Inhibition of *myogenin* expression by activated Raf is not responsible for the block to avian myogenesis.

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Running Title: Raf inhibition of myogenin promoter activity

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SUMMARY

Activated Raf is a potent inhibitor of skeletal muscle gene transcription and myocyte formation through stimulation of downstream MAPK. However, the molecular targets of elevated MAPK with regard to myogenic repression remain elusive. We examined the effects of activated Raf on myogenin gene expression in avian myoblasts. Overexpression of activated Raf in embryonic chick myoblasts prevented myogenin gene transcription and myocyte differentiation. Treatment with PD98059, an inhibitor of MEK, restored myogenin expression but did not reinstate the myogenic program. Using a panel of myogenin promoter deletion mutants, we were unable to identify a region within the proximal 829 bp promoter that confers responsiveness to MEK. Interestingly, our experiments identified MEF2A as a target of Raf-mediated inhibition in mouse myoblasts but not in avian myogenic cells. Embryonic myoblasts overexpressing activated Raf were unable to drive transcription from a minimal myogenin promoter reporter, containing a single E-box and MEF2 site, to levels comparable to controls. Unlike mouse myoblasts, forced expression of MEF2A did not synergistically enhance transcription from the myogenin promoter in chick myoblasts indicating that additional molecular determinants of the block to myogenesis exist. Results of these experiments further exemplify specie differences in the mode of Raf-mediated inhibition of muscle differentiation.
INTRODUCTION

Negative regulation of skeletal myogenesis can be accomplished through growth factor stimulation of several intracellular signaling pathways. The majority of these signaling cascades are associated with the activation of the membrane localized GTPase, Ras 1. Ras enzymatic activity is a critical component of many ligand-induced responses including those initiated by the myogenesis inhibitory growth factor, FGF2 2;3. In addition, sustained Ras activity represents one of the most potent myogenic repressive agents known 4;5. The means by which activated Ras inhibits skeletal myocyte formation and muscle gene expression remains elusive. However, it is unlikely that repression is mediated by the simple activation of one of the downstream mitogen activated proteins kinases (MAPK), such as Jun N-terminal kinase (JNK), p38 or ERK1/2 6-8.

One of the downstream kinase cascades responsible for transmission Ras function include the archetypical signaling axis, Raf/MEK/MAPK 9. Initiation of this pathway through mis-expression of activated Raf leads to repression of the myogenic gene program and inhibition of myocyte formation 10. The biochemical rationale for suppression of avian skeletal myogenesis by activated alleles of Raf appears to be independent of perturbations in the inherent transcriptional activities of the myogenic regulatory factors (MRFs). However, Raf-initiated morphological transformation of the myoblasts is a direct consequence of increased AP-1 function 11.

The myogenic regulatory factors (MRFs) have long been recognized as master control
switches for induction of the skeletal muscle phenotype. Each of the MRFs is required at distinct stages during myogenesis with myogenin assuming a critical role during terminal differentiation and myoblast fusion. Mice devoid of myogenin direct a full complement of cells that are located in the appropriate muscle forming regions. However, the majority of the cells fail to fuse into the multinucleate myocytes capable of contractile protein synthesis. Control of myogenin gene expression requires both E-box binding factors such as MyoD and myocyte enhancer-binding factor 2C (MEF2C). Cis elements for these two transcription factors reside within the minimal promoter region of the myogenin gene and deletion of either site in the mouse gene results in a severe reduction in myogenin expression.

Because myogenin is critical for full differentiation of the myocyte and overexpression of activated kinases often leads to loss of MRF gene expression, we reasoned that Raf signaling pathways may inhibit skeletal myogenesis by disruption of myogenin expression and function. In this report, we find that activated Raf suppresses myogenin expression in avian myoblasts and this repression is achieved by MEK activation. One of the downstream inhibitory targets of Raf signaling is the transcription factor, MEF2. Overexpression of MEF2A reverses the negative effects of activated Raf on myogenin reporter gene expression in mouse myoblasts. However, additional transcriptional intermediates are involved in full repression of myogenin gene expression by Raf kinase as MEF2C overexpression is unable to fully overcome the detrimental effects of the Raf/MEK/MAPK signaling module.
METHODS AND MATERIALS

Plasmids

Expression plasmids coding for myc-tagged activated human Raf [pCS2+MT shuttle Raf BXB (CMV-Raf BXB), pCS2+MT shuttle Raf CAAX (CMV-Raf CAAX)] have been described. RCAS(A)-Raf BXB and RCAS(A)-Raf CAAX are replication competent retrovirus containing the coding sequence for activated alleles of human Raf. Mammalian expression plasmids encoding activated MEK1 (pBABE MEK E217/E221) or dominant inhibitory MEK1 (pBABE MEK A221) were a generous gift from Dr. M.H. Cobb (University of Texas-Southwestern, Dallas, TX). Myogenin promoter deletions were constructed by PCR of genomic chick DNA. In brief, genomic DNA isolated from embryonic day 10 myocytes was amplified with Pfu DNA polymerase (Stratagene, Loyola, CA) and the primers 5’-CGAGGTCGACGGTATCGATAAG (5’ primer) and 5’-GGGAGGACGTGTGCGCGGCTC (3’ primer), which span the region from 829 nucleotides upstream of the transcriptional start site (+1) of the myogenin gene to the translational start site, respectively. The fidelity of the amplified DNA fragment (−829/+40) was confirmed by automated sequencing (Davis Sequencing, Davis CA). The myogenin promoter fragment was inserted into the vector pGL2-basic (Promega, Madison, WI) to create −829mgn-Luc. Five prime deletion mutants of −829mgn-Luc were created by PCR using Pfu DNA polymerase (Stratagene, Loyola, CA), the 3’ primer (see above) and the following 5’ primers: −829, 5’-CCCTCGAGGTCGACGGTATCGATAAG; −668, 5’-CTCCGCTGGAATCTGGCCCAG; −545, 5’-GAAGGGTGAAAACCCATCCC; −470, 5’-GAAGGTGAAAAACCATCCC; −470, 5’-GAAGGGTGGAAAAACCATCCC; −228, 5’-AGGGCAGCTCCCACCACATGCC.
An Xba restriction site was included at the beginning of the 5’ primer and a Bgl II restriction site was included on the 3’ primer to allow for directional cloning into pGL2. The fidelity of the reporters was verified by automated DNA sequencing.

Cell culture and transient transfection

Myoblasts were isolated from the hind limbs of embryonic day 10 (ED10) chicks (SPAFAS, Preston, CT) as described previously. The cells were seeded onto gelatin-coated tissueware and cultured in growth media composed of Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2% chicken serum, 1% penicillin-streptomycin and 20 µg/ml Geneticin (Gibco BRL, Grand Isle, NY). Eighteen hours post-plating, the myoblasts (2X10^5) were transiently transfected by calcium phosphate precipitate formation with 0.5 µg of CMV-Raf BXB, CMV-Raf CAAX, 0.5 µg of CMV-LacZ or 0.1 µg of pRL-CMV and 1.0 µg of 829mgn-Luc or an equimolar amount of the respective myogenin promoter deletion reporter plasmids. The cells were incubated with the DNA precipitates for 4 hours followed by a brief osmotic shock (20% glycerol in DMEM). The cultures were maintained in growth media for an additional 48 hours prior to lysis and measurement of luciferase and β-galactosidase (β-gal) or Renilla luciferase (renilla) activities. The luciferase values were normalized to the β-gal or renilla to correct for transfection efficiencies. Statistical significance between the treatments was determined by ANOVA.

RNA Isolation and RT-PCR
Chick ED10 myoblasts (1X10^6) were transduced with RCAS, RCAS-Raf CAAX and RCAS-Raf BXB and treated for 48 hours with 20 µM PD98059 (NEB, Beverly MA) or an equivalent amount of DMSO. The cells were washed with PBS and lysed with 5 ml of RNA STAT-60 (Tel-Test, Friendswood, TX). The lysates were processed according to the manufacturers directions and the total RNA pellet was dissolved in sterile water, quantified and stored frozen at 80°C until needed. One microgram of total RNA was reverse transcribed with MMLV reverse transcriptase (Promega, Madison, WI) in a reaction volume of 20 µl. Two microliters of the first strand reaction was amplified with Taq (Promega, Madison, WI) and the following primer sets: myogenin (F-GCAGCCTCAACCAGGAGGA, R-CTCCTCTGGGAACGTCACG) glyceraldehyde 3-phosphate dehydrogenase (GAPDH; F-AGTCATCCCTGGAGCTGAATG, R-AGGATCAAGTCCACAAACACG). The amplification products were separated through ethidium bromide containing agarose gels, visualized and photographed.

Western Analysis

ED10 myoblasts were transduced with RCAS, RCAS-Raf CAAX or RCAS-Raf BXB retroviruses as described previously 10. For the inhibition of MEK activity, the cultures were incubated with growth media supplemented with 20 µM PD98059 (Cell Signaling, Beverly, MA). Extracts of nuclear proteins were prepared and total protein content was determined by Bradford assay (BioRad, Hercules, CA). Ten micrograms of nuclear proteins were electrophoretically separated through 12% denaturing gels and transferred to nitrocellulose. The blots were incubated for 60 minutes in 10 mM TRIS, pH 8.0, 150
mM NaCl, and 0.1% Tween-20 (TBST) containing 5% nonfat dry milk. Subsequently, the blots were incubated for one hour at room temperature with anti-myogenin (1:3,000, a kind gift of Dr. Bruce Paterson, NIH). The blots were washed extensively with TBST prior to incubation for one hour with anti-rabbit peroxidase conjugated secondary antibodies (Vector Labs, Burlingame, CA). The blots were further washed with TBST and the myogenin immunoreactive complexes were visualized by chemiluminescence (ECL; Amersham, Piscataway, NJ). Myoblasts (2X10^5) transiently transfected with 5 µg of pBABE MEK E217/E221, pBABE MEK A221, CMV-Raf BXB or CMV-Raf CAAX were analyzed in a similar fashion. Briefly, the cells were lysed with SDS-PAGE sample buffer three days after transfection. Total cellular proteins were separated through 12% polyacrylamide gels, transferred to nitrocellulose and analyzed using anti-myc (1:5,000; 9E10 ascites, Developmental Hybridoma Bank, University of Iowa, Iowa City, IA) for the detection of Raf proteins and anti-MEK1 (1:500; C-18, Santa Cruz Biotech, Santa Cruz, CA) for the detection of MEK A221 proteins. Visualization of immunocomplexes was achieved using the appropriate peroxidase conjugated secondary antibody and chemiluminescence.

RESULTS

Activated Raf inhibits myogenin expression. Early work demonstrated that constitutive Ras activity leads to a loss of MRF expression in myocytes, which may contribute to the inhibitory effects of the kinase 21. Because Raf signaling is a product of Ras action and Raf is an inhibitor of myogenesis, we examined the relative amounts of myogenin protein in
Raf transformed chick myoblasts 10. In brief, equal amounts of nuclear proteins were analyzed by Western using a polyclonal myogenin antibody. Chemiluminescent detection revealed equivalent amounts of myogenin in RCAS and RCAS-Raf BXB transduced myoblasts (Figure 1A). By contrast, the level of myogenin protein synthesized by RCAS-Raf CAAX transformed myogenic cells is significantly reduced. To determine the effects of MEK inactivation on myogenin production, RCAS, RCAS-Raf BXB and RCAS-Raf CAAX transduced cells were treated with 20 μM PD98059 and analyzed by Western blot. PD98059 is a chemical agent with preferential specificity for MEK that acts to suppress activation of this kinase leading to an inhibition of MAPK activity 23-25. Results demonstrate that the relative levels of myogenin protein synthesized by RCAS and RCAS-Raf BXB transduced myoblasts are unaffected by the MEK inhibitor. However, treatment of RCAS-Raf CAAX cells with the chemical agent restored myogenin protein expression. The re-establishment of myogenin expression is not a reflection of altered protein translation. Semi-quantitative RT-PCR for myogenin mRNA expression in the Raf transformed cells is analogous to the Western blot findings. RCAS and RCAS-Raf BXB transduced cells transcribe abundant amounts of myogenin message, while RCAS-Raf CAAX transduced myoblasts direct a fraction of the amount (Figure 1B).

Myogenin promoter-reporters are responsive to Raf activity. Myogenin expression is affected by MEK activity and re-establishment of gene expression to levels comparable to controls is achieved by inhibition of MEK. However, the restoration of myogenin does
not remove the Raf imposed block to myoblast differentiation. To better understand the means by which MEK contributes to Raf induced repression of \textit{myogenin}, a panel of \textit{myogenin} promoter deletions was constructed. The largest \textit{myogenin} promoter fragment was amplified from chicken genomic DNA with primers designed from the published \textit{myogenin} promoter sequence. The 829 bp \textit{myogenin} promoter region was analyzed by TFSEARCH, a web-based search engine for the identification of putative \textit{cis} elements, and the results were used to develop the deletion mutants. As shown in Figure 2, several DNA binding sites exist within the proximal promoter that are known to be affected by MAPK activity including Ras response element binding protein (RREB), cAMP response element binding protein (CREB), E-boxes and MEF2 binding sites. ED10 chick myoblasts were transiently transfected with \textit{-829mgn-luc}, \textit{-668mgn-luc}, \textit{-545mgn-luc}, \textit{-470mgn-luc}, \textit{-228mgn-luc} or pGL2-basic, CMV-\textit{β}-galactosidase and CMV or CMV-Raf CAAX. After 48 hours the cells were lysed and analyzed for luciferase and \textit{β}-galactosidase (\textit{β}-gal) activity. As shown in Figure 3, all of the \textit{myogenin} promoters are capable of supporting strong luciferase expression. The smallest promoter fragment, \textit{-228mgn-luc}, drives reporter gene expression to levels comparable to the largest \textit{myogenin} promoter-reporter (-829mgn-luc) indicating that all of the elements necessary for transcription are present in the proximal 228 base pairs. Additionally, constitutive expression of activated Raf dramatically inhibits each of the \textit{myogenin} promoters to levels approximating 70% of control cells lacking Raf CAAX expression. No differences were found between the various reporters with regard to the extent of inhibition. Therefore, the \textit{cis} elements affected by Raf signaling likely are contained within the
minimal promoter reporter, -228mgn-luc.

**-829mgn-Luc does not contain MEK responsive elements.** To identify the region of the *myogenin* promoter that responds to MEK signaling, the panel of *myogenin* promoter reporters was tested in Raf-expressing myoblasts treated with the MEK inhibitor, PD98059. Embryonic day 10 chick myoblasts were transiently transfected with equimolar amounts of -829mgn-Luc or -228mgn-Luc, pRL-renilla and CMV or CMV-Raf CAAX. After 48 hours, the cells were lysed and luciferase and Renilla luciferase activities were measured. As shown in Figure 4A, both the full-length (-829mgn-Luc) and minimal (-228mgn-Luc) promoter reporters efficiently activate luciferase expression in the absence of Raf signaling and the levels of luciferase are significantly reduced in the presence of activated Raf. Moreover, treatment of Raf CAAX myoblasts with 20 µM PD98059 did not restore 829mgn-Luc or 228mgn-Luc transcriptional activities to levels comparable to those found in control myoblasts. Similar findings were found for the remaining deletion mutants (data not shown). Because the chemical inhibitor increases basal myogenin promoter activity, ED10 myoblasts were transiently transfected with either the full-length reporter or the minimal promoter and expression plasmids encoding Raf CAAX and MEK-A221, a kinase-defective MEK. After 48 hours, the cells were lysed and luciferase activity measured. The amount of luciferase activity was corrected for transfection efficiency by normalizing for β-gal activity. Myoblasts expressing activated Raf directed low levels of 829mgn-Luc and 228mgn-Luc reporter activity, as expected (Figure 4B). Cells cotransfected with CMV-Raf CAAX and pBABE-MEK-
A221 failed to direct transcription from 829mgn-luc to levels comparable to myoblasts transfected with reporter-only. In an analogous fashion, the dominant inhibitory MEK A221 protein was unable to restore full activity to the −228mgn-Luc reporter in the presence of activated Raf.

The inability of the MEK inhibitors to restore *myogenin* promoter function in the presence of Raf CAAX is not a product of insufficient MEK-A221 protein expression as determined by Western (Figure 4C). In brief, myoblasts were transfected with the expression plasmids coding for MEK-A221 and Raf CAAX. After 48 hours, the cells were lysed with SDS-PAGE buffer and equal amounts of protein were electrophoretically separated and transferred to nitrocellulose. The blots were probed with anti-MEK1. Chemiluminescent detection revealed that abundant amounts of MEK-A221 were produced in the presence of Raf CAAX. Thus, reestablishment of myogenin gene expression by inhibition of MEK activity occurs independent of the *cis* DNA elements located in the 829 bp promoter region.

*Activated Raf inhibits myogenesis by MEF2-dependent and independent mechanisms.*

Recently, it was reported that an inducible Raf BXB inhibits L6 myoblasts from terminal differentiation and the block to myogenesis was a consequence of MEF2 localization to the cytoplasm 28. Raf CAAX inhibits the minimal myogenin promoter (−228mgn-Luc) and the region contains a single MEF2 DNA binding site (Figure 2). To examine the effects of activated Raf on MEF2 and E-box function, each of the sites were mutated in
the minimal promoter reporter to create –228mMEF2mgn-Luc and –228mEmgn-Luc, respectively. The promoter-reporters were transfected into ED10 myoblasts with pRL-CMV, CMV or CMV-Raf CAAX. After 48 hours, the cells were harvested and luciferase activities were measured. As shown in Figure 5, both the wild type –228mgn-Luc and –228mEmgn-Luc direct abundant levels of luciferase activity in the absence of Raf signals. Mutation of the single MEF2 site within the context of the minimal promoter completely abolishes transcriptional activity, as reported previously. Cotransfection of CMV-Raf CAAX significantly repressed activation of both wild type and the E-box deficient promoter reporters. Moreover, cotransfection of pMT2-MEF2A with –228mgn-Luc or –228mEmgn-Luc significantly enhanced the level of reporter activity compared to controls. Unexpectedly, cotransfection of the ED10 myoblasts with expression plasmids coding for Raf CAAX and MEF2A did not restore the levels of luciferase activity to levels comparable to control muscle cells transfected with –228mgn-Luc or –228mEmgn-Luc plus MEF2A. In addition, coexpression of activated Raf with MEF2 does not result in sequestration of the transcription factor in the cytoplasm (Figure 6). These results are attributable to differences between primary avian muscle cells and C3H10T1/2 myoblasts as we were able to duplicate the results of Winter and Arnold (2000). Fibroblasts were transfected with expression plasmids encoding avian MyoD (CMD), activated Raf, MEF2A and –228mgn-Luc, -228mEmgn-Luc or –228mMEF2mgn-Luc. After 48 hours in differentiation permissive media, the cells were harvested and reporter activities were measured. Activated Raf suppressed transcription from the wild-type reporter (Figure 7). The transcriptional block was
effectively removed by coexpression of MEF2 with Raf CAAX, as reported previously. Interestingly, CMD was unable to activate transcription from –228mMEF2mgn-Luc, which contains a viable E-box. MEF2A readily activated –228mEmgn-Luc and the levels of activity were significantly enhanced by coexpression with activated Raf. Thus, activated Raf inhibits avian myogenesis through a mechanism that is not entirely reflected in disrupted MEF2 function.

**DISCUSSION**

Constitutive activation of components of the Ras/Raf/MEK/MAPK signaling axis consistently has demonstrated a strong repressive effect on skeletal myogenesis 4;10;20;28;29. However, the mechanism by which chronic activity of the archetypical signaling module inhibits muscle gene expression is less well understood. Members of the myogenic regulatory factors retain their inherent DNA binding and transcriptional activation capacities thus, suggesting that the molecular means for the repression of gene function is levied at alternate transcriptional mediators 4;10. Our work demonstrates that loss of myogenin gene expression accompanies elevated Raf kinase signaling in a MEK-dependent manner. Constitutive expression of Raf CAAX, a full-length membrane-localized Raf protein, dramatically reduces the number of myogenin mRNA transcripts and subsequent protein synthesis. Surprisingly, RCAS-Raf BXB transduced myoblasts transcribe abundant amounts of myogenin mRNA yet the cells remain differentiation defective. Moreover, restoration of myogenin gene transcription by treatment of the RCAS-Raf CAAX myocytes with PD98059 does not reverse the negative effects of the kinase on
morphological or biochemical differentiation. From these results, we conclude that loss of myogenin expression is not the molecular target of activated Raf that leads to repression of the muscle differentiation program.

The predominant signaling cascade induced by Raf is the sequential activation of MEK and MAPK 1. However, Raf kinase interacts with additional kinase intermediates that may serve to amplify or instigate alternate, MEK-independent signaling30;31;32;32. A direct physical association occurs between Raf and MEKK1, a dual-specific kinase that signals primarily through downstream JNK with minimal activation of MAPK 30. Protein complexes comprised of MEKK1, MEK and MAPK also can be found suggesting that the kinase may serve as a scaffold protein for the Raf/MEK/MAPK module. Moreover, a mutated Raf protein that fails to interact with MEK and induce MAPK activity retains its ability to transactivate NFκB reporter genes further supporting the growing body of evidence that Raf represents a signaling bifurcation point32. Our previous work indicates that the membrane localized form of constitutively active Raf signals through downstream kinases that are MEK-independent 11. Here, we report that MEK is present in embryonic chick myoblasts transduced with RCAS-Raf CAAX and the chemical inhibitor PD98059 suppresses the functional activity of MEK in these cells. Thus, Raf CAAX disrupts transcription from the myogenin gene in a MEK-dependent manner. We chose to look for MEK-responsive cis elements within the promoter of myogenin in an effort to identify targets of Raf/MEK/MAPK signaling that contribute to repression of myogenesis. Serial deletions of the largest promoter fragment did not reveal the
presence of elements that are solely regulated by Raf/MEK actions. Interestingly, overexpression of a kinase defective MEK (MEK A221), disruption of the Raf/MEK interface (RKIP-N60) or treatment with PD98059 did not restore full transcriptional activity to –228mgn-Luc in the presence of Raf CAAX. These findings illustrate that the MEK responsive cis element lies outside of the boundaries of the myogenin promoter regions examined.

Recently, MEF2 was identified as a critical myogenic regulator whose function was disrupted as a consequence of activated Raf signaling. L6 myoblasts overexpressing activated Raf fail to differentiate into mature myocytes partially owing to the cytoplasmic localization of MEF2. Forced expression of MEF2 resulted in restoration of myoblast fusion and muscle gene transcription. In a similar manner, we found that activated Raf suppresses transcription from the minimal myogenin promoter in C3H10T1/2 myoblasts and the block to reporter gene activation is overcome by compulsory MEF2A expression. Thus, MEF2 likely is a target of Raf/MEK/MAPK signaling that lends to the inhibitory effects of the kinase in mouse myogenic cells. However, additional transcription factors also are altered in response to activated Raf signaling that contribute to the block to avian myogenesis. Embryonic chick myoblasts transfected with the minimal promoter (–228mgn-Luc) and expression plasmids coding for activated Raf and MEF2A did not direct reporter gene activity levels comparable to those found in avian myoblasts expressing MEF2 alone. While MEF2 does partially restore transcription from the myogenin promoter in the presence of Raf kinase, the levels fail to reach those directed by
the synergistic actions of endogenous MRF and MEF2. Moreover, immunostaining for MEF2 failed to demonstrate a localization of the factor to the cytoplasm in the presence of constitutive Raf activity. These results argue that additional inhibitory mechanisms are initiated by activated Raf in avian myoblasts that contribute to the block to myocyte formation.

The role of the MEK/MAPK signaling with regard to myogenic repression remains controversial. Recently, it was reported that a nuclear localized, activated MEK can inhibit skeletal muscle gene transcription. Moreover, the repressive actions of MEK involved a physical association of the kinase with the transcriptional activation domain (TAD) of MyoD and were independent of the bHLH and chromatin remodeling domains of the MRF. A direct interaction of the MRFs and MEK may partially explain the repressive effects of Raf that are not attributable to disrupted MEF2 function. Under moderate levels of Raf activity (Raf BXB), myogenesis is inhibited by sequestration of MEF2 in the cytoplasm in a MAPK-dependent manner. Extreme levels of Raf activity (Raf CAAX) prevent muscle gene transcription by mechanisms that involve MAPK-mediated MEF2 translocation to the cytoplasm and MEK:MRF complex formation and repression. The validity of these inferences remains to be experimentally tested.

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REFERENCES


FIGURE LEGENDS

Figure 1. Activated Raf inhibits myogenin gene expression. Chick moblasts transduced with RCAS(A), RCAS(A)-Raf CAAX or RCAS(A)-Raf BXB were treated for 48 hours with DMSO or 20 µM PD98059. Cells were lysed and analyzed by Western using anti-myogenin (A). Total RNA was isolated from RCAS(A), RCAS(A)-Raf CAAX and RCAS(A)-Raf BXB myoblasts and analyzed by RT-PCR for myogenin and GAPDH mRNA expression (B).

Figure 2. Creation of avian myogenin promoter reporters. The full-length chick myogenin promoter was amplified from genomic DNA, sequenced and used as a template for generation of subsequent deletion mutants. The promoter fragments were cloned upstream of the luciferase gene in pGL2 basic. Several cis DNA elements are noted in the promoter sequences including ras responsive elements (RREB), cAMP response element binding sites (CREB), E-boxes and MEF2 sites.

Figure 3. Activated Raf inhibits myogenin promoter reporter activity. Chick myoblasts (2X10^5) were transfected with 1 µg of 829mgn-Luc or an equimolar amount of 5 deletion reporter, 0.5 µg CMV-LacZ, 0.5 µg CMV or CMV-Raf CAAX. After 48 hours, cells were lysed and luciferase and β-gal activities were measured. Normalized luciferase activity produced by pGL2 was set to 1. Means and standard errors are from 3 independent experiments.
**Figure 4. Activated Raf inhibits myogenin reporter activities independent of MEK.**

Chick myoblasts (2X10^5) were transiently transfected with 1 µg 829mgn-Luc or an equimolar amount of 228mgn-Luc and pRL-CMV. Cells were treated with DMSO or 20 µM PD98059 for 48 hours prior to lysis and measurement of luciferase and Renilla activities (A). Alternatively, myoblasts were transfected with 1 µg pBabe or pBabe-MEK A221 and the plasmids described above. Luciferase and Renilla activities were measured after 48 hours (B). Means and standard errors represent 3 independent experiments. Lysates from chick myoblasts overexpressing Raf CAAX or Raf CAAX and MEK A221 were analyzed by Western (C). Abundant levels of Raf (α-myc) and MEK A221 (α-MEK) were detected by chemiluminescence.

**Figure 5. MEF2 is a potential target of activated Raf-directed myogenic inhibition.**

Chick myoblasts (2X10^5) were transiently transfected with 1 µg 228mgn-Luc reporters, 0.1 µg pRL-CMV, 0.5 µg pMT2-MEF2A and CMV or CMV-Raf CAAX. Luciferase and Renilla activities were measured after 48 hours. 228mEmgn-Luc lacks a functional E-box binding site and 228mMEF2mgn-Luc lacks a functional MEF2 DNA binding site. Means and standard errors are from 3 independent experiments.

**Figure 6. MEF2A localizes to the nucleus in the presence of Raf CAAX.** Chick myoblasts were transiently transfected with pMT2-MEF2A (A) or pMT2-MEF2A and CMV-Raf CAAX (B). Cells were fixed and immunostained for MEF2A protein expression. Arrows indicate MEF2-containing nuclei. Representative photomicrographs
Figure 7. MEF2A restores myogenin promoter reporter activity to Raf expressing myoblasts. C3H10T1/2 fibroblasts were transfected with 1 μg 228mgn-Luc reporter plasmids, 0.1 μg pRL-CMV, 0.5 μg CMV-CMD, and 0.5 μg pMT2-MEF2A, CMV-Raf CAAX or both plasmids. Luciferase activities were normalized to Renilla activity. 228mEmgn-Luc and 228mMEFmgn-Luc lack a functional E-box and MEF2 site, respectively.
Figure 1

A. 

RCAS(A) 

- Raf CAAx 
- Raf BXB 

-PD98059

+PD98059

α-Myogenin

B. 

RCAS(A) 

- Raf CAAx 
- Raf BXB 

myogenin

GAPDH