A Transforming Growth Factor-\(\beta\) Control Element Required for SM \(\alpha\)-Actin Expression \textit{in Vivo} Also Partially Mediates GKLF-dependent Transcriptional Repression*

Received for publication, February 24, 2003, and in revised form, July 29, 2003 Published, JBC Papers in Press, September 10, 2003, DOI 10.1074/jbc.M301902200

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We previously demonstrated that a conserved transforming growth factor-\(\beta\) control element (TCE) within the 5′-region of the smooth muscle cell (SMC) differentiation marker gene SM \(\alpha\)-actin could mediate both transcriptional activation and repression in cultured SMCs through interaction with members of the zinc finger Kruppel-like transcription factor (KLF) family. The aims of the present studies were to: 1) determine the role of the SM \(\alpha\)-actin TCE \textit{in vivo} through mutagenesis studies in transgenic mice and 2) further characterize the possible role and mechanisms by which the TCE-binding factor GKLF/KLF4 induces repression of SMC marker genes in various SMC model systems \textit{in vitro}. Our results showed that the TCE was required for SM \(\alpha\)-actin promoter activity \textit{in transgenic mice \textit{in vivo}.} Results of transient transfection studies showed that GKLF-induced repression of a SM \(\alpha\)-actin promoter/luciferase reporter gene partially depended on the TCE. Furthermore, a GKLF overexpressing adenovirus inhibited whereas GKLF morpholinoo antisense oligos increased expression of endogenous SMC marker genes. Results of chromatin immunoprecipitation assays showed GKLF binding to TCE-containing regions of various SMC marker gene promoters within intact chromatin. Finally, results of co-transfection studies showed that overexpression of IKLF/KLF5 reversed GKLF-dependent repression thus supporting a model of reciprocal activation-repression of SMC gene expression by different members of the KLF gene family.

Regulation of SMC\(^{1}\) differentiation is known to play a critical role in blood vessel formation during embryogenesis (1). Moreover, in pathological states such as atherosclerosis, restenosis, and hypertension, SMCs “dedifferentiate” by reducing expression of SMC marker genes and increasing their proliferation rate and synthesis of extracellular matrix proteins (2). As such, there is considerable interest in identifying extracellular signals, signal transducing molecules and transcription factors involved in regulating SMC differentiation. Many different environmental cues are known to affect SMC differentiation including endothelial cell-SMC interactions, SMC-matrix contacts, mechanical forces, and various cytokines such as platelet-derived growth factor BB (PDGF-BB), activin, and transforming growth factor \(\beta\) (TGF-\(\beta\)). Of particular interest, TGF-\(\beta\)-1 has been implicated in control of SMC differentiation/proliferation and synthesis of extracellular matrix, processes that are important for blood vessel development/maturation and/or progression of vascular disorders such as atherosclerosis and restenosis after balloon angioplasty (3, 4). TGF-\(\beta\)-1 has also been shown to induce expression of SMC differentiation marker genes in a variety of cell types \textit{in vitro}, including multipotent embryonic 10T1/2 cells (5), neural crest cells (6), myoblasts (7), and pericytes (8). The spatial and temporal expression pattern of TGF-\(\beta\)-1 and TGF-\(\beta\)-\(\beta\) type II receptor expression in mesenchymal cells is consistent with the possibility that TGF-\(\beta\)-1 plays an important role in stimulating vascular development (9). In addition, targeted disruption of the TGF-\(\beta\)-1 gene, or TGF-\(\beta\)-receptor genes resulted in early embryonic lethality due to defects in development and/or vascular maturation as manifested by reduced investment of endothelial tubes by presumptive SMCs. For example, 50\% of mice deficient in both alleles of TGF-\(\beta\)-1 died in utero between 9.5 and 10.5 dpc from abnormalities in yolk sac blood vessel development (10). The vessel defects observed included impaired contacts between layers of endothelial and mesenchymal cells. Similarly, disruption of the TGF-\(\beta\)- type II receptor caused embryonic lethality around E10.5 due to defects in yolk sac vasculogenesis, including enlargement and reduced SMC investment of yolk sac blood vessels (11). Knockout of endoglin, a TGF-\(\beta\)-type III receptor, also led to embryonic lethality by 11.5 dpc from defects in angiogenesis including failed VSMC development (12). However, since the mice with disrupted genes died before SMC differentiation began during embryogenesis, the role of TGF-\(\beta\)-1 in regulating SMC differentiation \textit{in vivo} remains unclear.

In contrast to its clearly documented effect in promoting SMC differentiation \textit{in vitro}, the role of TGF-\(\beta\)-1 in atherogenesis and restenosis is controversial and complex. For example, TGF-\(\beta\)-1 is not only released by platelets (13) and macrophages (14) at the site of vascular injury, but its mRNA and protein are also elevated in neointimal vascular SMCs following balloon injury to rat carotid arteries (15, 16). Furthermore, TGF-\(\beta\)-1 expression was shown to be higher in restenotic tissue than in primary plaque tissue from human atherectomy specimens (16). In addition, reduction of TGF-\(\beta\)-1 levels in the blood vessel wall by neutralizing antibodies led to a small but significant

* This study was supported by National Institutes of Health Grants P01HL19242, P01HL38854, and R37HL57353 (to G. O.) and an American Heart Association postdoctoral fellowship (to S. S.). The costs of publication of this article were defrayed in part by the payment of page charge. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: SMC, smooth muscle cell; TGF, transforming growth factor; TCE, TGF-\(\beta\)-control element; ANOVA, analysis of variance; pfu, plaque-forming unit; ChIP, chromatin immunoprecipitation assay; KLF, Kruppel-like transcription factor; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus.

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reduction in neoantima formation, whereas overexpression of TGF-β1 by adenoviral gene transfer resulted in transient neoantima formation (17). On the other hand, it was recently reported that administration of TGF-β1, 2, and 3 neutralizing antibody accelerated the development of atherosclerotic lesions in apoE-deficient mice (18).

Previously our laboratory identified a novel cis-element referred to as TGF-β control element or TCE in the SM α-actin promoter that is highly conserved across species in multiple SMC marker genes including smooth muscle myosin heavy chain (SM MHC), SM22α, and h1-calponin (19). Mutation of the TCE within the SM22α promoter in transgenic mice showed that the TCE is critical for expression of SM22α in all three muscle types during embryogenesis and in SMC containing tissues in adult mice (20). To identify possible TCE binding factors, a 17.5-day mouse embryo cDNA library was screened with a yeast one-hybrid system, using three repeats of TCE as bait sequence. One of the candidate TCE-binding factors identified was GKLF/KLF4, which belongs to a family of Kruppel-like zinc finger transcription factors hallmarked with three CXXC finger transcription factors hallmarkcd with three CXC_CXXF;_XXHX;_H zinc finger motifs separated by a highly conserved 7-amino acid interfinger spacer, TGEKP(Y/F) (21).

Studies from our laboratory showed that GKLF specifically bound to the TCE of the SM α-actin or SM 22α promoters and overexpression of GKLF repressed expression of SM22α and SM α-actin promoter-reporter constructs in cultured SMCs. Paradoxically, TGF-β has been shown to up-regulate various SMC marker genes (20, 22). To reconcile this apparent contradiction, we postulated that the TCE could act as an activator or repressor of expression of SMC marker genes, and that activation or repression would dominate depending on the stoichiometry of specific binding factors. Consistent with this hypothesis, we found that: 1) GKLF was not expressed in TGF-β-treated differentiated SMCs but was present in phenotypically modulated cultured SMCs; 2) TGF-β1 could down-regulate GKLF RNA and protein in cultured SMCs; and 3) BTEB2/IKLF/KLF 5 could stimulate the activity of SM22α and SM α-actin promoters in cultured cells (20).

Whereas the preceding studies have provided evidence implicating a possible role of the TCE/GKLF in transcriptional regulation of SM22α and SM α-actin in cultured SMCs, the previous studies have not: 1) addressed if GKLF could repress expression of endogenous SMC marker genes as opposed to transiently transfected promoter-reporter constructs; 2) directly tested if GKLF is an endogenous repressor for SMC differentiation using loss-of-function approaches; 3) determined if GKLF is directly associated with promoters of SMC marker genes within intact chromatin, and 4) directly tested whether IKLF can antagonize GKLF induced repression of SM α-actin promoter activity in cultured cells. The present studies address each of these questions, and provide compelling evidence that GKLF is a very potent repressor of multiple SMC differentiation marker genes at least in cultured cell systems.

MATERIALS AND METHODS

Generation and Analysis of Mutation of the SM α-Actin TCE in Transgenic Mice—-Mutation of the SM α-actin TCE was made by PCR mutagenesis in the context of a full-length SM α-actin promoter/enhancer from −2560 to +2784 that we have previously shown is sufficient to recapitulate expression of the endogenous SM α-actin in transgenic mice in vivo (23). A 1.1-kb fragment containing the TCE mutation was verified by sequencing and used to replace its wild type counterpart within the SM α-actin promoter (Fig. 1). The mutated promoter was then linked to a LacZ reporter to make the transgenic construct PPTTCE-LacZ. The transgenic mice were then generated by the Transgenic Core Facility at the University of Virginia as described previously (23). Positive founder lines were identified by PCR genotyping, and bred for assessment of transgene expression in embryonic and adult mice. Animals were euthanized by CO2 asphyxiation, and tissues or embryos stained for β-galactosidase activity as previously described (23). Multiple independent founder lines were analyzed to assess possible locus and transgene copy number effects on expression patterns.

Cell Cultures—Rat aortic SMCs were cultured in Dulbecco’s modified Eagle’s medium + F12 (Invitrogen) supplemented with 10% fetal bovine serum (Hyłrones), 200 µg/ml l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) as previously described (19). NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyłrones), 0.075% sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 200 µg/ml l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen).

Generation of Constructs and Virus—The SM α-actin promoter region was amplified with primers 2960 to +2784 without TCE mutation was subcloned into the pGL3 vector to generate PPTTCE-Luc and PPI-Luc reporter constructs, respectively. GKLF cDNA was subcloned into a pcDNA3.0 mammalian expression vector (pcDNA-GKLF). The SRF expression plasmid pcDNA-SRF was generated as previously described (24). The mouse IKL cDNA was a generous gift from Dr. Masahiko Kurabayashi at the Gumm University (25) and was subcloned into pcDNA3.0 vector (pcDNA-IKLF). A FLAG epitope tag was included on the N terminus of GKLF to generate pcDNA-Flag-GKLF. The FLAG-tagged GKLF was then subcloned into pShuttle (Clontech), and used to make an Adeno-GKLF construct according to the manufacturer’s protocol. Purified GKLF adenoviruses (Ad-CMV-GKLF) were prepared by Gene Transfer Vector Core (Gene Transfer Program, University of California, San Francisco). Control virus (Ad-CMV-EGFP) were also obtained there. A GKLF construct lacking the binding domain (pcDNA-GKLF-NB) was made by deleting 93 bp on the C terminus of the GKLF open reading frame.

Transient Transfections/Reporter Assays and Infections by Adenoviruses—SMCs were transfected with PPI-Luc or PPITCE-Luc and pcDNA-GKLF or pcDNA-GKLF-NB, and NIH 3T3 cells were transfected with PPI-Luc or PPITCE-Luc, pcDNA-SRF, pcDNA-GKLF and pcDNA-IKLF in triplicate using Superfect reagent (Qiagen) according to the manufacturer’s protocol. The cells were incubated 48 h before being harvested with Passive Lysis Buffer (Promega). Luciferase activity was measured with Luciferase Assay Substrate (Promega), and normalized to total protein (Bio-Rad Protein Assay Kit). Transfections were repeated at least three times, and the relative luciferase activities were presented as mean ± S.E.

SMCs were infected with GKLF Adeno virus (Ad-CMV-GKLF) or control virus (Ad-CMV) in triplicate 3 days after plating at multiplicity of infection ( moi) of five plaque forming unit (pfu) per cell. The infection efficiency was demonstrated to be almost 100% based on infection with an EGF expression adenovirus construct, i.e. Ad-CMV-EGFP (data not shown). RNA samples were prepared with Trizol reagent (Invitrogen) according to the manufacturer’s instructions 24 h after infection. RNA samples were then subjected to real time RT-PCR analyses, and the relative changes are presented as mean ± S.E. The experiments were repeated three times.

Loss-of-Function Assay Using Morpholino Oligos—A GKLF antisense oligonucleotide was designed to target at the initiation site for GKLF translation (agactcgccaggtggctgcctcatt) and was synthesized by Gene Tools (Fig. 5A). Morpholinos were transfected into SMCs with ethoxylated polyethylenimine (EPEI) according to the manufacturer’s instructions 1 day after plating. The transfection efficiency was verified to be higher than 80% with fluorescent control oligos (data not shown). The specificity of the antisense oligo was validated by employing two rigorous control oligos: GKLF-Inv, ttaacctcgctggcagcctga and GKLF-Mis, agttcagcggagcctgtcctt. RNA samples were prepared from cells 48 h after transfection with Trizol reagent (Invitrogen) according to the manufacturer’s instruction. The purified RNA was then subjected to real time RT-PCR, and the relative changes are presented as mean ± S.E. The experiments were repeated seven times.

Real Time RT-PCR—RNA samples were run on a 1% agarose gel to check quality. 1 µg of RNA was reverse-transcribed as follows: dH2O was mixed with 1 µg of RNA, made to 10 µl, heated at 68 °C for 10 min, then cooled on ice for 3 min, and mixed with 2 µl of 5× buffer, 2 µl of 10× dNTP, 1 µl of random decamer (cat. 5722g, Ambion), 0.5 µl of RNAse inhibitor (40 units/µl), 0.5 µl of Superscript II (Invitrogen), and 2 µl of dH2O incubated at 42 °C for 90 min and heated up at 95 °C for 5 min. 0.5 µl of cDNA was used for each real time PCR reaction. The PCR primers were performed in the iCycler (Bio-Rad), and unknown samples were qualified by reference to serial dilutions of a known standard. For each sample, SMC-specific gene expression was normalized to 18 S RNA or GAPDH level to correct for differences in RNA extraction and reverse transcription efficiencies. The primer and probe sequences are described here: 18 S rRNA-For,
FIG. 1. Schematic representation of the wild-type and TCE mutant constructs used to generate transgenic mice. 4-bp mutations were made in the TCE (underlined) by PCR mutagenesis as described under “Materials and Methods.”

evgetaccaacctcaagga; 18 S rRNA-Rev, agctggaattaccgcggc; 18 S probe, ttcggcactagcctgct; Rat SM α-actin-For, atcttcctctagtcgtcctaggt; Rat SMMHC-For, cgcggcactagcctgct; SMMHC-Rev, atcttcctctagtcgtcctaggt; SMMHC probe, caaatatacaacagactaagggagc; GAPDH-For, ggggctagaggctgtcatcatc; GAPDH-Rev, ggtgggcaagcttgcatcttt; and GAPDH probe, ggtgggcaagcttgcatcttt (IDT).

Chromatin Immunoprecipitation (ChIP)—Three days after plating, SMCs were infected with Ad-CMV-GKLF or Ad-CMV at 5 M.O.I. After a 24-h incubation, ChIP assays were performed as described previously (26). In brief, chromatin samples were immunoprecipitated with no antibody, anti-acetylated histone 4 (Upstate), anti-SRF antibody (Santa Cruz Biotechnology, Inc.), or anti-FLAG antibody (Sigma). Immunoprecipitated chromatin samples were reverse-cross-linked, purified, and subjected to PCR amplification using primers specific to TCE-containing promoter regions of SM α-actin, SM MHC, or transin. The super-natant of the no antibody immunoprecipitation reaction was used as total input DNA. The sequences of the PCR primers were as follows: SM α-actin 5, atcttcctctagtcgtcctaggt; Rat SM α-actin-Rev, atcttcctctagtcgtcctaggt; Rat SM α-actin-For, cgcggcactagcctgct; SMMHC-For, cgcggcactagcctgct; SMMHC-Rev, atcttcctctagtcgtcctaggt; SM MHC 5, atcttcctctagtcgtcctaggt; SM MHC 3, atcttcctctagtcgtcctaggt; SM MHC 2, atcttcctctagtcgtcctaggt; GAPDH-Rev, atcttcctctagtcgtcctaggt; and GAPDH probe, ggtgggcaagcttgcatcttt (IDT).

RESULTS

The SM α-Actin TCE Was Required for SM α-Actin Promoter Activity in Vivo—Our initial goal was to determine if a 4-bp mutation of the SM α-actin TCE altered expression in transgenic mice (Fig. 1). Our earlier studies showed that the TCE mutation employed prevented TGF-β-dependent TCE binding activity in EMSA and abolished SM α-actin promoter activity in cultured SMCs (20). In the current study, four independent transgenic founder lines carrying the PPI/PPITCE-LacZ transgene were obtained, and three of them successfully transmitted the transgene over multiple generations. Embryos at 13.5 dpc and 16.5 dpc or adult tissues from 6–10 weeks old mice were stained with X-gal and compared with embryos or tissues carrying the wild-type SM α-actin promoter from -2560 to +2784/ LacZ transgene (PPI-LacZ), which was previously shown to recapitulate the same spatial and temporal expression pattern as the endogenous SM α-actin gene (23). Our results showed that the TCE mutation completely abolished SM α-actin promoter activity in all three muscle types in embryos (Fig. 2A). In adult tissues, the TCE mutation abolished LacZ expression in aorta, coronary vessels, and bronchi, while it dramatically decreased LacZ expression in bladder, intestine, and stomach (Fig. 2B). These results thus provide compelling evidence that the TCE is required for expression of the SM α-actin gene in vivo in most SMC tissues, although results showing partial detection of β-gal activity in bladder and intestine suggest that there may be different regulatory paradigms for SM α-actin in various SMC subtypes in a manner similar to the SM MHC (26) and SM22α genes (27). Virtually identical results were found in all three independent TCE mutant transgenic founder lines thus indicating that loss of expression was a function of mutation of the TCE and not due to random locus-dependent gene silencing.

A major long term goal of our laboratory has been to understand mechanisms that down-regulate expression of SMC marker genes associated with phenotypic modulation. However, since results of our TCE mutant studies in transgenic mice showed virtually complete absence of SM α-actin promoter activity in various SMC tissues in vivo, it was not possible to further examine role of TCE in phenotypic modulation of SMC using these transgenic founder lines. As an alternative, we employed cultured SMCs, which are well documented to be phenotypically modulated (28), to study the possible role of the TCE in expression of SM α-actin gene expression.

GKLF-induced Repression of the SM α-Actin Promoter in Cultured SMCs Was Partially Dependent on a Conserved TGF-β Control Element (TCE)—Our previous studies showed that the zinc finger Kruppel-like factor GKLF bound to SM22α and SM α-actin TCEs in EMSA and repressed the activity of the SM22α and SM α-actin promoters in co-transfection studies of TGF-β1-treated 10T1/2 cells (20). In this study, we tested whether the GKLF-mediated repression of the SM α-actin promoter activity was TCE-dependent. As shown in Fig. 3A, at a low concentration of GKLF plasmid, repression of SM α-actin expression was highly TCE dependent, whereas at higher concentrations of GKLF plasmid, the relative dependence on the TCE was virtually lost. Since transcription factors are usually present at low copy number in cells, it is tempting to speculate that the TCE dependence exhibited at lower concentrations of GKLF plasmid might better reflect what occurs in vivo under physiological circumstances, and that the effects seen at higher concentrations of GKLF plasmid may represent gene squelching. However, due to the absence of antibody that is completely specific for GKLF the actual concentration of GKLF protein that exists in vivo is unknown, and it is equally plausible that GKLF may play a key role in modulating SMC gene expression via both TCE dependent and independent mechanisms. The results shown in Fig. 3A indicate that a significant component of GKLF-induced suppression of SM α-actin was TCE-independent. Indeed, the proximity of the TCE and the CArG box, a conserved SRF binding element that is required for expression of multiple SMC genes (23, 26, 27) suggests that TCE-independent GKLF mediated repression might depend on both...
protein-DNA and/or protein-protein interactions. To test if DNA binding was required for GKLF-induced repression, we made a GKLF construct with two zinc fingers of the DNA binding domain deleted (designated GKLF-NB). This mutation completely abrogated GKLF’s ability to repress the SM/H9251-actin promoter (Fig. 3B). However, we could not rule out the possibility that destroying the GKLF DNA binding domain might also inactivate its effector domain or its activity to interact with other protein involved in regulation of SMC marker genes, although studies by others have shown that GKLF has a modular structure, and that its DNA binding domain and effector domain can function independently of each other (29, 30). In summary, the preceding results indicate that GKLF can potentially inhibit transcription of SM/H9251-actin through both TCE-dependent and -independent mechanisms. However, it is unclear whether TCE-independent activity is mediated through binding to another cis-element in the SM/H9251-actin promoter and/or is mediated through protein-protein interactions.

A GKLF-expressing Adenovirus Repressed Endogenous SM α-Actin and SM MHC Expression in Cultured SMCs—Because of low transfection efficiency in cultured SMCs, to directly test if GKLF could alter expression of endogenous SMC marker genes, a GKLF-expressing adenovirus (Ad-CMV-GKLF) was generated. Cultured SMCs were infected by Ad-CMV, Ad-CMV-EGFP, or Ad-CMV-GKLF at 5 pfu/cell. The infected cells appeared normal, and no apparent cell death was observed by phase-contrast microscopy. Total RNA was prepared from infected cells, and subjected to reverse transcription and real time RT-PCR analyses of SM α-actin and SM MHC, which represent markers of early and late stages of SMC differentiation respectively. As shown in Fig. 4, expression of SM α-actin and SM MHC mRNAs were substantially reduced in GKLF virus infected cells as compared with cells infected with control viruses. Results thus indicate that GKLF can potential inhibit expression of multiple endogenous SMC differentiation marker genes.

Inhibition of Endogenous GKLF with Antisense Morpholinos Increased Expression of Endogenous SM α-Actin and SM MHC in Cultured SMCs—Our earlier studies showed that GKLF was expressed in phenotypically modulated cultured SMCs (20). To test whether GKLF is an endogenous repressor of SMC marker gene expression, morpholino antisense oligos were used to knockdown GKLF in cultured SMCs. Morpholino oligos consist of four different morpholino subunits, each of which contains one of the four genetic bases linked to a 6-member morpholino ring, and the subunits are joined in a specific order by non-ionic phosphorodiamidate intersubunit linkage. As such, morpholino oligos are highly resistant to DNase and RNase degradation (31). Morpholinos knockdown target gene expression in a RNase H-independent manner by forming a duplex with the initiation region of targeted mRNA. In contrast to other antisense oligos, morpholinos solely bind to target RNA but do not induce target RNA degradation. Since only the oligos bound near the translation initiation site have an effect on target protein level, the specificity of morpholino is intrinsically 30–40-fold higher than other antisense oligos (32). Our present studies showed that transfection of specific GKLF morpholinos into cultured SMCs increased expression of endogenous SM α-actin and SM MHC as opposed to control oligo-treated cells. A rigorous set of control morpholinos were employed to validate the specificity of effect of the GKLF morpholino, including an inversion control (GKLF-Inv), and a mismatch control (GKLF-
KLF5 activated expression of both the SM22α and SM α-actin promoters in 10T1/2 fibroblasts, and that TGF-β1 reduced GKL expression. To further test this hypothesis, we tested the ability of IKLF/KLF5 to rescue GKL-induced suppression. Of major interest, IKLF overexpression reversed GKL-mediated repression in NIH3T3 cells induced to express SM α-actin by overexpression of SRF (Fig. 7). This reversal was concentration-dependent, and indeed at high IKLF/KLF5 plasmid concentration there was net stimulation of SM α-actin expression. Taken together, these results support a model of reciprocal activation-repression of SM α-actin expression via altering the ratio of IKLF versus GKL.

**DISCUSSION**

The results of the present studies are the first to show that the TCE plays a critical role in positive regulation of SM α-actin expression *in vivo*. Previously, our laboratory demonstrated that mutation of the TCE in the SM22α promoter from −445 to +60 also abolished expression of SM22α promoter activity *in vivo* in transgenic mice (20). Moreover, the promoters of several additional SMC marker genes including SM MHC and h1 calponin also contain TCEs (19). Although the latter TCEs have not yet been tested directly by mutagenesis, the fact that they have been highly conserved through evolution suggests that this will be the case. Taken together, the preceding observations provide strong evidence in support of the hypothesis that the TCE contributes to coordinate regulation of multiple SMC marker genes. As summarized in a recent review (Kumar and Owens, Ref. 34), this regulation is likely to involve...
complex combinatorial interactions of many cis-elements/transcription factors including E-Box/E12/HEB, CArG/SRF/myocardin, and TCE/KLF4/KLF5. However, a unique aspect of the TCE is that results of present and previous studies in our laboratory provide several lines of evidence that this TCE cis-element can mediate both transcriptional activation and repression: 1) mutation of the TCEs of SM22α/H9251 and SMα-actin promoters (Fig. 2) abrogated expression of β-galactosidase in transgenic mice in vivo; 2) GKLF specifically bound to SM22α and SM α-actin TCE in EMSA (20), and GKLF specifically bound to TCE-containing regions of SM α-actin and SM MHC promoter within intact chromatin in ChIP assays (Fig. 6); 3) GKLF markedly repressed the activity of SM22α and SM α-actin promoter in cultured cells (20); 4) Ad-CMV-GKLF downregulated (Fig. 4) but GKLF morpholino antisense oligo up-regulated (Fig. 5) expression of multiple endogenous SMC marker genes; and 5) IKLF/KLF5 reversed GKLF-induced suppression in NIH3T3 cells induced to express SM α-actin by overexpression of SRF (Fig. 7). Taken together, the preceding studies support a model in which the TCE controls transcriptional activation or repression of multiple SMC marker genes via recruiting GKLF and other member(s) of the Kruppel-like zinc finger transcription factor family.

We have previously used transgenic mice with a SMC specific promoter driving a LacZ reporter to study a transcriptional repressor cis-element, a G/C-rich region. Our studies showed that mutation of this G/C-rich element dramatically attenuated repression of a SM22-LacZ transgene in response to arterial injury model (35). Unfortunately since the TCE mediates both activation and repression, the almost total loss of

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**FIG. 5.** GKLF antisense morpholino oligo (GKLF) and control oligos (GKLF-Inv and GKLF-Mis) were synthesized by Gene Tools. A, morpholino oligos were transfected into cultured SMCs as described under “Materials and Methods.” Expression of endogenous SM α-actin and SM MHC were assayed by real time RT-PCR. As shown in B, specific abrogation of endogenous GKLF increased expression of endogenous SM α-actin by 165% or 157% (p < 0.01 by ANOVA), and SM MHC by 135% or 138% (p < 0.01 by ANOVA) compared with inverse control or mismatch control morpholinos, respectively (B).

**FIG. 6.** Direct association of GKLF with the TCE-containing region of the SM α-actin, SM MHC, and transin promoters was studied using chromatin immunoprecipitation assays. Confluent cultured SMCs were infected with GKLF adenovirus (Ad-CMV-GKLF) or control adenovirus (Ad-CMV), and incubated for 24 h before harvesting. Cells were fixed with 1% formaldehyde, and sheared by sonication and subjected to precipitation with no antibody, anti-FLAG, anti-acetylated H4, or anti-SRF antibody. Attached DNA was reverse cross-linked, washed, and subjected for PCR assays. Input DNA was derived from no antibody control reaction, and diluted 100-fold for PCR. Results showed that GKLF was specifically bound to TCE-containing regions of the SM α-actin and SM MHC promoters, but not to the non-SMC selective transin gene.
baseline activity resulting from mutation of the SM 22α and SM α-actin TCEs precludes further studies of the potential repressor activity of the TCE in vivo in injury or atherosclerosis models associated with SMC phenotypic modulation. As such, further advances in our understanding in this area will depend on first identifying candidate TCE repressors in simpler in vitro systems and then eventually testing the contribution of these factors in vivo using various loss- and/or gain-of-function approaches. Given the observations in the present study that GKLF acts as a potent TCE repressor in cultured cells, it will be of interest to conduct further studies of GKF in vivo. However, GKF is relatively widely expressed in various tissues including the epidermal layers of intestine (21) and skin (36), and it is also thought to play multiple roles including: transcriptional activation (37, 38), transcriptional repression (39, 40), and proliferation inhibition or stimulation (41, 42). Knockout of GKF resulted in early death 1 day following birth due to poorly developed skin (36). Although no overt vascular abnormalities were found, this finding is not unexpected since we postulate that GKF acts as a repressor for SMC marker gene expression and thus is unlikely to be expressed early in embryonic vascular development. Alternatively, since the GKF knockout mice have not been closely examined with respect to SMC differentiation, we cannot rule out the possibility that GKF disruption might lead to precocious expression of SMC marker genes. In any case, the role of GKF in vivo in SMC phenotypic modulation during atherogenesis and restenosis cannot be addressed with conventional knockout approaches due to early lethality, and conditional and/or SMC-targeted knockout and/or overexpression systems will be needed.

Although the role of GKF/KLF4 in SMC differentiation regulation in vivo is not clear, other Kruppel-like factors, IKL/KLF5 and LKLF/KLF2 have been identified as candidates that positively regulate SMC differentiation in vivo. For example, genetic ablation of IKLF led to embryonic death at 8.5 dpc (43). Analysis of heterozygous animals showed that the medial and adventitial layers of aorta wall were thinner in heterozygous mice than in wild-type mice. Moreover, in response to injury, there was less intimal and medial layer thickening in IKLF+/— mice than in the wild-type controls (IKLF+/+). In addition, the present studies showed that IKLF was able to antagonize GKF-induced repression in vitro (Fig. 7). Similarly, disruption of LKLF/IKLF2 led to embryonic death between 12.5 and 14.5 dpc due to defects in blood vessel formation caused by deficient recruitment of pericytes and SMCs (44). In addition, the LKLF—/— aorta was surrounded by poorly organized tunica media, and LKLF—/—VSMCs displayed a cuboidal morphology and expressed less SM α-actin than wild-type counterparts. Although the greatest LKLF expression in this study was seen in endothelial cells by in situ hybridization, we have detected LKLF expression by RT-PCR in cultured SMCs (data not shown). As such, these in vitro studies indicate that IKLF and LKLF may be activators for SMC marker genes. However, it remains unclear whether this is due to a direct effect on the promoters of SMC marker genes. Furthermore, given the similar DNA binding domain possessed by GKF, IKLF and LKLF (45), IKLF and LKLF may act via binding to TCE. Taken together, results support a model wherein GKF represses and IKLF and/or LKLF stimulate SMC marker gene expression via binding to the TCE. However, the overall effect will likely depend on the stoichiometry of candidate repressor and activator factors. Furthermore, since there exist multiple cis-elements including E-Box, CArG box, and TCE in many SMC marker gene promoters (34), it is also possible that interactions between GKF and other cis-element binding factors such as E12/HEB or SRF/Myocardin may be important in the control of SMC differentiation. In any case, the present studies add to a growing body of evidence showing that regulation of SMC differentiation is complex and requires coordinative interaction of multiple cis-elements and their binding factors.

In summary, the preceding studies support a model in which the TCE controls transcriptional activation or repression of multiple SMC marker genes via recruiting GKF and other member(s) of the KLF family. Although GKF has been clearly shown to be a potent repressor for SMC marker gene in cultured SMCs, the role of GKF in vivo in regulation of SMC differentiation remains to be elucidated.

Acknowledgments—We thank Dr. Masahiko Kurabayashi at Gunma University for providing the mouse IGF2 cDNA. We thank M.S. Kumar, and Dr. F. Dandre for useful discussions. We gratefully acknowledge the expert technical assistance of Margaret Ober, Diane Raines, Doug Mullinex, and Rupande Tripathi.

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