Terminal differentiation of muscle cells results in opposite effects on gene promoters: muscle-specific promoters, which are repressed during active proliferation of myoblasts, are turned on, whereas at least some proliferation-associated promoters, such as c-fos, which are active during cell division, are turned off. MyoD and myogenin, transcription factors from the basic-helix-loop-helix (bHLH) family, are involved in both processes, up-regulating muscle genes and down-regulating c-fos. On the other hand, the serum response factor (SRF) is involved in the activation of muscle-specific genes, such as c-fos, as well as in the up-regulation of a subset of genes that are responsive to mitogens. Upon terminal differentiation, the activity of these various transcription factors could be modulated by the formation of distinct protein-protein complexes. Here, we have investigated the hypothesis that the function of SRF and/or MyoD and myogenin could be modulated by a physical association between these transcription factors.

We show that myogenin from differentiating myoblasts specifically binds to SRF. In vitro analysis, using the glutathione S-transferase pull-down assay, indicates that SRF-myogenin interactions occur only with myogenin-E12 heterodimers and not with isolated myogenin. A physical interaction between myogenin, E12, and SRF could also be demonstrated in vivo using a triple-hybrid approach in yeast.

Glutathione S-transferase pull-down analysis of various mutants of the proteins demonstrated that the bHLH domain of myogenin and that of E12 were necessary and sufficient for the interaction to be observed. Specific binding to SRF was also seen with MyoD. In contrast, Id, a natural inhibitor of myogenic bHLH proteins, did not bind SRF in any of the situations tested. These data suggest that SRF, on one hand, and myogenin bHLH, on the other, could modulate each other's activity through the formation of a heterotrimeric complex.

Proliferation and cell differentiation are mutually exclusive processes, as best exemplified in muscle precursor cells. Proliferation inhibition is a crucial step that precedes muscle-specific gene expression and cell fusion into myotubes in the process of muscle cell terminal differentiation (1). Indeed, terminal differentiation of myoblastic cell lines in vitro is triggered by the accumulation of the precursor cells (myoblasts) in a G0 state (1). This step is a prerequisite, and a number of mitogens (2–5) or oncogenes (6–10) inhibit terminal differentiation.

Two distinct families of transcription factors, MEF-1 and MEF-2, are instrumental to the muscle cell differentiation process. The MEF-1 family includes the myogenin bHLH proteins MyoD (11–13), myogenin (14, 15), Myf5 (16) and MRF4/herculin/Myf6 (17). These proteins are all able to elicit in vitro a muscle determination program in a number of nonmuscle cell types (18, 19). These muscle-restricted proteins share a domain of homology, the bHLH, which is also common to ubiquitous transcription factors such as the products of the E2A gene, E12 and E47 (20, 21), with which myogenic factors form heterodimeric complexes. Heterodimers between myogenin bHLH and E12 or E47 (22–26) bind to upstream regulatory sequences of the form CANNTG (E boxes) (27) in muscle-specific gene promoters. MyoD, myogenin, and Myf5 are all able to transactivate these promoters efficiently (28), a function that involves a common motif in the basic domain (29).

MEF-2 transcription factors are members of the MADS box protein family. MADS box proteins are widely expressed, from plants to man and including yeast (30). MADS box proteins exert a wide range of functions from development in plant cells to muscle differentiation or growth factor responses in mammalian cells. In yeast, the MADS box protein MCM1 is involved in the mating-type phenotype (31). A highly homologous protein, the serum response factor (SRF), is instrumental to immediate early gene mitogenic responses in mammals (32, 33). The sequences that are recognized by the two proteins are homologous (34). The two proteins function in a similar manner: although both proteins are able to transactivate transcription (35, 36), they mainly function by recruiting ternary complex-forming factors through a protein motif located in the MADS box (37, 38).

The MEF-2 subset of MADS box proteins includes four members, MEF-2A, MEF-2B, MEF-2C, and MEF-2D, some of which are ubiquitous and some restricted to differentiated muscle cells. These proteins are related to SRF and are often referred to as rSRF proteins (39). They bind to and transactivate CTA(A/T)₄TAG DNA elements, a consensus sequence that is homologous, but not identical, to the CArG boxes (CC(A/T)₆GG), which are recognized by SRF. In fact, SRF and rSRF (MEF-2) cannot heterodimerize with each other (39), and thus, they belong to distinct subsets of MADS box proteins.
The factors that control the balance between proliferation and differentiation in muscle are not fully understood at present. However, myogenic factors of the bHLH family seem to be involved in this delicate control. In particular, MyoD is both a target for mitogens that are inhibiting differentiation (2, 7, 10) and a negative regulator for cell proliferation in vitro (40, 41). Part of the mechanism by which MyoD blocks cell proliferation in vitro seems to be the repression of proliferation-associated genes, and indeed, most of the proliferation-associated genes are silent in differentiated muscle cells (42). In particular, immediate early genes such as c-fos are repressed on terminal differentiation (42, 43). In fact, the transcription factor AP1, which is formed by heterodimers between members of the Fos-related proteins and members of the Jun-related factors, is a prominent target for MyoD: MyoD interferes with AP1 function by forming specific complexes with Jun proteins (44). In addition, MyoD (43) and myogenin (45) both act as repressors for the c-fos promoter through the inhibition of its main element, the serum response element (SRE) (46). The SRE includes a CArG box, the binding site for SRF, which is repressed on muscle promoters, SRF needs to interact physically with other factors for optimal transactivation. A tempting hypothesis would thus be that, in order to be active on muscle promoters, SRF needs to interact physically with other factors.

In this paper, we have tested the hypothesis that SRF interacts with the myogenic bHLH proteins. We demonstrate that a heterotrimeric complex forms between bHLH proteins and SRF both in vitro and in cultured cells. The formation of this complex could be one of the means by which SRF activity is deviated from proliferative to differentiating genes upon muscle cell terminal differentiation.

**MATERIALS AND METHODS**

**Plasmids**—pEMSV-E12, pGST-SRF, and pSRE-CAT were as described in Ref. 43. pEMSV-myogenin was a kind gift of Dr. E. Olson. pEMSV-JunH1-myogenin (in which amino acids 71-163 have been deleted) and pEMSV-JunH1-myogenin (in which amino acids 71-163 have been deleted) were constructed using a polymerase chain reaction amplification method. The internal primers were TGGCAGGCTCAGGCCTCTCGCAG and GGGCCGTGCAACTTGCATGCCCCACG for pEMSV-JunH1-myogenin and TGGACGTTGGAATGAGCCCTTGGAAGG and TCATTCCACCTCTCGTCCCATCCGCA for pEMSV-JunH1-myogenin. The forward external primer included a consensus translation start site (63), and both forward and reverse external primers included an EcoRI restriction site for cloning convenience. The sequences of these primers were GAGATCATCATGGAGCTGTAT- GAGACATCCC (forward) and GGGGGGATTAGTGCCATGTT- TCG (reverse). PhIV-SRF, used in in vitro translation experiments, was constructed by replacing the chloramphenicol acetyltransferase gene in a pGEM-HIV-CAT construct (62) with a sequence encoding the complete SRF protein obtained by polymerase chain reaction amplification (forward primer sequence, 5'-CCCCAAGCTTACATTTGTTAGCCACG-3'; and reverse primer sequence, 5'-CTTACCTGCTTTGCTGTTTTGGGCCGT- GTTG-3'). Plasmids pGAD424 and pGBT9 were a kind gift of Dr. S. Fields (63). Plasmid pRS313 has been described by Sikorski and Hieter (64). Plasmids pGAD-E12 and pGAD-myogenin were constructed by inserting the corresponding complete coding sequence, obtained by polymerase chain reaction amplification using the above-described primers for myogenin, into the EcoRI site of pGAD424. Plasmid pGB-SRF was constructed by inserting the complete coding sequence of SRF, obtained by polymerase chain reaction using the above-described primers and subcloned into a Bluescribe vector, between the EcoRI and SalI sites of pGBT9. pRS-E12 and pRS-myogenin were constructed by subcloning an SpHl-Sphl insert from the corresponding pGAD construct into the SmaI site of pRS313. These constructs were controlled by partial sequence (which did not reveal any mutations), and results obtained with these constructs were confirmed using two independent clones.

pEMSV-MyoD, pEMSV-MyoD-NHβ, pEMSV-MyoD-βE, and pEMSV-MyoD-bt4 were kind gifts of Dr. H. Werntraub (27). pEMSV-myogenin, pEMSV-myogenin, and pEMSV-JunH1/myogenin were kind gifts of Dr. E. Olson (65).

CDs and Transfections—NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with antibiotics (a mixture of penicillin and streptomycin) (Life Technologies, Inc.) used according to the manufacturer's recommendations and 5% FCS. Cells were transfected by electroporation as described previously (43). Briefly, cells were harvested by scraping, washed, and resuspended in 150 μl of Dulbecco's modified Eagle's medium supplemented with 0.5% FCS. 2 μg of SRE- CAT; the indicated doses of pEMSV-E12, pEMSV-myogenin, or myogenin, and 1 μg of RSV- luc (as an internal control for transfection efficiency) were added. After electrical shock (using a Bio-Rad apparatus) each microfuge tube (100 μl), each sample was divided in two aliquots, and cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.5% FCS for 48 h, after which one of the aliquots was treated with 20% serum for 4 h. Cells were harvested, and extracts were standardized based on the luciferase activity of the non-serum-treated sample (samples from the same transfection were standardized based on the protein content, as measured by a Bio-Rad kit following the manufacturer's recommendations. The programed lysates (10 μl) were incubated with the GST or GST-SRF beads, and bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis using standard procedures.

Analysis of Myoblastic Proteins—C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Confluent dishes were induced to differentiate by switching the medium to 0.5% FCS. Differentiating cells were harvested by scraping, and nuclear proteins were prepared according to the method of Dignam et al. (67). 200 μg of extracts were incubated for 1 h at room temperature with GST or GST-SRF beads, and bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by dry transfer. Filters were probed with an anti-myogenin monoclonal antibody, a kind gift of Dr. W. E. Wright (68), and revealed by chemiluminescence using an ECL kit (Amersham). Plasminogen activator, plasminogen activator, and plasminogen activator were as described (69). 20 μl of extract were assayed for β-galactosidase activity using a chemiluminescent detection procedure (Tropix Inc.) according to the manufacturer's instructions.
RESULTS

Myogenin Physically Interacts with SRF—A biochemical approach was used (71) to test the hypothesis of a physical interaction between SRF and myogenin bHLH. GST or GST-SRF covered beads were incubated with \(^{35}\)S-labeled, in vitro translated myogenin and/or E12 (Fig. 1A). These experiments were controlled using an irrelevant translation product (luciferase) as a negative control and standardized using SRF core (DNA binding and dimerization domain) as an internal positive control (data not shown). The results demonstrated that neither myogenin (lane 6) nor E12 (lane 4) was able to bind to SRF when isolated. However, a significant level of binding could be detected when both proteins were cotranslated (lane 8), suggesting that formation of heterodimers between myogenin and E12 results in SRF recognition. The converse experiments, in which beads were coated with a mixture of GST-E12 and GST-myogenin and used to retain in vitro translated SRF, did not give clearly interpretable data, most likely due to inefficient heterodimerization of E12 and myogenin on the beads. However, when myogenin-coated beads were incubated sequentially with in vitro translated E12 and then with SRF, a significant level of SRF retention could be observed (data not shown).

These results were confirmed in vivo using a double-hybrid approach in yeast (63). Indicator yeast cells (permanently transfected with a GAL1-lacZ reporter construct) were transfected with expression vectors encoding fusion proteins between the GAL4 DNA-binding domain (pGB series) or trans-activating domain (pGAD series) and SRF, myogenin, or E12 in all combinations. These experiments showed that expression of fusion products between the GAL4 DNA-binding domain and E12 or myogenin resulted in a high background, indicating that the transactivation domains of these proteins are active in yeast (data not shown). Therefore, these constructs could not be used in subsequent assays. When yeast cells were transfected with pGB-SRF and pGAD-myogenin or pGAD-E12, no \(\beta\)-galactosidase could be detected, suggesting that a heterodimer does not form between SRF and E12 or myogenin, as expected from the results of GST pull-down assays. We next introduced, into the same yeast indicator cells, three expression vectors bearing three distinct selectable markers and encoding a fusion protein between the GAL4 DNA-binding domain and SRF and fusion proteins between the GAL4 activation domain and myogenin or E12, respectively. Yeast that had received the three expression vectors expressed significant amounts of \(\beta\)-galactosidase (Fig. 1B), indicating that a heterotrimer had formed in the cells, resulting in the activation of the GAL4-responsive promoter. In contrast, galactosidase activity was hardly detectable in yeast transfected with SRF and E12 or with SRF and myogenin (lanes 1 and 2), confirming that complex formation between SRF and isolated E12 or myogenin was not very efficient. All other combinations, including pGAD-myogenin (or pGAD-E12), pGB-SRF, and pR5 as a backbone vector, resulted in background or low expression of \(\beta\)-galactosidase (data not shown). Taken together, these data indicate that heterodimers of myogenin and E12 physically interact with SRF in vitro and in vivo.

A complex between SRF and myogenin was also detected when nuclear extracts from differentiating myoblasts were used (Fig. 1C). C2C12 cells were or were not induced to differentiate in low serum. Nuclear proteins were extracted at different time points and incubated with GST- or GST-SRF-covered agarose beads. Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting using an anti-myogenin monoclonal antibody. These experiments demonstrated that cellular myogenin, amounts of which increased during differentiation, was specifically retained on SRF-coated beads in differentiating myoblasts.
Taken together, these data demonstrate that myogenin, in its physiological heterodimeric form, binds to SRF.

Physical Interaction between SRF and Myogenin Occurs through Dimerization/DNA-binding Domains—Myogenin and E12 are structurally organized in domains. To test which parts of the molecules were involved in the interaction with SRF, deletion mutants of both proteins were tested in the GST pull-down assay (Fig. 2). Removing the C- or N-terminal part of myogenin or E12 was not detrimental to SRF recognition (Fig. 2B). In fact, both regions of the molecules could be deleted, and the resulting minimal bHLH domain was sufficient to observe specific binding to SRF-covered beads. Indeed, myogenin mutants in which the bHLH domain was partly (ΔbH1) or entirely (ΔH) deleted could not bind specifically to SRF (Fig. 2D, lanes 6–9). Note that these mutants were also unable to heterodimerize with E12 (lanes 10–12). These data indicate that the bHLH domains of myogenin and E12 are both necessary and sufficient to observe the interaction.

On the other hand, experiments performed with deletion mutants of SRF indicated that, similarly, core SRF (a minimal domain of SRF that includes the DNA-binding and dimerization domains) was sufficient to observe a specific interaction with myogenin (Fig. 3). No interaction could be detected with the N- or C-terminal moiety of SRF.

Takentogether,theseresultsindicatethattheDNA-binding domains of SRF, E12, and myogenin are both necessary and sufficient to observe the interaction, raising the possibility that the interaction occurs through nonspecific binding of these proteins to contaminating DNA during the GST pull-down assay. However, the interaction among the three proteins was still observed when contaminating DNA was degraded using nuclease or when the GST pull-down assay was performed in the presence of ethidium bromide, which prevents DNA-protein interaction (data not shown). The results of these control experiments rule out the possibility that the SRF:bHLH complex could be due to artifactual nonspecific binding to DNA.

SRF Recognition Is Restricted to a Subset of bHLH Proteins—To further analyze the interaction between bHLH proteins and SRF, we have tested a variety of bHLH proteins in the GST pull-down assay. The results indicate that specific binding to SRF is not a feature restricted to myogenin, but is also observed for MyoD (Fig. 4B). MyoD was not retained on SRF-covered beads, but the products of MyoD and E12 cotranslation were specifically retained. MyoD-SRF interaction requires the MyoD bHLH domain since a mutant that has lost this domain cannot recognize SRF (Fig. 4B).

However, the ability to physically interact with SRF was not
have also tested several mutants of MyoD or myogenin (Fig. 4). We observed for all members of the bHLH family. In particular, Id, a functional antagonist of MyoD or myogenin, did not bind to SRF, either alone or in combination with E12 (Fig. 4C). We have also tested several mutants of MyoD or myogenin (Fig. 4D). MyoD-bE12 (a mutant of MyoD in which the basic domain has been replaced by the E12 basic domain) and MyoD-bT4 (in which the basic domain has been replaced by that of the T4 achaete scute Drosophila protein) both bind to DNA efficiently (27), but do not transactivate muscle genes. Whereas MyoD-bE12 retains a high affinity for SRF, MyoD-bT4 has lost SRF recognition.

**DISCUSSION**

Terminal differentiation of myoblasts is accompanied by drastic changes in the pattern of gene expression: muscle-specific genes are up-regulated, while mitogen-responsive promoters are repressed. Paradoxically, some enhancer elements are involved in the activation of both mitogen-responsive and differentiation-associated genes. In particular, CARG boxes or related elements are active in a number of muscle promoters (51, 55, 60, 72–76) as well as in immediate early genes such as c-fos or cytoskeletal actin (33, 77). Furthermore, the CARG box-binding protein SRF, a prominent factor in the immediate early response to mitogens, seems also to be indispensable for muscle cell terminal differentiation (57). Thus, SRF is involved in the up-regulation of both mitogen-responsive genes (such as c-fos) and differentiation-associated genes (such as those encoding muscle-specific proteins), although these two sets of genes are regulated in an opposite manner. A tempting hypothesis to explain this apparent paradox is that SRF activity is regulated through the formation of distinct protein complexes. In particular, SRF activity, during muscle terminal differentiation, could be modulated by a physical interaction with factors involved in this process. We have thus tested the hypothesis that such a physical interaction could occur between the myogenic bHLH proteins and SRF and found that complexes could indeed be detected between MyoD or myogenin using various assays in vitro or in vivo, including GST pull-down and hybrid assays in yeast. Interestingly, this interaction requires that myogenic bHLH be in a heterodimeric form: no complex could be detected between myogenin or MyoD and SRF in the absence of E12. Accordingly, complex formation required the integrity of the bHLH domain on both E12 and MyoD or myogenin. A likely interpretation of these results is that heterodimeric formation between E12 and MyoD or myogenin induces a conformational change in the proteins that unmasks a site of interaction with SRF. The bHLH domain is also sufficient for the interaction to occur, suggesting that this conformational change occurs in the bHLH domain, which is not unexpected. Interestingly, core SRF is also necessary and sufficient for complex formation with bHLH. This suggests that core SRF is involved in a wide variety of functions, including dimerization and DNA binding (32), interaction with p62 (78), transcriptional activation in response to some signal transduction pathways (36), and interaction with members of distinct transcription factor families (this study). Furthermore, this interaction might also be observed with other members of the MADS box family that share large domains of homology with core SRF. Indeed, a similar physical interaction has been demonstrated for MEF-2 (79), although for MEF-2, the formation of the complex did not require the presence of E12.

From a biochemical point of view, we do not know if the interaction between SRF and the heterodimeric bHLH proteins involves amino acids from the three proteins or if only two of the proteins are physically involved. It should be noted, however, that some mutations in MyoD bHLH domain result in inactivation of SRF recognition, suggesting that MyoD and myogenin are directly involved in SRF binding. Our experiments have been performed in various systems, from reticulocytes to yeast, and thus, it is likely that the interaction among the three proteins is direct. However, we cannot rule out the...
Heterodimers of myogenic basic helix-loop-helix (bHLH) proteins, such as SRF, interact with DNA through CArG boxes (51, 58, 59), although it seems instrumental for E boxes to achieve full activation. SRF is a ubiquitous protein that is a key participant in immediate early responses, entering muscle-specific transactivators that proceed to turn SRF, a ubiquitous protein, on and off. It seems that this process is instrumental for E boxes to achieve full activation. SRF is a ubiquitous protein that is a key participant in immediate early responses, entering muscle-specific transactivators that proceed to turn SRF, a ubiquitous protein, on and off. It seems that this process is instrumental for E boxes to achieve full activation.

In this regard, it seems that the formation of this trimeric complex is associated with the terminal step of muscle differentiation since 1) it is detected only in cells that have entered the differentiation process; 2) it requires heterodimerization of MyoD or myogenin with E12, an event that occurs only at this step of the process, even if MyoD pre-exists in myoblasts; and 3) Id-E12 complexes, which are present in proliferating myoblasts, do not bind to SRF. The formation of the complex on terminal differentiation might turn SRF, a ubiquitous protein, on and off. It seems that this process is instrumental for E boxes to achieve full activation.

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However, we cannot rule out the possibility that the formation of this complex also results in MyoD/myogenin modulation by SRF. In fact, for some muscle promoters, the transactivation of E boxes by MyoD or myogenin requires that an intact CArG box also be present on the promoter (52). This suggests some cooperation between myogenic bHLH and proteins binding to the CArG box. Clearly, however, the interaction with SRF is not sufficient for myogenic activity of bHLH proteins: MyoD induces the formation of this trimeric complex is associated with the terminal step of muscle differentiation since 1) it is detected only in cells that have entered the differentiation process; 2) it requires heterodimerization of MyoD or myogenin with E12, an event that occurs only at this step of the process, even if MyoD pre-exists in myoblasts; and 3) Id-E12 complexes, which are present in proliferating myoblasts, do not bind to SRF. The formation of the complex on terminal differentiation might turn SRF, a ubiquitous protein, on and off. It seems that this process is instrumental for E boxes to achieve full activation.

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Myogenic bHLH and SRF

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Physical Interaction between the Mitogen-responsive Serum Response Factor and Myogenic Basic-Helix-Loop-Helix Proteins
Regina Groisman, Hiroshi Masutani, Marie-Pierre Leibovitch, Philippe Robin, Isabelle Soudant, Didier Trouche and Annick Harel-Bellan

doi: 10.1074/jbc.271.9.5258

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