Involvement of the p38 Mitogen-activated Protein Kinase α , β , and γ Isoforms in Myogenic Differentiation

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We and others previously showed that p38 mitogen-activated protein kinase is indispensable for myogenic differentiation. However, it is less clear which of the four p38 isoforms in the mouse genome participates in this process. Using C2C12 myogenic cells as a model, we showed here that p38 α , β , and γ are expressed with distinct expression patterns during differentiation. Knockdown of any of them by small interfering RNA inhibits myogenic differentiation, which suggests that the functions of the three p38 isoforms are not completely redundant. To further elucidate the unique role of each p38 isoform in myogenic differentiation, we individually knocked down one p38 isoform at a time in C2C12 cells, and we compared the whole-genome gene expression profiles by microarrays. We found that some genes are coregulated by all three p38 isoforms, whereas others are uniquely regulated by one particular p38 isoform. Furthermore, several novel p38 target genes (i.e., *E2F2*, *cyclin D3*, and *WISP1*) are found to be required for *myogenin* expression, which provides a molecular basis to explain why different p38 isoforms are required for myogenic differentiation.

INTRODUCTION

Myogenic differentiation is one of the best models to study the molecular mechanisms by which the cellular programs switch from proliferation to differentiation. This is largely due to our extensive knowledge of key myogenic factors and the availability of both cell culture and animal models. Two families of transcription factors, namely, the myogenic regulatory factors (MRF) and myocyte enhancer binding factor 2s (MEF2s), act cooperatively as master transcription factors to control the expression of an array of cellular genes that collectively contribute to the initiation of the differentiation program and maintenance of the differentiated state (Molkentin and Olson, 1996; Puri and Sartorelli, 2000). MRFs consist of four basic helix-loop-helix (bHLH) proteins (i.e., Myf5, MyoD, myogenin, and MRF4), each heterodimerizing with E proteins (E12 or E47) to efficiently bind to the E box (i.e., CANNTG) in promoters of many muscle-specific genes (Tapscott, 2005). MEF2s consist of four MADS-box-containing proteins (i.e., MEF2A, 2B, 2C, and 2D) that are capable of forming both homo- and heterodimers to bind to a consensus AT-rich sequence (i.e., the MEF2 site) (Black and Olson, 1998).

Both MRFs and MEF2 are in turn regulated by distinct intracellular signaling pathways. We and others previously showed that the p38 mitogen-activated protein kinase (MAPK)-mediated signaling pathway is indispensable for myogenic differentiation (Cuenda and Cohen, 1999; Zetser *et al.*, 1999; Wu *et al.*, 2000b). Accumulating evidence indicates that p38 MAPK can regulate myogenic differentiation

through multiple mechanisms. p38 MAPK directly phosphorylates MEF2 and enhances its transcriptional activity (Han *et al.*, 1997; Zhao *et al.*, 1999; Wu *et al.*, 2000a). p38 MAPK also potentially enhances the transcriptional activity of MyoD, which is probably due to an increased association of MyoD with E47, because the latter can be directly phosphorylated by p38 MAPK (Wu *et al.*, 2000b; Lluís *et al.*, 2005). Through MyoD and MEF2, which directly bind to the *myogenin* promoter, p38 MAPK also critically controls the expression of *myogenin* gene, which is absolutely essential for execution of the differentiation program (Xu *et al.*, 2002). Recently, p38 MAPK was also found to directly phosphorylate BAF60, a component in the SWI/SNF complex, and to target the complex to selected muscle-specific loci (Simone *et al.*, 2004). p38 MAPK also facilitates the recruitment of MyoD and MEF2D to selected promoters of muscle-specific genes (Penn *et al.*, 2004). In addition, p38 MAPK can promote activation of the quiescent muscle satellite cells, and it is also required for proliferation of myoblasts (Jones *et al.*, 2005).

Four p38 isoforms, namely, p38 α , β , γ , and δ , exist in both the human and mouse genomes (Lluís *et al.*, 2005; Keren *et al.*, 2006). So far, many studies on the role of p38 MAPK in myogenic differentiation have relied on the use of SB203580 and SB202190, two pyridinyl imidazole-based small-molecule inhibitors that mainly inhibit p38 α and β , but they have no effect on p38 γ and δ (Kumar *et al.*, 1997). As both SB203580 and SB202190 potentially inhibit myogenic differentiation (Cuenda and Cohen, 1999; Zetser *et al.*, 1999; Wu *et al.*, 2000b), it suggests that p38 γ and δ do not play any significant role in myogenic differentiation. In contrast, a previous report showed that p38 γ is required for myogenic differentiation, because overexpression of a wild-type p38 γ promotes, whereas that of a dominant-negative form of p38 γ inhibits myogenic differentiation in C2C12 cells (Lechner *et al.*, 1996). It remains unclear whether p38 δ plays any role in

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myogenic differentiation. To clarify the confusion about the role of p38 γ and to clearly understand the role of each p38 isoform in myogenic differentiation, we decide to take the short interfering RNA (siRNA) approach by individually knocking down each p38 isoform followed by assessment of the knockdown effect on myogenic differentiation. Except for p38 δ , which is barely detectable in C2C12 cells, we found that p38 α , β , and γ are all required for myogenic differentiation. Microarray analysis reveals that some genes are uniquely controlled by a particular p38 isoform, whereas others are coregulated by multiple p38 isoforms. Importantly, several novel p38 target genes were indeed found to be involved in myogenic differentiation, which provides a molecular basis to explain why different p38 MAPKs are required for myogenic differentiation.

MATERIALS AND METHODS

Cell Culture and Antibodies

C2C12 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 20% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (growth medium, or GM) in a humidified incubator at 37°C with 5% CO₂. To induce differentiation, near confluent C2C12 cells were grown in DMEM supplemented with 2% horse serum (differentiation medium, or DM). Antibodies used in this work were from the following sources: anti-sarcomeric myosin heavy chain (MHC) (MF20) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); anti-p38 α (Cell Signaling Technology, Danvers, MA); anti-p38 β (Zymed Laboratories, South San Francisco, CA); and cyclin D3 (C-16), E2F2 (C-20), anti-myogenin (F5D), anti- β -actin (C-2), and anti-hemagglutinin (HA) (Santa Cruz Biotechnology, Santa Cruz, CA). The polyclonal anti-p38 γ was a kind gift from Dr. M. A. Bogoyevitch (University of Western Australia).

DNA Constructs, Transfection, and Cell Lysis

Expression vectors encoding Flag-p38 α , Flag-p38 β , and Flag-p38 γ were kindly provided by Dr. J. Han (Scripps Research Institute, San Diego, CA). G133-luc, 4RE-luc, 3MEF2-luc, and HA-MKK6EE were described previously (Wu *et al.*, 2000b; Xu *et al.*, 2002). Flag-Wnt-induced secreted protein 1 (WISP1) was amplified from C2C12-derived cDNAs by polymerase chain reaction (PCR), and it was cloned into pcDNA3.0 vector. Transient transfection was performed by using LipofectAMINE/PLUS reagents following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells were lysed in the lysis buffer (50 mM HEPES, pH 7.6, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 100 mM NaF, 20 mM bis(2-chloroethyl)ammonium salt, 20 mM β -glycerol phosphate, 2 mM dithiothreitol, 50 μ M sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, 0.5 μ g/ml leupeptin, and 0.7 μ g/ml pepstatin) for 10 min on ice. Soluble whole cell extracts (WCEs) were prepared by centrifugation to remove cell debris.

RNA Interference (RNAi)

The following siRNAs (only the top-strand sequence is shown) were synthesized at either Dharmacon RNA Technologies (Lafayette, CO) or RIBOBIO (Guangzhou, China): enhanced green fluorescent protein (5'-GCT GAC CCT GAA GTT CAT C-3'); p38 α (1#: 5'-AGC CCA GCA ACC TAG CTG T-3'; 2#: 5'-GAG CCT GAC CTA TGA TGA A-3'); p38 β (1#: 5'-TGC TGG TAC TAG ACA CGC A-3'; 2#: 5'-ATT GAG CAG TGA GGC ATT G-3'); p38 γ (1#: 5'-GCA TGA GAC CCT GAG TGA A-3'; 2#: 5'-CCT GTT CTT CGG CTT TCG A-3'); E2F2 (1#: 5'-GCA CCT GAC CGA AGA TAA T-3'; 2#: 5'-GTG ACC TCT TCG ACT CCT A-3'); cyclin D3 (1#: 5'-CCA TTC ACC TGT AGC

TTG A-3'; 2#: 5'-CTG CTT AGC TTC TGT GAT T-3'); and WISP1 (1#: 5'-CGG CAG GTC CTA TGG ATT A-3'; 2#: 5'-CCA CTA GAG GAA ACG ACT A-3'). For siRNA transfection, 40–60% confluent C2C12 cells were transfected with 100 nM siRNA by LipofectAMINE 2000 following the manufacturer's instructions. Four to 6 h later, the siRNA-liposome mixtures were removed, and fresh GM was added into cell culture plates. Generally, 24 h after transfection, GM was replaced with DM to induce myogenic differentiation.

Reporter Assays

For reporter assays, 50–70% confluent C2C12 cells in 12-well dishes were cotransfected with 0.5 μ g of reporter plasmids and 100 nM siRNA together by LipofectAMINE 2000. All the samples were prepared in duplicate or triplicate. Luciferase activity was determined with a LB9507 luminometer (Berthold Technologies, Bad Wilbad, Germany) by adding 10 μ l of WCE to 150 μ l of freshly made luciferase buffer [0.4 μ M luciferin, 13.3 mM ATP, 0.1 M Tris-HCl, pH 7.8, 1 mM EDTA, pH 8.0, and 10 mM Mg(OAc)₂]. Luciferase units were normalized against total protein amount present in each sample determined by Protein Assay Reagent (Bio-Rad, Hercules, CA).

Immunostaining

Cells were first fixed in 4% paraformaldehyde for 15 min, and then they were permeabilized in 0.2% Triton X-100 for 15 min and blocked in 5% bovine serum albumin/phosphate-buffered saline (PBS) for 1 h. Cells were then incubated with the mouse anti-MHC antibody overnight, washed three times with PBS, and reincubated with the rhodamine-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Then, 100 ng/ml 4,6-diamidino-2-phenylindole (DAPI) was then added for another 10 min. Fluorescence microscopy was performed using an Olympus IX70 microscope linked to a charge-coupled device digital camera (Spot RT; Diagnostic Instruments, Sterling Heights, MI).

Gene Expression Analysis by Microarrays

Duplicate C2C12 cells were transfected separately with siRNAs targeting p38 α , p38 β , p38 γ , or green fluorescent protein (GFP) (control). Twenty-four hours after transfection, cells were switched to DM for another 18 h before harvest. Total RNA was extracted using TRIzol reagent following the manufacturer's suggestions. The GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA) were used for hybridization, which provide 45,000 probe sets to analyze the expression levels of >39,000 transcripts and variants of mouse genes. The raw data were normalized and analyzed using dChip (www.dchip.org) (Li and Hung Wong, 2001). To obtain p38 isoform-specific genes, the combined comparisons were carried out. For example, the combined comparison (control vs. p38 α and not control vs. p38 β and not control vs. p38 γ) was used to identify genes uniquely regulated by p38 α with the criteria of group mean fold change >1.5, absolute group mean differences >20, and $p \leq 0.05$ for testing equal group means using Student's t test. To identify genes controlled by all three p38 isoforms, we carried out the combined comparison (i.e., control vs. p38 α AND control vs. p38 β AND control vs. p38 γ) by using the same criteria as mentioned above.

Semiquantitative Reverse Transcription (RT)-PCR and SYBR Green-based Quantitative (q)RT-PCR

RT-PCR was performed by a two-step method. Briefly, cDNA was generated from 0.5 μ g of total RNA by ImProm-II reverse transcription system (Promega, Madison, WI) with oligo(dT)₁₅ as a primer according to the manufacturer's instruction. PCR was performed in a reaction of 25 μ l containing 40 ng of cDNA. PCR products were analyzed by 2% agarose gel electrophoresis. For qRT-PCR, 2 $\times$ SYBR Green Supermix from Bio-Rad (Hercules, CA) was added to 25 μ l of PCR reactions according to the manufacturer's instructions. Triplicate samples were subjected to qPCR using a Stratagene Mx3000P real-time PCR system with the maximum cycle number of 40. GAPDH was used as an internal control. The relative abundance of genes of interest was calculated

Figure 1. Expression profiles of p38 isoforms in mature muscles and C2C12 cells. (A) Detection of different p38 isoforms in selected mouse tissues by semiquantitative RT-PCR analysis. GAPDH was used as a loading control. (B) 293T cells were separately transfected with plasmids encoding Flag-tagged p38 α , β , γ , and δ . Different Flag-p38 isoforms were immunoprecipitated (IP) from cell extracts with the anti-Flag antibody, and the immunoprecipitates were subjected to SDS-polyacryl-

amide gel electrophoresis (PAGE) and examined by Western blotting (WB) by using isoform-specific p38 antibodies. (C) C2C12 cells were let to grow and differentiate in cell culture. Cells were harvested at different time points as indicated, and WCEs were subjected to SDS-PAGE followed by Western blotting analysis.

after normalized to *GAPDH*. Three independent batches of RNA samples were used for qRT-PCR analysis, and data were presented as mean $\pm$ SD and analyzed by student *t* test ($p < 0.05$ was considered statistically significant). Primer pairs used were as follows: myogenin (forward: 5'-GAC TCC CCA CTC CCC ATT CAC ATA-3'; reverse: 5'-GGC GGC AGC TTT ACA AAC AAC ACA-3'); *GAPDH* (forward: 5'-CCC ACT CTT CCA CCT TCG-3'; reverse: 5'-TCC TTG GAG GCC ATG TAG GCC AT-3'); *p38 α* (forward: 5'-GGA GGG ACC TTC TCA TAG AT-3'; reverse: 5'-GAG GGA TAG CCT CAG ACC-3'); *p38 β* (forward: 5'-CTG CAA GGA AAG GCC CTC-3'; reverse: 5'-CAG GCA ATG CCT CAC TGC-3'); *p38 γ* (forward: 5'-GAT TAC TGG GAA GAT CCT G-3'; reverse: 5'-CGT CAC AGA GCC GTC TCC-3'); *p38 δ* (forward: 5'-GAC ACT CTT CAA GGG CAA G-3'; reverse: 5'-GCC ATC AAT CAC TGC AGC-3'); *E2F2* (forward: 5'-GGT TCC TGT GGT CAG GAG-3'; reverse: 5'-CAG TTC CTG AGG GTG AAC-3'); and *WISP1* (forward: 5'-GTC CAG GAC TTC ACA ATT GAG C-3'; reverse: 5'-CCA GGC TTT GCT TCC ATT G-3').

RESULTS

p38 α , β , and γ Are Expressed in Both Mature Muscles and C2C12 Myogenic Cells and Display Distinct Expression Profiles during Myogenic Differentiation

To explore the roles of p38 isoforms in myogenic differentiation, we first examined the mRNA expression patterns of four p38 isoforms in mouse muscle tissues by RT-PCR. The mouse brain, liver, and kidney tissues were used as controls. In agreement with previous reports, *p38 α* and β were abundantly expressed in both muscles and many other tissues, whereas *p38 γ* was preferentially expressed in muscles (Figure 1A) (Lechner *et al.*, 1996). In contrast, *p38 δ* was barely detectable in muscles, even though it was abundantly expressed in kidney tissues (Figure 1A) (Hu *et al.*, 1999). Similarly, we found that *p38 α , β , and γ* , but not δ , were also expressed in C2C12 myogenic cells as judged by RT-PCR (data not shown). To examine the protein levels and expression patterns of p38 isoforms during C2C12 differentiation, we first characterized isoform-specific p38 antibodies. As shown in Figure 1B, each p38 isoform-specific antibody was indeed highly specific for that particular isoform and did not cross-react with other p38 isoforms. Using these isoform-specific antibodies, we found that, during C2C12 differentiation, the levels of *p38 α* remained unchanged and the levels of *p38 γ* gradually increased, whereas the levels of *p38 β* gradually decreased (Figure 1C). Myogenin expression was used here as a marker to monitor the progression of differentiation, whereas β -actin levels were used as a loading control.

Knockdown of Any of the Three p38 Isoforms Inhibits C2C12 Differentiation

Since early 1990s, pyridinyl imidazole-based p38 inhibitors (e.g., SB203580 or SB202190) have been very popular research tools to implicate p38 MAPK in various biological processes, including myogenic differentiation (Cuenda and Cohen, 1999; Zetser *et al.*, 1999; Wu *et al.*, 2000b). However, there are several obvious drawbacks with these inhibitors: first, they only inhibit *p38 α* and β but not γ and δ (Kumar *et al.*, 1997); and second, they may inhibit cellular targets other than p38 MAPK, especially at higher concentrations (Davies *et al.*, 2000; Lali *et al.*, 2000). To reveal a role for each p38 isoform in C2C12 differentiation without resorting to p38 inhibitors, we used the siRNA-based RNAi approach. To reduce the "off-target" effect, we used different siRNAs for each p38 isoform. After siRNA transfection, cells were then allowed to differentiate for various times. As shown in Figure 2A, our siRNAs were very specific and effective in knocking down specific p38 isoforms. Importantly, compared with cells transfected with GFP-siRNA, cells transfected with two different sets of siRNAs against either *p38 α* , β , or γ were all defective in differentiation as evidenced by reduced expression of myogenin and myosin heavy chain

Figure 2. *p38 α , β , and γ* are all required for C2C12 myogenic differentiation. (A) C2C12 cells were separately transfected with siRNAs targeting either GFP (control) or different *p38* isoforms. After growing in GM for 24 h, cells were induced to differentiate in DM for another 24 or 48 h (for detection of MHC only). Cell extracts were harvested and subjected to Western blot analysis. 1# and 2# denote two different siRNAs against each *p38* isoform. (B) After differentiating in DM for 48 h, C2C12 cells were fixed and immunostained with an anti-MHC antibody (MF20). Cell nuclei were counterstained with DAPI. The fusion index was calculated as the ratio of the number of MHC-positive cells with two or more nuclei over that of DAPI-positive nuclei in three randomly chosen fields. The results are presented as mean $\pm$ SD. Phase, phase-contrast images. (C) C2C12 cells were cotransfected with various siRNAs as indicated together with 4xRE-luc or 3xMEF2-luc. After growing in GM for 24 h, cells were switched to DM for another 24 h before harvest followed by luciferase assays. All experiments were done in triplicate, and the results are shown as mean $\pm$ SD. Fold change was the ratio of the luciferase activity in cells transfected with p38-siRNAs over that with GFP-siRNA.

(Figure 2A) and a drastically reduced number of multinucleated myotubes (Figure 2B). Consistently, when C2C12 cells were cotransfected with various siRNAs together with either

Table 1. Selected genes coregulated by all three p38 isoforms

Probe set ID	Gene	GenBank accession no.	Fold change		
			p38 α	p38 β	p38 γ
1450448_at	Stanniocalcin 1	BQ032752	-5.57	-3.32	-5.85
1419391_at	Myogenin	NM_031189	-3.42	-4.93	-1.88
1449178_at	PDZ and LIM domain 3	NM_016798	-4.28	-4.31	-2.57
1415927_at	Actin, α , cardiac	NM_009608	-3.71	-6.22	-2.33
1452651_a_at	Myosin, light polypeptide 1	AK003182	-3.57	-4.62	-1.81
1419606_a_at	Rroponin T1, skeletal, slow	NM_011618	-3.42	-2.79	-2.31
1415999_at	Hairy/enhancer-of-split related with YRPW motif 1	NM_010423	-3.24	-5.25	-2.18
1456242_at	Hypothetical LOC433110	AV101904	-3.13	-3.64	-2.39
1427735_a_at	Actin, α 1, skeletal muscle	M12233	-2.96	-3.94	-1.92
1417614_at	Creatine kinase, muscle	NM_007710	-2.61	-3.06	-1.98
1455203_at	RIKEN cDNA A930003A15 gene	BB522820	-1.96	-2.79	-1.89
1422340_a_at	Actin, γ 2, smooth muscle, enteric	NM_009610	-1.92	-2.25	-2.86
1418509_at	Carbonyl reductase 2	BC010758	-1.79	-1.95	-2.10
1455790_at	E2F transcription factor 2	BB543028	-1.83	-1.57	-1.66

The negative numbers in the last three columns denote fold changes in gene repression compared with the reference values of the same set of genes in control cells (i.e., cells transfected with GFP-siRNA).

Table 2. Selected genes uniquely regulated by p38 α

Probe set ID	Gene	GenBank accession no.	Fold change
1458813_at	Sodium channel, voltage-gated, type V, α	BB516098	-1.86
1418578_at	Diacylglycerol kinase, α	BC006713	-1.69
1428623_at	Plexin A1	AK011193	-1.68
1438661_a_at	ADP-ribosylation factor 2	AV023312	1.66
1457827_at	RIKEN cDNA 9330196J05 gene	BB084936	1.75
1418417_at	Musculin	NM_010827	2.44
1418105_at	Stathmin-like 4	NM_019675	4.18

The negative and positive numbers in the last column denote fold changes in gene repression and induction respectively compared with the reference values of the same set of genes in control cells.

4RE-Luc or 3xMEF2-Luc (i.e., the MRF- and MEF2-dependent luciferase reporter genes, respectively), the cells transfected with any of the three p38 isoform-specific siRNAs displayed significantly reduced reporter activities compared with those transfected with the control GFP-siRNA (Figure 2C). Our above-mentioned data showed that p38 α , β , and γ are all required for normal myogenic differentiation.

Genome-wide Search for Genes Regulated by Each p38 Isoform during Early Myogenic Differentiation

Because each of the three p38 isoforms (i.e., α , β , and γ) is required for myogenic differentiation, we hypothesized that these p38 isoforms have nonredundant roles in myogenic differentiation. To reveal downstream genes regulated by each p38 isoform, we individually knocked down one particular p38 isoform at a time in C2C12 cells and let cells differentiate for 18 h, which represents a time point during early differentiation when p38 activity is known to be induced (Wu *et al.*, 2000b). Total RNAs from duplicate samples were then extracted and subjected to microarray analysis using the GeneChip Mouse Genome 430 2.0 array (Affymetrix). By comparative and statistical analysis with the dChip software (www.dchip.org) (Li and Hung Wong, 2001), we obtained gene sets that were either induced or repressed by >1.5-fold compared with the control (i.e., cells transfected with GFP-siRNA) when one particular p38 isoform was knocked down. Some of these p38 target genes

were further confirmed by RT-PCR and the representative results were shown in Tables 1–4. From these tables, it was obvious that a subset of genes was coregulated by all three p38 isoforms as knockdown of any of the isoforms had a similar effect on these genes (Table 1). In contrast, other subsets of genes were differentially regulated by different p38 isoforms (Tables 2–4). Apparently, many p38 target genes may not play a direct role in myogenic differentiation. To understand why different p38 isoforms are required for early differentiation, we aimed to identify those p38 target genes which could contribute to early myogenic differentiation. To do so, we tried to avoid genes encoding obvious structural proteins (e.g., skeletal muscle α -actin), proteins with unknown function (e.g., RIKEN cDNA E130014H10 gene), and metabolic enzymes (e.g., carbonyl reductase 2). Instead, we mainly focused on genes encoding nuclear factors and signaling molecules. siRNAs against these p38 target genes were designed and their effects on *myogenin* induction were examined. As a result, three novel p38 target genes including *E2F2*, *cyclin D3* and *WISP1* were identified which met the criteria mentioned above. They were selected for further in-depth analysis.

E2F2 Is Coregulated by p38 α , β , and γ and Essential for Myogenin Expression

As revealed by microarray analysis (Table 1) and confirmed by both RT-PCR and qRT-PCR, *E2F2*, a member of the E2F

family (Trimarchi and Lees, 2002; Dimova and Dyson, 2005), was coregulated by p38 α , β , and γ , because individual knockdown of either p38 α , β , or γ all led to reduced expression levels of *E2F2* (Figure 3A). As SB202190 inhibits both p38 α and β , as expected, the addition of SB202190 to C2C12 cells reduced the expression levels of both *E2F2* and myogenin (Figure 3B). It was interesting to note that *E2F2* was already present at low levels before differentiation; however,

upon differentiation, both mRNA and protein levels of *E2F2* further increased (Figure 3, C and D). Based on qRT-PCR analysis, by 48 h after differentiation, the amount of *E2F2* mRNA was nearly eightfold higher than that in proliferating myoblasts (Figure 3C). Importantly, knockdown of *E2F2* by two different sets of siRNAs significantly inhibited myogenic differentiation as judged by reduced myogenin expression (Figure 3E), and a drastically reduced number of

Figure 3. *E2F2* is coregulated by p38 α , β , and γ and required for myogenic differentiation. (A) C2C12 cells were separately transfected with various p38-siRNAs and induced to differentiate in DM for 24 h. Total RNA was extracted and the *E2F2* mRNA level was analyzed by both RT-PCR (bottom) and qRT-PCR (top). (B) Near-confluent C2C12 cells were induced to differentiate for 12 and 24 h with or without SB202190 (10 μ M) (added at the time of medium change). WCEs were subjected to Western blotting analysis. + and - signs denote the presence and absence of SB202190, respectively. (C and D) Total RNA (C) and WCEs (D) were harvested from C2C12 cells at different times as indicated. The mRNA and protein levels of *E2F2* were analyzed by RT-PCR, qRT-PCR, and Western blotting, respectively. (E-G) C2C12 cells were separately transfected with *E2F2*-siRNA and GFP-siRNA, and they were induced to differentiate for various times as indicated. In E and G, WCEs were prepared and analyzed by SDS-PAGE and Western blotting. In F, cells were fixed after growing in DM for 48 h followed by immunostaining with an anti-MHC antibody (MF20). Cell nuclei were counterstained with DAPI. The calculation of fusion index and data presentation were essentially the same as described in the legend of Figure 2B. (H) C2C12 cells were cotransfected with various siRNAs as indicated together with 4xRE-luc or 3xMEF2-luc. After growing in GM for 24 h, cells were switched to DM for another 24 h before harvest followed by luciferase assays. All experiments were done in duplicate, and the results are shown as mean $\pm$ SD. Fold change was the ratio of the luciferase activity in cells transfected with *E2F2*-siRNAs over that with GFP-siRNA.

Table 3. Selected genes uniquely regulated by p38 β

Probe set ID	Gene	GenBank accession no.	Fold change
1426852_x_at	Nephroblastoma overexpressed gene	X96585	-4.26
1416164_at	Fibulin 5	NM_011812	-2.47
1415907_at	Cyclin D3	NM_007632	-2.45
1436458_at	Sema domain, transmembrane domain, and cytoplasmic domain, (semaphorin) 6A	AI606937	-2.23
1448737_at	Tetraspanin 7	AF052492	-2.12
1449315_at	Odd Oz/ten-m homologue 3 (<i>Drosophila</i>)	NM_011857	-2.1
1446554_at	Glycoprotein 6 (platelet)	BB139810	-2.1
1418012_at	Src homology (SH)3-domain GRB2-like B1 (endophilin)	BB221842	-2.02
1434944_at	Dystrophia myotonic-protein kinase	AW108486	-1.93
1448788_at	Cd200 antigen	AF004023	-1.91
1433924_at	Paternaly expressed 3	BM200248	-1.91
1419668_at	Sarcoglycan, β (dystrophin-associated glycoprotein)	AK014381	-1.87
1448398_s_at	Ribosomal protein L22	NM_009079	-1.86
1433477_at	Active BCR-related gene	AV325116	-1.81
1429459_at	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	BB499147	-1.8
1428785_at	Angiomotin-like 1	BG917015	-1.79
1457952_at	CD80 antigen	BM121947	-1.79
1448259_at	Follistatin-like 1	BI452727	-1.77
1418393_a_at	Integrin $\alpha 7$	NM_008398	-1.73
1418011_a_at	SH3-domain GRB2-like B1 (endophilin)	BB221842	-1.68
1418482_at	Cytochrome <i>b</i> -561 domain containing 2	NM_019720	1.71
1430780_a_at	Phosphomannomutase 1	BI739353	1.72
1424211_at	RIKEN cDNA 5730438N18 gene	BC011293	1.86
1450004_at	Thymic stromal lymphopoietin	NM_021367	2.16
1418741_at	Integrin $\beta 7$	NM_013566	2.24
1438405_at	Fibroblast growth factor 7	BB791906	2.7

The negative and positive numbers in the last column denote fold changes in gene repression and induction, respectively, compared with the reference values of the same set of genes in control cells.

multinucleated myotubes (Figure 3F). To understand why E2F2 is required for *myogenin* expression, we examined the effect of E2F2 knockdown on cell proliferation as well as expression and activity of several key cell cycle regulators and myogenic factors. We found that knockdown of E2F2 did not significantly affect cell cycle distribution or cell proliferation rate (Supplemental Figure). However, it increased the cyclin D1 level without affecting the expression levels of other cell cycle regulators (e.g., CDK4, Rb, p27, and p21) (Figure 3G; data not shown). In addition, knockdown of E2F2 did not affect the expression levels of MyoD and MEF2, but it significantly reduced both MRF- and MEF2-dependent gene transcription as judged by reporter assays (Figure 3H; data not shown). Our above-mentioned data indicated that E2F2 is a novel target of p38 α , β , and γ and that it mediates p38 MAPK-induced *myogenin* expression.

Cyclin D3 Is a Unique Target of p38 β and Contributes to Early Myogenic Differentiation

Our microarray data revealed that *cyclin D3* was specifically repressed by p38 β -siRNA (Table 3), which was independently confirmed by Western blotting (Figure 4A). Like E2F2, cyclin D3 was already present in proliferating myoblasts (Figure 4B). However, upon differentiation, the expression levels of cyclin D3 further increased, which correlated with increased expression of myogenin (Figure 4B). To evaluate the impact of cyclin D3 on myogenic differentiation, we designed two different cyclin D3-siRNAs. Both were effective and inhibited the expression of myogenin and MHC (Figure 4C). Similar to what was seen with E2F2,

knockdown of *cyclin D3* did not affect cell cycle distribution and cell proliferation (Supplementary Figure), yet it significantly enhanced the expression levels of cyclin D1 (Figure 4D). Unlike E2F2 which affected both MRF- and MEF2-dependent gene transcription (Figure 3H), knockdown of *cyclin D3* only affected MRF-dependent gene transcription as judged by reporter assays (Figure 4E).

WISP1 Is Specifically Regulated by p38 γ and Required for Myogenic Differentiation

Based on our microarray data, the gene encoding WISP1 was specifically repressed by p38 γ -siRNA (Table 4), which was confirmed by both RT-PCR and qRT-PCR (Figure 5A). In C2C12 cells, we found that WISP1 mRNA was already expressed in proliferating myoblasts and that its levels gradually increased during differentiation (Figure 5B). To assess the contribution of WISP1 to differentiation, we designed two different siRNAs against WISP1. When transfected into C2C12 cells, both siRNAs were able to inhibit the expression of myogenin and MHC (Figure 5C). To understand why WISP1 is required for *myogenin* induction, we examined the effect of WISP1 knockdown on cell proliferation and expression and activity of key cell cycle regulators and myogenic factors. No obvious effect was seen on cell proliferation or expression levels of several cell cycle regulators (e.g., cyclin D1, p21, p27, CDK4, and Rb). However, a significant inhibition on MRF- and MEF2-dependent gene transcription was observed (Figure 5D). Because WISP1 is a secreted protein (Brigstock, 2003), our data suggested that WISP1 could affect myogenic differentiation in an autocrine manner. To test this hypothesis, *Flag-WISP1* and an empty vector

Figure 4. Cyclin D3 is uniquely regulated by p38 β and required for myogenic differentiation. (A) C2C12 cells were transfected with various siRNAs and induced to differentiate for various times. (B) C2C12 cells were induced to differentiate for various times. (C and D) C2C12 cells were separately transfected with either GFP-siRNA or two different cyclin D3-siRNAs, then induced to differentiate for various times. WCEs from A–D were subjected to SDS-PAGE and Western blotting. (E) C2C12 cells were cotransfected with siRNAs and reporter plasmids as indicated. The measurement of the luciferase activity, calculation of the fold change and data presentation were the same as described in the legend of Figure 3F.

(control) were separately overexpressed in C2C12 cells and the conditioned medium were collected and added to C2C12 cells transfected with either GFP-siRNA or WISP1-siRNA. As shown in Figure 5E, in cells transfected with GFP-siRNA, addition of the conditioned medium from WISP1-overexpressing cells only slightly elevated myogenin expression levels (compare lanes 1 and 2). This could be due to the presence of the endogenous WISP1 secreted in the conditioned medium from the vector-transfected cells. In cells transfected with WISP1-siRNA, the myogenin expression was inhibited compared with cells transfected with GFP-siRNA (compare lanes 1 and 3), which was in agreement with the results in Figure 5C. However, addition of the conditioned medium from WISP1-overexpressing cells efficiently rescued the inhibitory effect of WISP1-siRNA on myogenin expression (compare lanes 3 and 4). Thus, our above-mentioned results indicate that WISP1 positively regulate myogenic differentiation in an autocrine manner.

DISCUSSION

All Three p38 Isoforms Are Involved in Normal Myogenic Differentiation

Since the initial findings about the indispensable role of the p38 MAPK pathway in myogenic differentiation (Cuenda

and Cohen, 1999; Zetser *et al.*, 1999; Wu *et al.*, 2000b), many research groups have carried out in-depth studies to elucidate the underlying molecular mechanisms. Because four p38 isoforms exist in the human and mouse genomes, a pertinent question to address is whether all four p38 isoforms are involved in myogenic differentiation. Using C2C12 cells as a model, we show here that three p38 isoforms (i.e., α , β , and γ) are expressed in C2C12 cells and that all three are required for C2C12 differentiation. A recent report by Perdiguero *et al.* (2007) also addressed the same question using primary myoblasts isolated from mice deficient in one of the four p38 isoforms. Although both their studies and ours agree that p38 α is required for myogenic differentiation, their findings differ from ours in that they found that myoblasts deficient in either p38 β or γ proliferate and differentiate normally compared with the wild-type counterparts (Perdiguero *et al.*, 2007). It is likely that there is an active compensatory mechanism in animals deficient in either p38 β or γ , which may mask the normal physiological effects of p38 β and γ . In this regard, it would be very informative to reveal the levels and activities of the remaining p38 isoforms in primary myoblasts deficient in a particular p38 isoform. Apparently, such a compensatory mechanism does not efficiently operate in C2C12 cells, because the expression levels of the remaining p38 isoforms in C2C12 cells did not change significantly when one particular p38 isoform was transiently knocked down (Figure 2A). We do not think that the results from our siRNA approach are artifacts, because different siRNAs targeting distinct regions of either p38 β or γ displayed similar effects. In addition, our results about the involvement of p38 γ in myogenic differentiation were consistent with a previous report (Lechner *et al.*, 1996). Furthermore, our whole-genome gene expression profiling clearly revealed that there are indeed subsets of genes uniquely controlled by either p38 β or γ . Importantly, some of these p38 β - or γ -controlled genes (e.g., *cyclin D3* and *WISP1*) are required for myogenic differentiation. Thus, we think that the difference between our study and that by Perdiguero *et al.* (2007) is due to different approaches used and that the two approaches are complementary to each other.

Identification of Novel p38 Target Genes Involved in Myogenic Differentiation

Many novel p38 target genes have been identified through our genome-wide gene expression profiling, and some of them are found to be required for early myogenic differentiation, which provides a mechanism to explain why different p38 isoforms are required. E2F2, a member of the E2F family, is coregulated by p38 α , β , and γ . Although E2F2 is normally involved in cell proliferation in many cellular systems (Lukas *et al.*, 1996; Wu *et al.*, 2001), unexpectedly, we find that it also plays an important role during early myogenic differentiation (Figure 3, E and F). Because E2F2 is already present in proliferating myoblasts (Figure 3, C and D), and it is required for *myogenin* induction upon differentiation, it suggests that, in addition to MEF2, MyoD, and Six proteins (Xu *et al.*, 2002), E2F2 is another key mediator involved in p38 MAPK-mediated *myogenin* expression. However, it is noteworthy that these mediators are differentially regulated by p38 MAPK: whereas MEF2 serves as a direct substrate of p38 MAPK (Han *et al.*, 1997; Wu *et al.*, 2000b), E2F2 is regulated by p38 MAPK at either the transcriptional or posttranscriptional level. For MyoD and Six, it remains unclear how exactly p38 MAPK activates them. At present, we do not know whether E2F2 regulates *myogenin* expression through direct binding to the *myogenin* promoter

Table 4. Selected genes uniquely regulated by p38 γ

Probe set ID	Gene	GenBank accession no.	Fold change
1438556_a_at	Tropomodulin 3	BB224629	-3.79
1416342_at	Tenascin C	NM_011607	-3.33
1423089_at	Tropomodulin 3	AK017725	-2.57
1419149_at	Serine (or cysteine) proteinase inhibitor, clade E, member 1	NM_008871	-2.55
1437385_at	Transcribed locus	AV264768	-2.49
1451407_at	Junction adhesion molecule 4	BC004806	-2.34
1435083_at	Cortexin	BI155559	-2.27
1449133_at	Small proline-rich protein 1A	NM_009264	-2.16
1423088_at	Tropomodulin 3	AK017725	-2.1
1450171_x_at	Granzyme E	NM_010373	-2.08
1422520_at	Neurofilament 3, medium	NM_008691	-1.99
1438769_a_at	Thymocyte protein thy28	BF719766	-1.82
1416022_at	Fatty acid binding protein 5, epidermal	BC002008	-1.8
1435077_at	Additional sex combs like 1 (<i>Drosophila</i>)	BE956516	-1.76
1448593_at	WISP-1	NM_018865	-1.74
1428942_at	Metallothionein 2	AA796766	1.69
1443977_at	RIKEN cDNA E130014H10 gene	BB538930	1.94
1435264_at	Elastin microfibril interfacier 2	BB811788	1.98
1451191_at	Cellular retinoic acid binding protein II	BC018397	2.37
1435504_at	Restin-like 2	BM217861	2.38
1428662_a_at	Homeobox only domain	AK009007	2.59
1439630_x_at	Suprabasin	AI844734	2.62
1417932_at	Interleukin 18	NM_008360	2.94
1452031_at	Solute carrier family 1 (glial high-affinity glutamate transporter), member 3	BB357585	3.08

The negative and positive numbers in the last column denote fold changes in gene repression and induction respectively compared with the reference values of the same set of genes in control cells.

or through other intermediate molecules that in turn bind to the *myogenin* promoter. Our initial analysis suggests that E2F2 is required for MRF- and MEF2-dependent gene transcription. In addition, E2F2 may facilitate cell cycle exit by promoting cyclin D1 down-regulation, because knockdown of E2F2 enhances cyclin D1 levels.

Unlike E2F2, *cyclin D3* is uniquely regulated by p38 β during myogenic differentiation. Consistently, the involvement of cyclin D3 in myogenic differentiation has been recognized in several previous reports (Kiess *et al.*, 1995; Rao and Kohtz, 1995; Cenciarelli *et al.*, 1999). Unlike cyclin D1, which is rapidly down-regulated upon differentiation, cyclin D3 levels gradually increase and peak in postmitotic myotubes (Figure 4B). Unphosphorylated Rb is found to interact with and stabilize cyclin D3 protein, which in turn forms a multiprotein complex containing inactive CDK2 and CDK4, p21, and proliferating cell nuclear antigen (Kiess *et al.*, 1995; Cenciarelli *et al.*, 1999). Such a complex is thought to contribute to irreversible cell cycle exit and maintenance of the differentiated state. In addition to these previous findings, our current study shows for the first time that cyclin D3 is required for *myogenin* induction. We suggest that cyclin D3 may facilitate cell cycle exit by promoting cyclin D1 down-regulation, because knockdown of *cyclin D3* prominently up-regulates cyclin D1 levels (Figure 4D). Furthermore, we show that cyclin D3 is required for MRF-dependent gene transcription. Because *cyclin D3* is a transcription target of MyoD (Cenciarelli *et al.*, 1999), this suggests the existence of a positive regulatory loop between MyoD and cyclin D3. It remains unclear how exactly cyclin D3 exerts its effect on cyclin D1- and MRF-dependent gene transcription.

WISP1, a known target gene induced by Wnt-1 (Pennica *et al.*, 1998; Xu *et al.*, 2000), is a member of the CCN family

proteins that consists of Cysteine-rich 61 (CCN1), connective tissue growth factors (CTGF or CCN2), nephroblastoma overexpressed (Nov or CCN3), WISP1 (CCN4), WISP2 (CCN5), and WISP3 (CCN6) (Brigstock, 2003). The receptors for members of the CCN family remain poorly characterized. In several cases, certain integrins are thought to be the receptors for CCN1, CCN2, and CCN3 (Brigstock, 2003). We show here that WISP1 is specifically induced by p38 γ during myogenic differentiation (Figure 5). As a secreted molecule, we show that WISP1 can regulate *myogenin* expression in an autocrine manner (Figure 6). Furthermore, WISP1 enhances both MRF- and MEF2-dependent gene transcription. Interestingly, CCN3, another member of the CCN family, is found to be specifically induced by p38 β (Table 3). However, the functional significance of CCN3 induction by p38 β remains unclear, because knockdown of CCN3 does not affect *myogenin* expression (data not shown). It also remains unclear how WISP1 affects *myogenin* expression. Because WISP1 is also induced by Wnt-1, it may serve as a converging point to integrate signals from both the p38 γ pathway and the Wnt pathway.

In summary, our current study demonstrates that different p38 isoforms can regulate myogenic differentiation through both common and unique target genes. It is less likely that perturbation of any one of these target genes would completely recapitulate the effect caused by that of a particular p38 isoform. However, an important point we try to make here is that different p38 isoforms may exert certain nonredundant effects on myogenic differentiation through distinct target genes. To understand precisely how different p38 isoforms contribute to myogenic differentiation, it is essential that we first understand how these

Figure 5. WISP1 is uniquely regulated by p38 γ and required for myogenic differentiation. (A and B) Total RNA was isolated from C2C12 cells either transfected with various siRNAs (A) or left untreated and induced to differentiate for various times (B). The expression of *WISP1* mRNA was analyzed by qRT-PCR (top) and RT-PCR (bottom). (C) C2C12 cells were separately transfected with various siRNAs as indicated. 24 h after transfection, cells were induced to differentiate in DM for another 18 or 36 h (for detection of MHC) before harvest. (D) C2C12 cells were cotransfected with various siRNAs together with reporter plasmids as indicated. After growing in GM for 24 h, cells were switched to DM for another 24 h before harvest followed by luciferase assays. All experiments were done in duplicate, and the results are shown as mean $\pm$ SD. (E) C2C12 cells were transfected with GFP-siRNA or WISP1-siRNA. Twenty-four hours after transfection, the culture media were replaced with conditioned media collected from C2C12 cells transfected with either an empty vector or *Flag-WISP1*. Cells were induced to differentiate in conditioned media for another 24 h before harvest. WCEs from C and E were subjected to SDS-PAGE and Western blotting.

Figure 6. Schematic on how different p38 isoforms regulate *myogenin* induction through distinct target genes. Three p38 isoforms can regulate *myogenin* expression through both common target genes (e.g., *E2F2*) and unique isoform-specific target genes (e.g., *cyclin D3* and *WISP1*). Unlike *E2F2* and *cyclin D3*, *WISP1* participates in *myogenin* regulation in an autocrine manner. G0, G1, G2, S, and M denote different phases of the cell cycle.

different p38 target genes function during myogenic differentiation.

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