Control of mitochondrial biogenesis during myogenesis


Control of mitochondrial biogenesis during myogenesis. Am J Physiol Cell Physiol 290: C1119–C1127, 2006; doi:10.1152/ajpcell.00463.2005.—We used expression and reporter gene analysis to understand how changes in transcription factors impinge on mitochondrial gene expression during myogenesis of cultured murine myoblasts (C2C12 and Sol8). The mRNA levels for nuclear respiratory factor-1 (NRF-1) and NRF-2α increased 60% by the third day of myogenesis, whereas NRF-1 and NRF-2 reporter gene activity increased by fivefold over the same period. Although peroxisome proliferator activated receptor (PPARs) mRNA levels increased almost 10-fold, the activity of a PPAR reporter was unchanged during myogenesis. The PPAR coactivator PPAR-γ coactivator-1α (PGC1α), a master controller of mitochondrial biogenesis, was not expressed at detectable levels. However, the mRNA for both PGC1α-related coactivator and PGC1β was abundant, with the latter increasing by 50% over 3 days of differentiation. We also conducted promoter analysis of the gene for citrate synthase (CS), a common mitochondrial marker enzyme. The proximal promoter (~2,100 bp) of the human CS lacks binding sites for PPAR, NRF-1, or NRF-2. Deletion mutants, a targeted mutation, and an Sp1 site-containing reporter construct suggest that changes in Sp1 regulation also participate in mitochondrial biogenesis during myogenesis. Because most mitochondrial genes are regulated by PPARs, NRF-1, and/or NRF-2, we conducted inhibitor studies to further support the existence of a distinct pathway for CS gene regulation in myogenesis. Although both LY-294002 (a phosphatidylinositol 3-kinase inhibitor) and SB-203580 (a p38-MAPK inhibitor) blocked myogenesis (as indicated by creatine phosphokinase activity), only SB-203580 prevented the myogenic increase in cytochrome oxidase activity, whereas only LY-294002 blocked the increase in CS (enzyme and reporter gene activities). Collectively, these studies help delineate the roles of some transcriptional regulators involved in mitochondrial biogenesis associated with myogenesis and underscore an import role for posttranscriptional regulation of transcription factor activity.

Citrate synthase; muscle; transcription factor; coactivator; metabolism

Myogenesis is central to the origins and maintenance of skeletal muscle fibers throughout the lifetime of an animal. Early in embryonic development, muscle progenitor cells undergo differentiation (myogenesis) and specialization into fiber types. Adult tissues also rely on recruitment of myoblasts to help remodel and repair mature muscle. In addition to the activation of suites of genes encoding contractile proteins, myogenesis is accompanied by a reorganization of intermediary metabolism (30, 34). Within the first 48 h of differentiation, the cells switch from glycolytic metabolism to oxidative phosphorylation as the sources of ATP. There is a rapid increase in mitochondrial content, as indicated by an increase in mitochondrial volume (34). Suites of mitochondrial enzymes more or less in parallel over this period. The levels of mRNA increase for mitochondrial enzymes (e.g., pyruvate dehydrogenase) increase about twofold within the first 3 days and remain elevated for the duration of differentiation (34). After 2 wk in culture, myotubes attain mitochondrial enzyme activities that approximate those demonstrated by skeletal muscle (34). In this study, we address the regulatory origins of the changes in gene expression that accompany mitochondrial biogenesis during myogenesis.

Mitochondrial biogenesis requires coordinated changes in the metabolic enzymes of oxidative phosphorylation, TCA cycle, and fatty acid oxidation. Expression of the hundreds of nuclear-encoded mitochondrial genes is coordinated by a few transcription factors that coregulate gene networks. Nuclear respiratory factor (NRF)-1 and NRF-2 regulate many of the genes encoding oxidative phosphorylation proteins (39, 40). Peroxisome proliferator activated receptors (PPARs) regulate genes encoding enzymes and transporters of fatty acid oxidation (4). Most studies of the control of mitochondrial gene expression implicate PPARγ coactivator PGC1α as a “master controller” of mitochondrial biogenesis, coactivating nuclear hormone receptors (PPARs, retinoic acid receptors, and thyroid hormone receptors) as well as NRF-1 (see Ref. 36). PGC1α may provide an important link between myogenesis and mitochondrial biogenesis. The myogenic transcription factor myocyte enhancer factor 2 induces PGC1α expression (10), and PGC1α overexpression can stimulate the formation of slow oxidative muscle (28). Two other members of the PGC1α gene family, PGC1β and PRC (PGC1α-related coactivator), have structural similarities to PGC1α and may play a similar role in coordinating mitochondrial gene expression (39, 40). These same transcriptional regulators (PGC1s, PPARs, and NRFs) also play vital roles in the maintenance and adaptive remodeling of striated muscle energetics (3, 33).

In this study, we investigate the pathways that regulate nuclear-encoded mitochondrial genes during myogenesis, focusing on the transcription factors that are most commonly implicated in control of mitochondrial biogenesis: PGC1α gene family of coactivators and DNA binding proteins including PPARs, NRFs, and Sp1.

Materials and Methods

Cell Culture

These experiments were conducted with murine immortalized myoblasts. Most experiments were conducted with both C2C12 and Sol8 cells, with few notable differences between lines. All cell culture media, antibiotics, and sera were supplied by Invitrogen. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 25 mM glucose, 4 mM glutamine, and 1 mM pyruvate supplemented with FBS (10% for C2C12, 20% for Sol8) at 10% CO2 and 37°C. Once the
cells had reached 90% confluence, differentiation was induced by changing the medium to DMEM supplemented with 2% horse serum. All media contained penicillin, streptomycin, and neomycin. Within 2–3 days of serum starvation, myotubes begin to form and the myogenic marker creatine phosphokinase increases (34). We studied regulatory events in this window of differentiation because this is the point at which mRNA levels for mitochondrial genes increase, typically doubling and remaining high for the duration of the myogenic program (34).

Plasmids

Transcriptional regulation of mitochondrial genes was assessed with the use of a series of reporter constructs.

Transcription factor and thymidine kinase-control reporters. Four reporter constructs were used to assess the influence of specific transcription factors on regulation of a minimal promoter. The reporters for NRF-1 (NRF-1-TKLuc) and NRF-2 (NRF-2-TKLuc), each with four tandem elements upstream of a thymidine kinase (TK) minimal promoter, were constructed in house (32). The PPAR reporter gene (PPAR-TKLuc), also with four tandem elements upstream of the TK minimal promoter, was a gift of Daniel P. Kelly of Washington University (17). All members of the PPAR gene family bind to the same element, and thus the PPAR reporter gene does not distinguish among family members. The Sp1-Luc reporter possesses a multimerized consensus Sp1 binding site (5'-CGCCCCCGgggggCCAGAC-3' repeated 9 times) cloned upstream of the albumin TATA box in a G-free construct containing the firefly luciferase gene digested from pGL2-Basic (Promega) (24).

CS promoter-reporter. We also explored a complex promoter from the citrate synthase (CS) gene, which appears to lack binding sites for NRF-1 and NRF-2. In the absence of a TATA box, there is no definitive transcription start site, but the longest 5' UTR available in the GenBank database includes ~165 bp upstream of the start codon. We chose to use the human gene for these studies because the murine gene has a large intron in the region of the gene encoding the 5' UTR. Because multiple transcription start sites are feasible, we defined our CS promoter as sequences upstream of the translation start codon. Figure 1 shows potential transcription factor binding sites.

To generate the CS reporter (CS-2113Luc), 2113 bp upstream of the human citrate synthase gene (Nucleotide accession no. NT_029419) translation initiation start site was amplified from human genomic DNA by PCR with restriction sites introduced using primers F1 and R1 (Table 1). The resulting PCR product was directionally cloned into the firefly luciferase reporter vector (pGL2-Basic, Promega) using the MluI and XhoI restriction sites.

CS deletions. A series of deletion constructs employed in this study are summarized in Fig. 1. The CS-1320Luc, CS-757Luc, and CS-223Luc constructs were generated by timed Exonuclease III digestion of the full-length reporter construct (CS-2113Luc). Briefly, plasmid was digested with SacI and MluI, followed by phenol-chloroform-isooamyl alcohol extraction. Purified plasmid (4 μg) was digested with 500 units of Exonuclease III (Promega) at 28°C in a 60-μl final volume of 1× buffer (supplied by manufacturer). At 30-s intervals, 2.5-μl aliquots were placed on ice in 7.5-μl S1 nuclease reaction mix [40 mM K+ acetate (pH 4.6), 340 mM NaCl, 1.35 mM ZnSO4, 6.8% glycerol, and 60 units of S1 nuclease (Promega)]. After 30 min on ice, stop buffer (0.3 M Tris, 0.05 M EDTA) was added and samples were placed at 70°C for 10 min. Digested DNA was precipitated, resuspended in TE buffer, blunted with Klenow fragment of T7 DNA polymerase (MBI Fermentas), and recircularized using T4 DNA ligase (MBI Fermentas).

The CS-559Luc construct was created by digesting CS-2113Luc with BssHII and KpnI. The resulting fragment was blunt-ended with Klenow fragment and ligated into blunt-ended pGL2-Basic at the MluI and KpnI sites. To generate CS-254Luc, the primers forward, F2 (Table 1), and reverse, GLprimer2 (Promega), were used to amplify a fragment from CS-757Luc that was digested using MluI and XhoI and ligated into pGL2-Basic. The CS-163Luc construct was generated by Smal digestion of CS-223Luc and subsequent recircularization of the plasmid.

CS Sp1 mutant. The CS-2113A8Sp1Luc reporter was created using the forward primer, F3 (Table 1), and the reverse primer, GLprimer2. Using CS-2113Luc as a template, we amplified a fragment that was then digested with ApaI and XhoI. The fragment was ligated back into CS-2113Luc that had been digested with ApaI and XhoI. The resulting plasmid contains a two-nucleotide substitution (GG to TT) in the proximal Sp1 site of the CS 5'-flanking sequence.

CS promoter-reporter for Renilla luciferase. The CS-757RL reporter plasmid was made in the following manner. The simian virus 40 (SV40) promoter-driven region of Renilla luciferase (pRL)-SV40 (Promega) was removed by digestion with BglII, followed by treatment with Klenow to create a blunt end, and then by digestion with HindIII. The 757-bp CS promoter insert was prepared with compatible ends by first digesting the luciferase vector (CS-757Luc) with KpnI, treating with Klenow, and then digesting with HindIII. The gel-purified insert and plasmid were then ligated together to produce CS-757Ren.

Transfections

Plasmids were prepared for transfection using Plasmid Maxi kit (Qiagen). Cells were transfected using FuGENE 6 (Roche). Briefly, cells grown to ~50% confluence in 24-well cell culture plates were transfected into fresh growth medium with 250 ng of firefly luciferase plasmid and 50 ng of pRL-TK, a TK promoter-driven Renilla luciferase expression vector used as an internal reference. After 24 h, either the cells were extracted for luciferase measurements (proliferating myoblasts) or the medium was changed to fresh DMEM with 2% horse serum. After 3 additional days, luciferase measurements were performed on the differentiated myocytes.

Luciferase expression was determined using the Dual Luciferase Assay System (Promega) according to the manufacturer’s instructions.

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Fig. 1. Human citrate synthase (CS) promoter analysis. The putative binding sites for selected transcription factors are shown in relation to specific deletion mutants used in these analyses. The CS promoter was analyzed for putative transcription factor binding sites with the use of TRANSFAC, version 4.0 (46) with MatInspector (37). Transcription element search software (TESS; see Ref. 41) was also used to manually search for the nuclear respiratory factor (NRF)-1 consensus binding site string YGGGCCAYGCCGCR (13) and a matrix (>NRF-1 111008000004, 43507030800, 14081018083, and 2020040001<) with MatInspector score filtering) derived from 8 NRF-1 binding sites (13) because no NRF-1 sites were included in the TRANSFAC database. Most transcription factor motif search programs misidentify NRF-1 as another unrelated transcription factor, nuclear factor (NF)-E2-related factor 1. To effectively search for the NRF-1 motif, a matrix was constructed based on known NRF-1 binding sequences identified by Evans and Scarpulla (12). CRE-BP, CCAAT/enhance-binding protein; CRE-BP, C/EBP, C/EBP, CCAAT/enhance-binding protein; CRE-BP, C/EBP, CCAAT/enhance-binding protein; MBD, myogenic regulatory factor D.

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on a luminometer (Lmax; Molecular Devices) with automatic injector. Briefly, cells grown in 24-well plates were harvested in 100 μl of 1X passive lysis buffer and frozen at −80°C. To measure firefly luciferase, 100 μl of luciferase assay reagent (LARI) was added to 20 μl of room-temperature cell lysate in a white 96-well plate. Relative light units were measured for 10 s, with a 2-s premeasurement delay. To measure Renilla luciferase, 100 μl of Stop & Glow solution (Promega) were then added to the same well, and relative light units were again measured for 10 s, with a 2-s delay.

**RNA Analyses**

Cells were harvested in guanidium thiocyanate and extracted as previously described (34). Northern blot analysis was used to measure RNA levels for PGC1β, PRC, and CS. From total RNA, 200 ng previously described (34). Northern blot analysis was used to measure RNA Analyses again measured for 10 s, with a 2-s delay.

**Table 1. Primer and response element sequences used in the construction of plasmids**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>AAAACGCGTGCTTTGCAATGGCCTTTATAAGC</td>
<td>MluI</td>
</tr>
<tr>
<td>R1</td>
<td>TTTTTTGAGGCGGCGGCGATCTCCGCGGAGC</td>
<td>XhoI</td>
</tr>
<tr>
<td>F2</td>
<td>ATGCCAAGGCTTCCATAAGGCGGACGCAGCCATTGCGG</td>
<td>MluI</td>
</tr>
<tr>
<td>F3</td>
<td>ATGCCAAGGCTTCCGAGAGACTCCCTGGAGAGGCGGCGGCTCGGTTCCGGGCAATGGTTCCGGGCTCCC</td>
<td>Apal</td>
</tr>
</tbody>
</table>

Restriction sites are underlined.

Table 2. Primers for real-time PCR or cDNA probe construction (5’ to 3’)

<table>
<thead>
<tr>
<th>Gene (Accession No.)</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRF-1 (NM010938)</td>
<td>GGAGCACTTACTGAGGATGCCC</td>
<td>CTGGCGGATTAGCTGGTGCTT</td>
</tr>
<tr>
<td>NRF-2x (AY419393)</td>
<td>GGAGAACAAGACAGGGAAAAAA</td>
<td>CGCTTTAGACGCTGCTAGCTT</td>
</tr>
<tr>
<td>PGC1β (NM133249)</td>
<td>TTGTGGTCAAGGTGGTGGGAC</td>
<td>CAGAAGGTCTTGCGGGTTTG</td>
</tr>
<tr>
<td>PPARα (NM011144)</td>
<td>TCACTATGTCAAGGCTGATAGGCTTG</td>
<td>ACTGCGCACAGTGGAGGAATC</td>
</tr>
<tr>
<td>PRC (AK122352)</td>
<td>AAGAGGCGCCATGAAAGA</td>
<td>TCTGTCGCGCTGGTTTCAC</td>
</tr>
<tr>
<td>TBP (U63993)</td>
<td>GCCGCTCTCGAAAAAGCATCTA</td>
<td>GCCGACGCGAAGCATAA</td>
</tr>
</tbody>
</table>

NRF, nuclear respiratory factor; PGC1β, peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1β; PRC, PGC1α-related coactivator; TBP, TATA binding protein.
than conducting both the drug treatment and the transient transfection simultaneously.

**Statistics**

Enzyme data and induction ratios (myocyte luciferase/myoblast luciferase) were assessed using ANOVA, followed by Dunnett’s post hoc test. Because of the variability in transfection efficiency between experiments, reporter gene data were analyzed relative to internal controls. For example, for each transcription factor reporter gene, the data for an experiment were expressed as the ratio of modified reporter relative to the minimal promoter (e.g., NRF-1-TKLuc/TKLuc). Likewise, the effects of the Sp1 mutant of the CS-757-Luc reporter were expressed as CS-757Sp1Luc/CS-757Luc from the same experiment. The internal control for CS deletion analyses was the full-length construct (CS-2113-Luc). The effects of the modified reporters were significant if the 95% confidence intervals around the mean of the ratio did not overlap with a value of 1.

**RESULTS**

**NRF-1, NRF-2, PPAR, and PGC1α Gene Family**

Luciferase reporter constructs for NRF-1, NRF-2, PPAR, and Sp1 elements were assessed in proliferating myoblasts (day 0) and differentiating myocytes (day 3). Luciferase activities were first corrected for transfection efficiency using the cotransfected Renilla plasmid. Analyses were conducted in both proliferating cells (24 h after transfection) and in differentiating cells (after 3 days of differentiation). In each case, the data are expressed relative to luciferase activity seen in cells transfected with TK-Luc.

NRF-1 and NRF-2 reporter genes displayed similar patterns with differentiation (Fig. 2A). Neither element had major effects in proliferating cells, enhancing TK-Luc expression by <50% (significant only for NRF-1). During differentiation, NRF-1 and NRF-2 elements enhanced TK-Luc expression 15-fold (similar results were seen in Sol8 cells; data not shown). The stimulation of NRF-1 and NRF-2 reporters coincided with an increase in expression of NRF-1 and NRF-2 genes, as indicated by mRNA levels (Fig. 2B).

The PPAR elements had no significant effect on TK-Luc expression in proliferating cells or in differentiated cells (Fig. 2A), in contrast to effects of NRF-1 and NRF-2. Paradoxically, the levels of PPARα mRNA rose ~10-fold by 3 days of differentiation (Fig. 2B).

We were unable to detect PGC1α using either poly(A)+ blots or real-time PCR, as has been shown in other studies on mouse myoblasts (1, 15). However, the other members of the PGC1α family were present in C2C12 cells. PRC mRNA was readily detectable via Northern blot analysis but did not change with differentiation. PGC1β mRNA was also present in cells and increased by 60% with 3 days of differentiation (Fig. 3).

**Sp1 and Citrate Synthase**

We incorporated into these analyses a complex promoter of the CS gene, which appears to lack elements for any of the transcription factors commonly implicated in coordinating mitochondrial gene expression (i.e., NRF-1, NRF-2, and PPAR).

The series of 5’ deletion constructs was used to assess the contribution of various elements in regulation of CS expression (Fig. 4). Similar results were seen in Sol8 and C2C12 myoblasts; only the C2C12 data are presented. Deletion of the region between −2113 and −757 nucleotides from the 5’ end of the promoter had little effect on the activity of the CS promoter. Deletion of the region between −757 and −559 nucleotides reduced luciferase activity by ~20%. The greatest loss in activity occurred on deletion of the elements between −559 and −254 nucleotides upstream of the translation initiation site. This deletion, which removed a Sp1 site, as well as elements for NF1, CAAT, and MyoD, reduced luciferase activity by 80% compared with the full-length promoter. Further reduction of the promoter by deleting another 31 bp to yield CS-223-Luc caused only a nominal loss of luciferase activity. Deletion of all but 163 bp upstream of the start codon removed a proximal pair of Sp1 sites and reduced reporter activity to barely detectable levels.
Overall, the pattern of expression seen with the deletion mutants in differentiated myocytes (Fig. 4B) was similar to that seen with proliferating myoblasts. Near-maximal activity was maintained with a promoter as short as 757 nucleotides. About 90% of the activity was lost when the promoter was reduced from 559 to 254 nucleotides. Another decrement in activity occurred when the promoter was shortened from 223 to 163 bp. Each of these critical regions possesses Sp1 sites, consequently, we constructed a site-specific mutant with a defective Sp1 site. The longest reporter construct (CS/H110022113Luc) was mutated to ablate the most proximal Sp1 site (5'-GGGCGG-3') leaving the second proximal Sp1 sites intact. The expression of the mutated construct (CS/H110022113Sp1Luc) was reduced in myoblasts by 39% and in myocytes by 48% (Fig. 5).

The Sp1 reporter, with multimerized Sp1 binding sites, is not a TK-based reporter. Thus expression levels cannot be directly compared with TK-Luc as was done for the NRF-1, NRF-2, or PPAR reporters. When cells transfected with the Sp1 reporter were induced to differentiate, luciferase activity increased 5.2-fold (SE = 1.3, n = 4). Thus the magnitude of the change in Sp1 reporter activity was less than that of NRF-1 and NRF-2.

**Signaling Pathways**

The activities of the mitochondrial enzymes CS and COX increased by about twofold after 3 days of differentiation, whereas CPK increased >20-fold. Thus the effects of the inhibitors are expressed in terms of how effective they were at preventing the increase in enzyme activity (Fig. 6). The LY-294002 blocked the increase in both CPK and CS to a similar extent at both 10 μM (~35–50%) and 50 μM (~90%). However, there was no effect of LY-294002 on the myogenic increase in COX. In contrast, the SB-203580 had no effect on CS activity, although it inhibited the increase in the activities of CPK and COX to a similar extent (Fig. 6).

CS reporter activity (CS-757Ren) was measured in stably transfected Sol8 cells in the presence or absence of these inhibitors (Fig. 7). Treatment with 10 μM LY-294002 reduced CS reporter activity by 50%, whereas 25 μM SB-203580 had no effect (Fig. 7), consistent with the enzyme analyses.
DISCUSSION

During mitochondrial biogenesis, hundreds of nuclear genes as well as the mitochondrial genome must be regulated in a coordinated manner. In many contexts, coactivators from the PGC1α/H925 gene family play central roles as master regulators of transcription, acting through a network of DNA-binding proteins, including NRF-1, NRF-2, and PPARs.

NRFs, PPARs, and the PGC1α Family

As in previous studies (1, 15), we found no evidence of a significant expression of PGC1α/H925 in either myoblasts or myocytes. However, both of the other family members were present. PRC has been shown to coactivate NRF-1 (1, 15), and PGC1α/H925 can interact with NRF-1 and PPARγ/H925 (27, 29).

Less is known about the role of the other PGC1α/H925 family members in mediating adaptive changes in mitochondrial content. In this study, the levels of PGC1α/H925 increased by 60% during myogenesis (Fig. 3). PGC1α/H925 has been shown to increase during differentiation of brown fat (27). The third family member, PRC, was also abundant, although the mRNA levels were similar in both myoblasts and myocytes. Thus, both PRC and PGC1α/H925 may functionally replace PGC1α/H925 in these cells, but only PGC1α/H925 mRNA changes in parallel with nuclear-encoded mitochondrial genes, as indicated by the pattern in CS mRNA.

The role of PPARs in control of mitochondrial gene expression is best established with the enzymes of fatty acid oxidation (4, 17). Although PPARα mRNA increased several-fold, there was no corresponding increase in PPAR reporter gene activity. Furthermore, PPAR elements cloned upstream of a minimal promoter did not enhance the reporter gene expression in either myoblasts or myocytes. The same plasmid construct (courtesy of D. Kelly) is a faithful reporter gene in other contexts (15); therefore, we conclude that the discrepancy between mRNA changes and reporter gene expression is related to other factors. First, on the basis of real-time PCR, PPARα mRNA levels are <5% of NRF-1 and NRF-2 mRNA levels and may be too low to yield a significant effect (PPARα protein levels were not

Fig. 5. Site-directed mutant of CS reporter gene. A mutant promoter was constructed with the most proximal Sp1 site ablated by a double point mutation. Sol8 cells were transfected and data are expressed in both myoblasts and myocytes, relative to the wild-type promoter. TK activities are included for reference. *Significantly different (P = 0.05).

Fig. 6. Mitochondrial and muscle enzyme changes in response to inhibition of phosphatidylinositol 3-kinase (PI3K) or p38 MAPK inhibition. The enzymatic activities of CS, cytochrome oxidase (COX), and creatine phosphokinase (CPK) were assessed in cells that had been treated for 3 days with vehicle (DMSO) or inhibitors of phosphatidylinositol 3-kinase (PI3K; LY-294002) or p38 MAPK (SB-203580). The data are expressed as the relative increase in activity, where a value of 1 is the increase observed in untreated cells and a value of 0 corresponds to the activity in myoblasts. Thus a value of 0.5 implies that only 50% of the normal increase in enzyme activity was observed. Actual mean values (in nmol·min⁻¹·mg protein⁻¹) for enzyme activity in myoblasts vs. myocytes were as follows: COX, 75 vs. 176; CS, 140 vs. 245; CPK 45 vs. 987. *Significantly different from control (P = 0.05).

Fig. 7. CS reporter gene activity in response to inhibition of PI3K or p38 MAPK inhibition. To construct stable transfectants, the CS promoter was subcloned into the Renilla-based reporter plasmid to construct (CS-757Ren). This construct was transfected in Sol 8 cells in combination with pPuro. After 10 days of selection, stable transfectants were differentiated and treated with vehicle or inhibitors. Data are expressed relative to untreated myoblasts or myoblasts. *Significantly different (P = 0.05) from the untreated cells.
measured). Second, PPARs rely upon ligands for activation, and though cells were grown in the presence of 2% horse serum, there may not have been enough natural ligand (e.g., fatty acids) to activate the existing receptors. Third, the low levels of the PPAR coactivator PGC1α may limit PPAR reporter activity. Although PGC1β was present, PPARα coactivation by PGC1β is strongly ligand dependent (29). This could explain why the PPAR reporter gene activity was unchanged despite an increase in PPARα synthesis, as indicated by transcript levels. PPAR regulation is very complex, with each of the gene family members exhibiting distinct kinetic properties, such as DNA binding and coactivator affinity (42).

NRF-1 and NRF-2 are believed to be key regulators of mitochondrial gene expression (39, 40). In this study, NRF-1 and NRF-2 elements enhanced the activity of a minimal promoter. In myoblasts, there was a minor increase in reporter gene activity, significant only for NRF-1. However, in myocytes, NRF-1 and NRF-2 reporter genes were both strongly induced (Fig. 2). These increases in activity coincided with an increase in the mRNA levels for both NRF-1 and NRF-2 (subunit α). The difference in the magnitude of the changes in reporter gene activity vs. mRNA levels may reflect posttranscriptional effects. There may be an increase in translation of existing mRNA. There may also be an increase in protein activity (e.g., DNA binding activity). For example, posttranslational modifications can alter the activity of NRF-1 (16) and NRF-2 (31, 43).

CS Gene Expression

Most studies of mitochondrial gene expression focus on enzymes of oxidative phosphorylation and fatty acid oxidation and have established roles for the regulation of mitochondrial genes by NRFs and PPARs. CS is a common marker of mitochondrial content, yet little is known of its regulation. In our study, 3 days of differentiation increased CS enzyme activity by 70% (Fig. 6) and mRNA by about 60% (Fig. 3). Likewise, the CS reporter gene demonstrated a marked increase in expression after 3 days of differentiation. The difference in the magnitude of the increase between endogenous gene products (CS mRNA and protein) and reporter gene (luciferase activity) can be attributed to two factors. First, a reporter gene in a transient transfection remains as naked DNA, with its promoter readily accessible to regulatory factors. In contrast, the context of the endogenous promoter within chromatin limits the accessibility of the regulatory elements to DNA binding proteins. Second, although the half-life of CS protein is unknown, it is likely much longer than the half-life of luciferase (3.5 h for the luciferase encoded by pGL2), and thus a longer period of elevated expression of the endogenous gene is needed to achieve an elevation in protein levels.

Given the importance of NRF-1 and NRF-2 in regulation of the genes of oxidative phosphorylation (12, 45), we were surprised to find no evidence of elements for either transcription factor in CS promoters of human or mouse. It is possible that NRF-1 and/or NRF-2 sites exist within the promoter but escaped detection. The 5′-flanking region of the rat gene for mitochondrial transcription factor also lacks typical NRF-1 binding motifs, yet responds to increased NRF-1 expression and binds NRF-1 in gel mobility shift assays (8). Whereas NRF-1 and/or NRF-2 regulate COX genes (39, 40), the data herein argue against a hidden role in myogenic induction of CS. Conditions that mediate a NRF-1-dependent upregulation of the cytochrome c gene do not appear to affect CS activity (18).

Further support for independent pathways of regulation of CS and COX can be found in our kinase inhibitor studies (Figs. 6 and 7). The myogenic increase in COX was curtailed by SB-203580, an inhibitor of p38-MAPK, the known targets of which include PPARα (5) and PGC1α (22). Conversely, SB-203580 had no effect on the normal increase in CS catalytic activity or CS reporter gene activity in differentiation. In contrast, LY-294002 had no effect on COX activity, yet it curtailed the normal increases in the CS catalytic activity (Fig. 6) and CS reporter gene activity (Fig. 7). It is important to acknowledge that these inhibitors can have effects on other signaling pathways. Whereas SB-203580 is fairly specific for p38-MAPK (11), LY-294002 is less specific for PI3K and can exert effects on other kinases, including mammalian target of rapamycin (7), casein kinase 2, and phosphorylase kinase (11). Regardless of the actual targets in our study, it was clear that the two inhibitors had distinct effects on CS and COX, reinforcing the conclusion that these mitochondrial genes are differentially regulated during myogenesis.

A variety of putative transcription factor binding sites are present in the 2113 nucleotide 5′ flanking region of the CS gene (Fig. 1), suggesting that regulation of this enzyme may involve the interaction of both cell-specific factors (i.e., myogenic factors such as MyoD via an E-box motif) and ubiquitous regulatory signals via Sp1, CAAT box, and NF1 sites. Sequential 5′ deletions of the CS promoter-reporter construct point to a subset of these putative transcription factor binding sites that might contribute to CS expression under differentiating conditions. The proximal 757 nucleotides upstream of the CS gene were sufficient to drive maximal expression of a luciferase reporter in both myoblasts and differentiating myotubes (Fig. 3). The primary decrease in basal promoter activity occurs upon deletion of the region from −559 to −254 bp. The main decrease in inducibility also occurs when this region is deleted. Subsequent studies implicated Sp1 sites, which occur in this critical region of promoter. First, the Sp1 reporter gene increased activity about fivefold during differentiation. Second, major decreases in CS promoter activity occur when regions possessing Sp1 sites are deleted. Third, specific mutation of one of three proximal Sp1 sites of the 2113-bp promoter caused a 40% reduction in luciferase expression.

While the potential role for Sp1 sites is clear, the exact mechanism of action is less certain. The action of Sp1 depends on the number of Sp1 sites and their context within the promoter. Many genes, including CS (Fig. 1), possess multiple Sp1 sites arranged in small clusters, as in the rat mitochondrial transcription factor A promoter (8), the rat cytochrome c promoter (12), and the rat calmodulin promoter (33). The context can alter the response of different Sp1-sensitive promoters, such that a given stimulus can enhance transcription at one Sp1 site and repress it at another (6, 35, 47). Sp1 sites are regulated through a large family of Sp1 site-recognizing proteins with varied expression (6). For example, an antagonistic transcription factor of the Sp1 family, Sp3, is expressed in at least three variants. The inhibitory variants act by interfering with DNA binding by other Sp1 family members (20, 21) and through intrinsic repressor activity (38). The DNA binding and
transactivation activity of Sp1 can also be influenced by post-translational modifications, such as phosphorylation (2, 6, 23) and glycosylation (19).

In general, the complexity of the mode of action of the members of the Sp1 family makes it difficult to predict their transcriptional effects. Several studies have characterized the pattern of change in the levels of Sp1 family members during myogenesis. Sp1 levels increase within the first 12 h of myogenesis (44), although Sp1 levels decline later in myotube formation (14, 44). Sp3 expression is upregulated early in IGF-stimulated myocytes (14), peaking as Sp1 expression returns to baseline levels (~24 h postdifferentiation). Whereas nuclear extracts appear to possess less Sp1 as myogenesis progresses (25), the profile of Sp1 family members is not known. The results from our inhibitor studies suggest that CS is regulated by a PI3K-dependent pathway, and it is possible that these effects are exerted via Sp1.

**Perspectives**

Collectively, these data provide some caveats about the omnipotence of PGC1α as a master regulator of mitochondrial biogenesis in all systems. First, there is clearly a role for the other PGC1α family members. Second, despite a central role for the coactivators, the DNA binding proteins themselves are also critical components of the transcriptional pathways. These transcription factors are subject to complex posttranscriptional, translational, and posttranslational regulation.

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