminal deletion to aa 560 or 700 nearly completely abolished activation by MKL2ΔN of a 1× SRE reporter gene (Fig. 5*A*). We found that each of these mutants could function as dominant negatives because, when coexpressed, they nearly completely abolished activation by wild type MKL2 (Fig. 5*A*). Because of the similarity and association of MKL1 and MKL2, we tested whether the mutants would also block activation by MKL1. Both of the C-terminal deletion mutants strongly inhibited activation by MKL1 (Fig. 5*B*). However, this inhibition does not require association of MKL1 and -2 because the MKL2ΔNΔC560 mutant does not contain the leucine zipper region.

These results show that the two MKL2 C-terminal deletion constructs can be used as dominant negatives to block activation by both endogenous MKL family members.

Dominant Negative MKL2 Blocks the Differentiation of C2C12 Myoblasts—The strong activation of the muscle-specific promoters in reporter gene assays and the elevated levels of MKL2 mRNA in skeletal muscle tissue, suggested a role for the MKL family proteins in skeletal muscle differentiation. To assess this possibility we have used the mouse C2C12 cell line. C2C12 skeletal myoblasts are a useful model system for differentiation because they differentiate to myotubes upon serum deprivation in tissue culture (54). It has also been shown that growth and differentiation of this cell line is dependent upon SRF (13). We first checked the expression of the MKL family members in C2C12 cells by semiquantitative RT-PCR. For comparison we used total mouse heart RNA. Myocardin was found in heart RNA but not in C2C12 RNA, whereas MKL1 and -2 were expressed in C2C12 cells (Fig. 6). There was no significant change in MKL1 or -2 expression upon differentiation. While MKL1 and -2 mRNAs were not initially detected in heart, a low level of expression was detected with greater cycle number (data not shown). As a control there was no change in GAPDH mRNA levels (Fig. 6). The expression of both MKL1 and MKL2 in C2C12 cells suggest the possible redundancy of these factors. However, the dominant negative MKL2 construct is particularly effective here because it blocks the activity of both factors.

We made C2C12 cell lines stably expressing the MKL2 dominant negative protein MKL2ΔNΔC700 (DN MKL2) using a retroviral vector. The expression of the protein was shown by immunoblotting with anti-epitope sera to the FLAG tag on the N terminus (Fig. 7*B*, *top*). These cells were induced to differentiate by switching them to a low serum medium for 3 days.
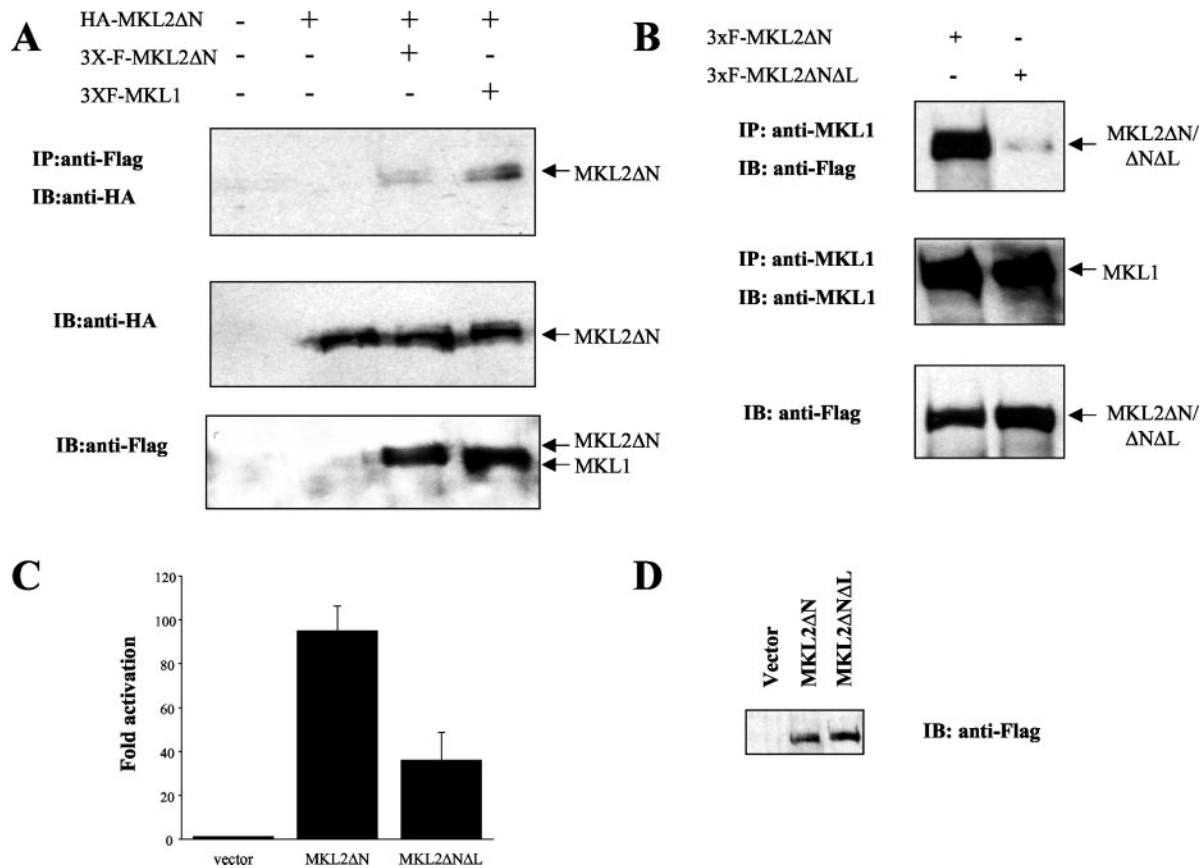
FIG. 4. **Oligomerization of MKL2 and MKL1.** *A*, HA-tagged MKL2ΔN was transfected into HeLa cells alone or with either FLAG-tagged MKL2ΔN or MKL1. Immunoprecipitates using anti-FLAG antibodies were immunoblotted (*IB*) with anti-HA antibodies (*top*). One-twentieth of the cell extract was directly immunoblotted with anti-HA antibody to detect the presence of HA-tagged MKL2ΔN (*middle*) and with anti-FLAG antibody to detect the presence of FLAG-tagged MKL1 and MKL2ΔN (*bottom*). *B*, co-immunoprecipitation of endogenous MKL1 and transfected MKL2. HeLa cells were transfected with FLAG-tagged MKL2ΔN or MKL2ΔNΔL (lacking the leucine zipper region, aa 574–604), immunoprecipitated with anti-MKL1 antisera and immunoblotted with anti-FLAG antibodies (*top*). The same blot was re-probed with anti-MKL1 to show the immunoprecipitation of endogenous MKL1 (*center*). One-twentieth of the cell extract was directly immunoblotted with anti-FLAG antibodies to detect the presence of the MKL2 mutants (*bottom*). *C*, effect of leucine zipper deletion on reporter gene activation. HeLa cells were transfected with MKL2ΔN (2 μg) or MKL2ΔNΔL (1 μg) (to normalize for protein expression levels) along with the 1× SRE reporter gene and luciferase assays were performed as described in the legend to Fig. 2. *D*, lysates of the samples used for luciferase assays in *C* were immunoblotted with anti-FLAG antibodies to show the normalized protein expression levels.

This treatment causes the fusion of cells to form myotubes that are very clear by 3 days (Fig. 7*A*, *top*). The cell line expressing DN MKL2 notably failed to form myotubes (Fig. 7*A*, *bottom*). An independent DN MKL2 cell line also failed to form myotubes (data not shown). To confirm the effect on differentiation we examined in immunoblots two differentiation markers that have CArG boxes in their promoters, skeletal α-actin and skeletal α-myosin heavy chain (6, 7). Each of these was induced in control C2C12 cells containing vector sequences, but not the DN MKL2 cell line (Fig. 7*B*). Because SRF is required to maintain the proliferation rate of C2C12 cells (13), we tested whether dominant negative MKL2 had an effect on cell growth. Both cell lines expressing DN MKL2 had a reduced rate of cell growth compared with C2C12 or C2C12 cells containing the retroviral vector (Fig. 7*C*). These results suggest that MKL family members, similar to SRF, are required for optimal cell growth and for myogenic differentiation of C2C12 cells.

Cellular Localization of MKL1—A recent study showed that MKL1 is localized primarily in the cytoplasm in NIH3T3 cells and moves to the nucleus in response to serum stimulation (34). We therefore tested whether MKL1 is nuclear or cytoplasmic in C2C12 cells and whether it moves during differentiation. We separated cells into nuclear and cytoplasmic fractions and immunoblotted for MKL1 and marker proteins. We found that the markers, hsp90 and topoisomerase I, localized to the cyto-

plasmic and nuclear fractions, respectively, as expected (Fig. 8). We also found that SRF localized predominantly to the nuclear fraction (data not shown). Surprisingly, we found that MKL1 localized primarily to the cytoplasm in C2C12 cells and did not significantly move to the nucleus during differentiation (Fig. 8, *lanes 5–10*). A comparison to NIH3T3 cells showed that MKL1 was also predominantly cytoplasmic in these cells and that there was little change in the distribution upon serum stimulation although there was a small increase in several experiments (Fig. 8, *lanes 1–4*, and data not shown). We have also found that MKL1 is predominantly cytoplasmic in HeLa cells and does not redistribute upon serum stimulation.[4] We cannot account for the differences with Miralles *et al.* (34) who observed the movement of MKL1 to the nucleus by immunofluorescence. Our antisera had too high a background to be effective in immunofluorescence.

More notable is the altered migration of MKL1 in response to serum in NIH3T3 cells. This was also observed by Miralles *et al.* (34) and was shown to be because of phosphorylation. We have detected a similar serum-induced change in mobility and phosphorylation in HeLa cells.[4] In C2C12 cells the mobility of MKL1 appears to be intermediary between that in serum-starved and serum-induced NIH3T3 cells (compare *lane 6* to *2*

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[4] B. Cen and R. Prywes, unpublished data.

FIG. 5. **Dominant negative MKL2 blocks activation by both MKL1 and MKL2.** *A*, expression plasmids for the deletion mutants of MKL2 (1 μg) were transfected into HeLa cells alone or together with wild type MKL2 (2 μg) and the α-cardiac actin reporter gene. The MKL2 C-terminal deletion mutants were expressed greater than 2-fold more highly than wild type MKL2 as determined in immunoblots (data not shown). ΔN, deletion of N-terminal 71 amino acids; $\Delta C700$, C-terminal deletion of amino acid 700 to 1049; $\Delta C560$, C-terminal deletion of amino acid 560 to 1049. *B*, expression plasmids for the deletion mutants of MKL2 (1 μg) were transfected alone or together with MKL1 (1 μg) and the α-cardiac actin reporter gene.
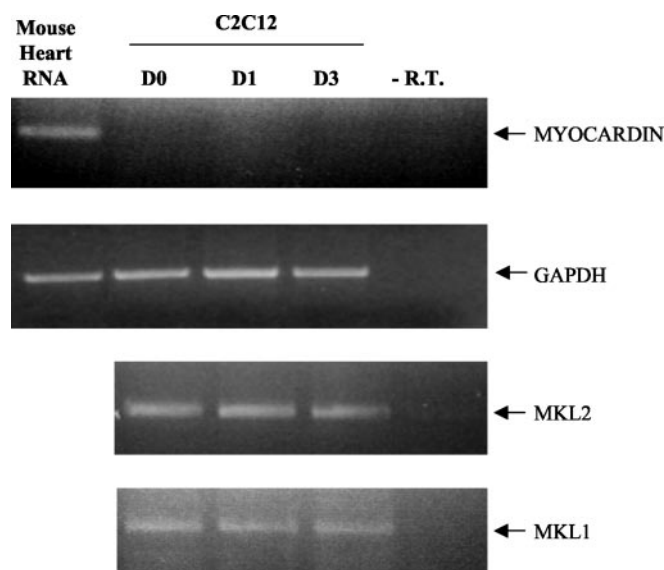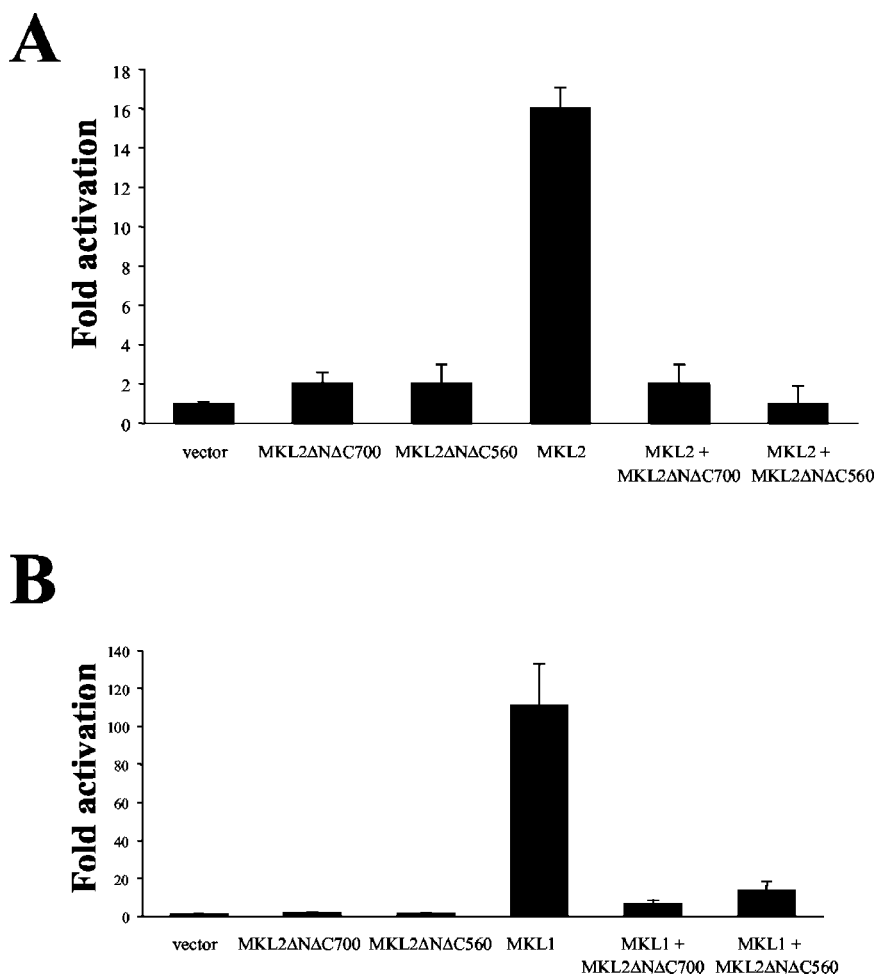
FIG. 6. **Expression of MKL family members in C2C12 cells.** Semiquantitative RT-PCR was performed with primers for mouse myocardin, MKL1, MKL2, and GAPDH. Total RNA (6 μg) of mouse heart tissue, undifferentiated C2C12 cells (*D0*), or C2C12 cells allowed to differentiate for 1 or 3 days (*D1* and *D3*). $-R.T.$, the RT-PCR procedure was performed without reverse transcriptase with heart RNA (*top two panels*) or undifferentiated C2C12 cells (*bottom two panels*).

and *4*). Unlike the change in NIH3T3 cells, there was little if any change in mobility during differentiation and a clear decrease in the amount of MKL1 was seen (Fig. 8, *lanes 5–10*). Further work is required to determine the significance of this

change. Although there is only a small proportion of MKL1 in the nucleus, because MKL1 acts as a positive coactivator with SRF, it still seems most likely that the nuclear portion of MKL1 is the active form.

DISCUSSION

We have identified MKL2 as the third member of the human myocardin/MKL1 family. Similar to the other family members, MKL2 binds to SRF and is a potent activator of CArG box-regulated promoters. While myocardin is expressed in heart and smooth muscle cells, MKL1 and MKL2 are more broadly expressed with notable expression in skeletal muscle. Expression of dominant negative MKL2 in the C2C12 myoblast system suggests that MKL family members are required for skeletal muscle differentiation.

MKL2, the Third Member of the Myocardin/MKL Family— We report here the cloning of human *MKL2*. During the course of this work, Wang and colleagues (36) cloned the mouse homolog of *MKL2*, *MRTF-B*. The mouse homolog is 82% similar to human MKL2. However, the first coding exons of *MRTF-B* and *MKL2* are different, *i.e.* they are different in their first 38 and 49 amino acids, respectively. This may be because of species-specific differences or the alternate expression of variant transcripts. Myocardin, MKL1, and MKL2 share common motifs including an N-terminal MKL homology domain with RPEL motifs, basic and glutamine-rich regions, a SAP domain, and a leucine zipper region. MKL2 can bind to SRF and activate numerous CArG box-containing promoters in reporter gene assays comparable with that of MKL1 at similar expression levels. These results contrast somewhat with those of Wang *et al.* (36) who found that MRTF-B did not activate CArG box-
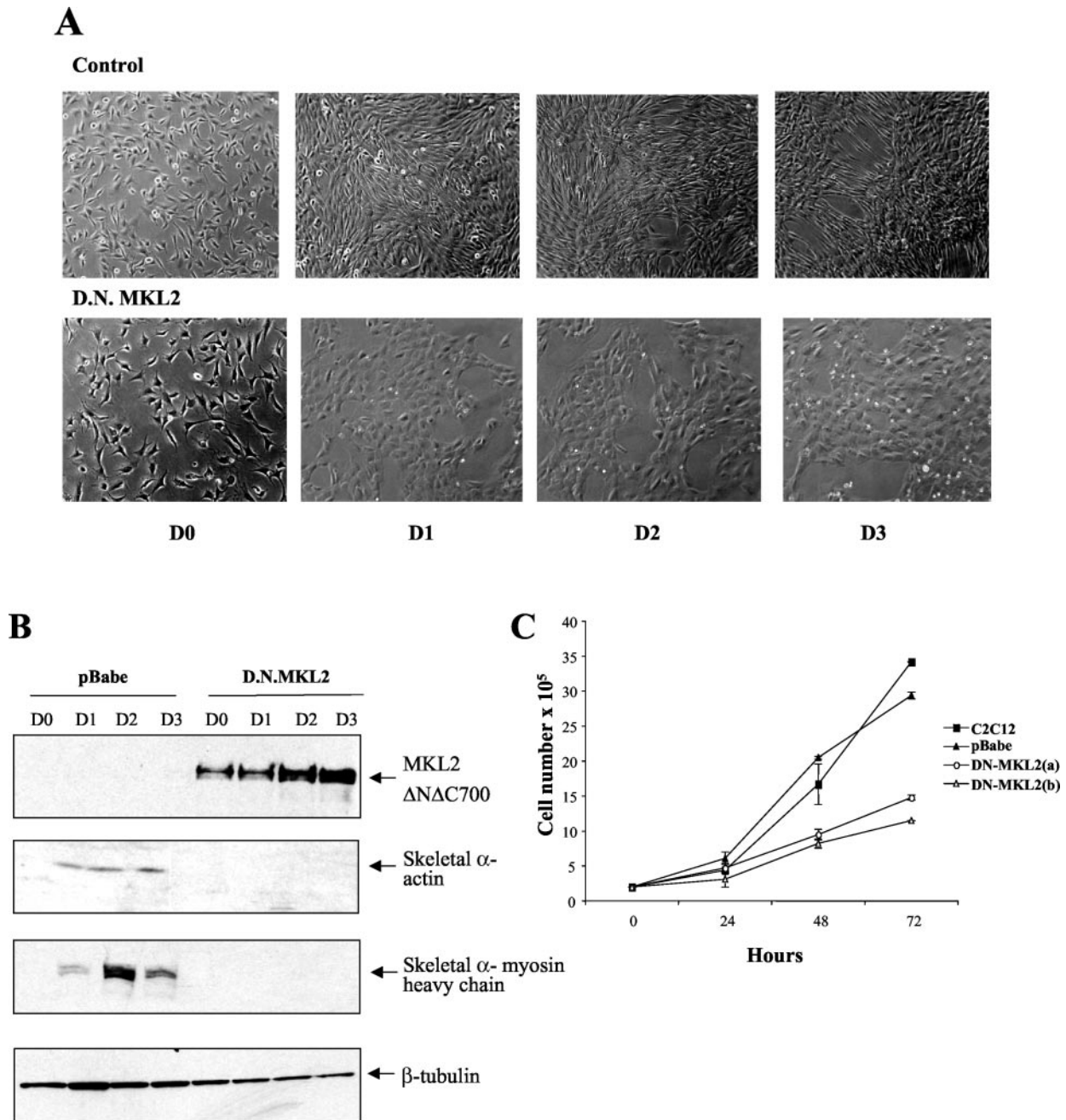
FIG. 7. **Differentiation of C2C12 cells is blocked by dominant negative MKL2.** *A*, stable C2C12 cell lines containing a retroviral vector (*Control*) or dominant negative MKL2 (MKL2ΔNΔC700) were grown in medium with 10% fetal bovine serum (*D0*), then changed to differentiation medium (2% horse serum) and phase-contrast pictures at ×100 magnification were taken every 24 h (*D1*, *D2*, and *D3*). *B*, expression of muscle-specific differentiation markers. The stable cell line expressing dominant negative MKL2 and the control cell line containing the vector pBabepuro (*pBabe*) were differentiated as in *A*. Lysates (20 μg) were immunoblotted with anti-FLAG, anti-skeletal α-actin, or anti-β-tubulin antibodies to detect DN-MKL2 (MKL2ΔNΔC700), striated α-actin and β-tubulin, respectively. For detection of skeletal muscle α-myosin heavy chain, 80 μg of lysates were immunoblotted with MF20 antibody. *C*, DN MKL2 inhibits C2C12 proliferation. Two different stable cell lines expressing DN MKL2, a cell line containing the retroviral vector (pBabe) and C2C12 cells were maintained in high serum medium, plated at 2 × 10^5 cells and counted every 24 h. The results of four repetitions are shown as the mean ± S.D.

reporter genes in COS cells or interact with SRF in gel mobility shift assays. Nevertheless, they did observe an interaction of MRTF-B and glutathione *S*-transferase-SRF *in vitro* and activation by MRTF-B of a reporter gene in ES cells. The differences likely involve the level of expression of the MKL2/MRTF-B proteins or the exact variants of the protein used.

The N-terminal region of MKL2, which is absent in MKL1, had a repressive effect on its interaction with SRF in gel mobility shift assays as well as the expression level of the protein in mammalian cells. The importance of this domain in MKL2 for SRF regulation remains to be determined. Because MKL2 has a much stronger transcriptional activation domain than MKL1, regulation of MKL2 activity may be more tightly controlled and the N-terminal region may provide an additional regulatory mechanism. After the unique MKL2 N-terminal region, there is a domain highly conserved with myocardin and MKL1 that contains RPEL motifs. It was recently shown that RPEL motifs in MKL1 can bind to actin, suggesting a regulatory link between the cytoskeleton and SRF regulation (34). Deletion of this region, however, did not affect activation of CArG box reporter genes by MKL2 (data not shown). We examined here the role of the leucine zipper-like region of MKL2.
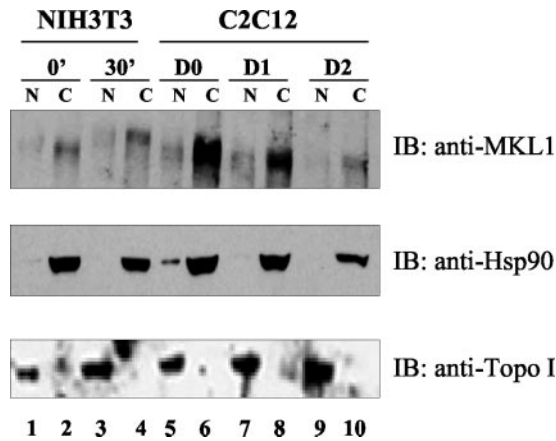
FIG. 8. **Cellular localization of MKL1 in C2C12 cells is not changed upon differentiation.** C2C12 or NIH3T3 cells were fractionated into nuclear (*N*) or cytoplasmic fractions (*C*) as described under "Experimental Procedures." NIH3T3 cells were serum starved for 24 h and either unstimulated (*0'*) or stimulated with 20% serum for 30 min (*30'*). C2C12 cells were undifferentiated (*D0*) or differentiated by growing in media containing 2% horse serum for 1 (*D1*) or 2 days (*D2*). Equal amounts of nuclear and cytoplasmic fractions normalized by volume were immunoblotted with anti-MKL1, anti-HSP90 (cytoplasmic marker), or anti-topoisomerase I (nuclear marker) antibodies, as indicated.

We found that MKL2 could oligomerize with itself or MKL1 and that the leucine zipper region was required for oligomerization with MKL1. However, deletion of this region only reduced transactivation by about half such that oligomerization does not appear to be required for its activity.

One of the unique features of MKL2 compared with myocardin and MKL1 is its long 3′-UTR. This region contains four CPEs that can promote cytoplasmic polyadenylation-induced translation (52). A binding protein for these elements, CPEB, can also be regulated during the cell cycle (55). It will be interesting to determine whether CPEB binds the MKL2 CPE sequences and whether this can control MKL2 translation.

The importance of the *MKL2* gene is further highlighted by its mutation in a gene trap experiment where homozygous mice with an *MKL2* insertion showed perinatal lethality (56). This suggests a critical developmental role for MKL2 distinct from that of myocardin and MKL1. However, in HeLa cells, we have found that MKL1 and MKL2 are redundant in their requirement for the serum induction pathway of SRF target genes (31). Because both MKL1 and MKL2 are expressed in C2C12 cells, it is likely that they are also redundantly required for differentiation in these cells.

Role of the MKL Family in Skeletal Muscle Differentiation— SRF and its upstream activator RhoA are required for skeletal muscle differentiation (13, 25). However, the factor(s) downstream of RhoA required for skeletal muscle differentiation have not been identified. MKL1/2 appears to be downstream of RhoA in HeLa and NIH3T3 cells because dominant negative MKL1 blocked RhoA activation of SRE reporter genes (31, 34). MKL1 and MKL2 transcripts are expressed in skeletal muscle tissue (Fig. 1*C*)[5] (32, 33) and these factors can strongly activate many muscle-specific promoters in reporter gene assays. We have studied the physiological significance of these proteins in the mouse skeletal myoblast C2C12 cell line using dominant negative MKL2. C2C12 cells expressing the dominant negative protein failed to differentiate as seen by the loss of myotube formation and the loss of expression of endogenous markers of muscle differentiation. These cell lines also showed a decreased proliferation rate when compared with the control cell lines suggesting that MKL1/2 also affects target genes required for cell growth. These may be the immediate early genes, such as

c-*fos* and *cyr61* that have been shown to be required for cell growth (57–59).

Smooth and skeletal muscle cells express distinct genes such as alternative forms of α-actin and myosin heavy chain (60–64). Both of these sets of genes contain CArG boxes and are regulated by SRF. Skeletal muscle and C2C12 cells express predominantly MKL1/2, rather than myocardin, such that these factors may help distinguish specific target genes. Overexpression of myocardin caused the activation of smooth muscle-specific genes in rat aortic smooth muscle cells, rat L6 myoblasts, and non-muscle 10T1/2 cells (28–30). It will be interesting to determine whether there are differences in the effects of the myocardin/MKL family on smooth muscle *versus* skeletal muscle genes.

Overexpression of myocardin in L6 myoblasts caused differentiation and reduced the growth potential of these cells (30). Consistent with these results, we found that inhibition of MKL1/2, using dominant negative MKL2, blocked myoblast differentiation. Surprisingly, however, dominant negative MKL2 reduced cell growth. This may reflect an effect on additional target genes, but also shows that inhibition of cell growth rate is not sufficient to cause differentiation.

Recent experiments suggest that RhoA activation of SRF is mediated by MKL1 and MKL2 (31, 34). The requirement of RhoA, SRF, and MKL1/2 for myogenic differentiation suggests that the RhoA to MKL1 to SRF pathway is critical for muscle differentiation. One mechanism of RhoA activation of SRF is through effects on actin treadmilling that causes a decrease in free G-actin levels (24). Actin was found to bind through the RPEL motifs of MKL1, causing it to be localized in the cytoplasm in NIH3T3 cells (34). However, we found that there was only a small serum-induced change of MKL1 distribution from the cytoplasm to the nucleus in NIH3T3 cells (Fig. 8) and no change in HeLa cells.[4] We similarly found that MKL1 was predominantly cytoplasmic in C2C12 cells and that there was no clear change in distribution during differentiation. Because MKL1/2 are positive coactivators of SRF that bind SRF and have strong transcriptional activation domains, it is likely that nuclear MKL1 is the active form of the protein. Cytoplasmic MKL1 may serve as a pool to move to the nucleus upon activation by certain pathways in specific cells. MKL1 may also be modified in the cytoplasm and then move very transiently to the nucleus. MKL1 phosphorylation was also induced by serum induction suggesting another possible regulatory mechanism (34).[4] There does not appear to be a similar change in MKL1 modification during C2C12 differentiation although we observed a subtle change in mobility of the MKL1 band. It will be important to determine whether MKL1/2 activity is regulated during myogenic differentiation and if so, which mechanism is used.

SRF associates with multiple factors in muscle cells and it is difficult to assess how these factors work together to regulate SRF activity and muscle-specific gene expression (14–19, 21). In skeletal muscle cells the Barx2B homeodomain protein, similar to MKL1/2, has been found to bind SRF and to be required for skeletal muscle cell differentiation (20, 21). However, in contrast to MKL1/2, Barx2B did not bind to SRF in gel mobility shift assays or activate SRE reporter genes (21). Different SRF-complexing factors may affect the expression of subgroups of SRF target genes in different cell types. Microarray analyses combined with gene inhibition in smooth, skeletal, or serum-induced cells should help clarify how each complexing factor contributes to SRF gene regulation.

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