Phosphorylation of MRF4 transactivation domain by p38 mediates repression of specific myogenic genes

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Skeletal myogenesis is associated with the activation of four muscle regulatory factors (MRFs): Myf5, MyoD, Myogenin and MRF4. Here we report that p38 mitogen-activated protein kinase represses the transcriptional activity of MRF4 (involved in late stages of myogenesis), resulting in downregulation of specific muscle genes. MRF4 is phosphorylated *in vitro* and *in vivo* by p38 on two serines (Ser31 and Ser42) located in the N-terminal transactivation domain, resulting in reduced MRF4-mediated transcriptional activity. In contrast, nonphosphorylatable MRF4 mutants display increased transcriptional activity and are able to advance both myoblast fusion and differentiation. We also show that expression of desmin and α -actin, but not muscle creatin kinase, decreased at late stages of muscle differentiation, correlating with the induction of MRF4 and p38 activation. Accordingly, inhibition of p38 during late myogenesis results in the upregulation of both desmin and α -actin. We propose that repression of MRF4 activity by p38 phosphorylation may represent a new mechanism for the silencing of specific muscle genes at the terminal stages of muscle differentiation.

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Introduction

The development of skeletal muscle is an ordered multistep process requiring the sequential activation of two groups of myogenic transcription factors. The first group includes the myogenic regulatory factors (MRFs), which belong to the basic helix–loop–helix (bHLH) protein family. This MRF family consists of four members: Myf5, MyoD, Myogenin

and MRF4, all of which are exclusively expressed in skeletal muscle. One of the unique properties of the MRFs is that their ectopic expression in nonmuscle cells can initiate the myogenic program and convert nonmuscle cells into myogenic derivatives (Tapscott *et al*, 1988; Rhodes and Konieczny, 1989). The MRF proteins contain one or two transactivation domains (at N- and C-termini), a conserved basic DNA-binding domain essential for sequence-specific DNA binding, and an HLH motif required for heterodimerization (Davis *et al*, 1990; Olson and Klein, 1994). Each of the MRFs has been shown to heterodimerize *in vitro* and *in vivo* with E proteins (like the E2A gene products E12 and E47) and to bind DNA in a sequence-specific manner at sites known as E-boxes (CANNTG) (Lassar and Munsterberg, 1994; Arnold and Winter, 1998). This leads to the transcriptional activation of muscle-specific genes, such as α -actin, muscle creatin kinase (MCK), troponin I (TnI), α 7 integrin or desmin, and eventually to the production of mature muscle fibers (Yutzey *et al*, 1990; Muscat *et al*, 1992; Li and Capetanaki, 1993; Ziober and Kramer, 1996). The second group of transcription factors important in myogenesis is composed of four different myocyte enhancer binding factor 2 (MEF2) proteins (A–D), which belong to the MADS box family, and bind to a consensus A/T-rich sequence found in the promoters of many muscle-specific genes. Muscle-specific gene transcription driven by the MRFs can be stimulated by MEF2 family members through interactions mediated by the basic region and the MADS domain, respectively (Molkentin *et al*, 1995; Black and Olson, 1998).

Because all four MRFs bind the same DNA sequence *in vitro*, it has been difficult to ascertain experimentally if they possess identical or distinct activities. MRF4 and MyoD contain N-terminal activation domains, yet MRF4 is considered a weak inducer of the expression of many muscle-specific genes despite its ability to bind E-box sequences (Braun *et al*, 1990; Mak *et al*, 1992; Schwarz *et al*, 1992; Moss *et al*, 1996). In this regard, when N-terminal MyoD and MRF4 sequences were exchanged in transfection experiments, any construct containing the MRF4 N-terminus was less capable of transactivating than those containing MyoD N-terminal sequences, demonstrating that the MRF4 N-terminal transactivation domain is unique (Moss *et al*, 1996). Consequently, the differential activities of the MRFs may be one of the mechanisms whereby diverse myogenic phenotypes are achieved. However, little is known about the cellular factors that could differently regulate the activities of myogenic transcription factors, especially those that are involved in receiving and transducing extracellular cues. Different intracellular signaling pathways have been implicated in the regulation of muscle differentiation. In particular, the p38 mitogen-activated protein kinase (MAPK) pathway is a critical regulator of this process. p38 activity increases during muscle differentiation (Cuenda and Cohen, 1999;

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Zetser *et al*, 1999; Wu *et al*, 2000), and it was shown to phosphorylate and increase the transcriptional activity of specific MEF2 isoforms (Han *et al*, 1997; Zetser *et al*, 1999; Zhao *et al*, 1999), providing a potential explanation for the promyogenic effect of p38. In addition, recent studies showed that p38 stimulates MyoD activity through an unknown indirect mechanism (Wu *et al*, 2000). To our knowledge, there is no evidence for a direct effect of p38 on the transcriptional activity of the MRFs.

In this study, we demonstrate that MRF4 is a novel substrate for p38 MAPK *in vitro* and *in vivo*, and that N-terminal Ser31 and Ser42 of MRF4 are key regulatory sites phosphorylated by p38. The transactivation potential of MRF4 was decreased by p38 phosphorylation, resulting in a reduced expression of specific muscle genes, while MRF4 mutated at Ser31 and Ser42 was resistant to downregulation by p38 and displayed increased myogenic potential. Moreover, inhibition of p38 activity at late stages of muscle differentiation resulted in the induction of specific genes, suggesting a novel inhibitory function for p38 in late myogenesis. In summary, this study demonstrates that p38 MAPK can directly phosphorylate a muscle regulatory transcription factor of the MRF family, resulting in downregulation of muscle-specific gene expression. Our results also suggest a potential mechanism for the specific silencing of muscle genes at the terminal stages of muscle differentiation.

Results

p38 MAPK activation accelerates skeletal muscle differentiation

Previous studies have shown that p38 MAPK is necessary for muscle differentiation (Cuenda and Cohen, 1999; Zetser *et al*, 1999; Wu *et al*, 2000). First, we confirmed that p38 MAPK was activated in C2C12 myoblasts undergoing differentiation. As shown in Figure 1A, p38 activity was elevated within 1 day of placing myoblasts in differentiation medium (DM), and continued to increase for at least 4 days. Myogenin expression was induced in C2C12 cells after 1 day in DM (Figure 1A), while after 3–4 days most myoblasts had formed well-differentiated myotubes (data not shown). If p38 contributes causally to myogenic differentiation, its constitutive activation should be sufficient for advancing or even inducing myoblast differentiation. For this purpose, we generated C2C12 cell lines that stably expressed the constitutive active form of MKK6, MKK6(b)E (from now on referred to as MKK6), and C2C12 cell lines expressing an empty vector. Both types of cells were cultured in growth medium (GM) and p38 activity was determined by immunoblotting using the anti-phospho-p38 antibody. As expected, p38 was activated in C2/MKK6 cells but not in control C2/vector cells (Figure 1B). C2/MKK6 cells, but not C2/vector cells, exhibited a pronounced induction of Myogenin levels in Western blotting analysis, even in proliferating conditions, which was strongly reduced by SB203580 treatment (Figure 1B, bottom), in agreement with previous reports (Wu *et al*, 2000). To investigate whether MRF4, the latest differentiation-induced MRF, also presented an advanced expression in response to activated p38, we performed RT-PCR analysis of those cells after 2 days in DM. As shown in Figure 1C, the expression of MRF4 was greatly induced in C2/MKK6 myocytes, with respect to C2/vector cells, in a p38-dependent manner,

Figure 1 Effect of p38 activity on myogenic differentiation. (A) p38 phosphorylation is induced during myoblast differentiation. C2C12 myoblasts were cultured in GM and then shifted to DM for 1, 2 and 4 days. Activation of p38 was determined using a specific anti-phospho-p38 antibody (P-p38). An anti-p38 antibody was used to confirm that the amount of p38 protein was unchanged in all lanes. C2C12 myogenic differentiation was confirmed by reprobing the membrane with an antibody against the early muscle differentiation marker Myogenin. (B) MKK6-expressing C2C12 cells show constitutive p38 phosphorylation. C2C12 cells stably transfected with either empty vector (C2/vector) or pcDNA3-MKK6 (C2/MKK6) were cultured in GM in the presence or absence of SB203580. p38 activation was analyzed using an anti-P-p38 and anti-p38 antibodies, respectively. (C) Constitutive p38 activity advances myogenic differentiation. The advanced expression of myogenic markers was analyzed by reprobing the membrane with an anti-Myogenin antibody (bottom panel in (B)), or by RT-PCR using MRF4- and GAPDH-specific primers. (D) Low-power views of representative fields. Magnifications are $\times 100$.

since this induction could be inhibited by SB203580 cell treatment. Myotube formation was also advanced in C2/MKK6-expressing cells in GM with respect to C2/vector cells, in a SB203580-dependent manner (Figure 1D). Altogether, these results indicate that p38 activation is sufficient for triggering myogenic differentiation and for advancing terminal muscle-specific gene expression, in addition to promoting myoblast fusion, suggesting a potential relationship between both events.

Different effect of activated p38 on MyoD and MRF4 transcriptional activities

Previous studies have shown that p38 modifies the transcriptional activity of MEF2 myogenic factors by phosphorylation (Han *et al*, 1997; Zhao *et al*, 1999). Here, we analyzed the effect of activated p38 on the transcriptional activity of the early-appearing MRF, MyoD, and the late-appearing MRF, MRF4. Accordingly, 10T1/2 fibroblast cells were transiently cotransfected with the promoter/reporter plasmid

p4RE-tk-Luc and MRF4 or MyoD expression plasmids, in the absence or presence of MKK6. After 48 h in differentiation-inducing medium, cell extracts were prepared and analyzed for luciferase activity. As shown in Figure 2B, MKK6 overexpression resulted in an increase in MyoD-mediated luciferase activity of p4RE-tk-Luc, in agreement with previous reports (Puri *et al*, 2000), in a SB203580-dependent manner. In contrast, MKK6 did not affect the MRF4-mediated activation of the E-box-dependent reporter plasmid (Figure 2A). Next, we analyzed whether the differential effect of p38 on the activity of these two MRFs was ascribed specifically to the heterologous E-box-tk promoter, or whether it could also be observed with wild-type myogenic promoters. Thus, similar transfection experiments were performed using cardiac α -actin and TnI promoters (245 and 2300 bp, respectively), which can be activated by MRF4 and MyoD (Chakraborty and Olson, 1991; Banerjee-Basu and Buonanno, 1993). Overexpression of MKK6 increased MyoD-dependent α -actin promoter activity but had no effect on TnI promoter activity (compare Figure 2D and 2F); in contrast, overexpression of

MKK6 decreased MRF4-dependent activity of both α -actin and TnI promoters (compare Figure 2C and E). Treatment of cells with SB203580 reversed these effects. Altogether, this indicates that during myogenesis p38 modifies differently the activity of MyoD and MRF4, in a promoter-specific manner.

p38 binds to and phosphorylates MRF4 *in vitro*

To determine whether the effect of p38 on MRF4 activity was direct or indirect, we performed *in vitro* binding and phosphorylation experiments. First, to analyze whether p38 and MRF4 were able to interact *in vitro*, recombinant GST-MRF4 bound to glutathione sepharose was incubated with 293T cell extracts. Immunoblotting revealed the binding of p38 to GST-MRF4-sepharose, but not to GST-sepharose (Figure 3A). Moreover, MRF4 was found to bind UV-treated 293T cells to a similar extent (data not shown), suggesting that phosphorylation of p38 is not necessary for MRF4 binding. Next, GST-MRF4 and GST-ATF2 fusion proteins (the latter being a well-known p38 substrate), and GST alone (negative control), were incubated with (γ - 32 P)ATP and with purified

Figure 2 Differential effect of MKK6-activated p38 on MRF4 and MyoD transcriptional activities. 10T1/2 fibroblasts were transiently transfected with p4RE-tk-Luc (A, B), p α -Actin-Luc (C, D) and pTnI-Luc (E, F) respectively, together with myc-tagged MRF4 (A, C and E) and MyoD (B, D and F), with either an empty vector (–) or an MKK6 expression vector, and in the presence or absence of SB203580. Luciferase activities are expressed relative to the activity found for each MRF expression plasmid, which were given a value of 100. Error bars represent the standard error of the mean value. Ectopic expression of MyoD and MRF4 in transfection assays was comparable, as detected by Western blotting using an anti-Myc (9E10) antibody (data not shown).

Figure 3 p38 binds to and phosphorylates MRF4 *in vitro*. (A) p38 MAPK interacts with MRF4 *in vitro*. 293T cell lysates were incubated with the indicated GST-fusion proteins bound to glutathione-sepharose beads. Total cellular proteins (lysate) and bound cellular proteins were analyzed using an anti-p38 antibody. The lysate lane contains 1/10th of the input used in incubations. Ponceau staining showed that comparable amounts of GST proteins were used in the pull-down assay. (B) p38 phosphorylates MRF4 *in vitro*. Increasing amounts of purified GST-MRF4, GST-ATF2 and GST were incubated with 200 ng of activated p38. (C) MRF4 is phosphorylated by p38 *in vitro*. In all, 200 ng of purified GST-MRF4 and GST-ATF2 were incubated with increasing amounts of MKK6-activated p38. All incubations were performed in the presence of γ -(32 P)ATP, and labeled proteins were separated by SDS-PAGE and detected by autoradiography. Coomassie staining showed that equal amounts of substrate proteins were used in the *in vitro* phosphorylation assays.

MKK6-activated p38 α (the major p38 isoform in most cell types). Proteins were resolved by SDS-PAGE and phosphorylated proteins were visualized by autoradiography. As shown in Figure 3B, GST protein alone was not phosphorylated by p38, whereas GST-MRF4 exhibited intense labeling, which augmented as increasing amounts of the fusion protein were used. The levels of GST-MRF4 labeling were comparable to those of GST-ATF2 (positive control). To analyze whether MRF4 phosphorylation depends on p38 concentration, we performed the same experiment using a constant amount of GST-MRF4 or GST-ATF2 and increasing amounts of activated p38. Figure 3C shows that both MRF4 and ATF2 were phosphorylated by p38 in a kinase concentration-dependent manner, even at the lower concentrations of kinase used in the assay. Altogether, these results clearly demonstrate the ability of p38 to bind and phosphorylate MRF4 *in vitro*.

Identification of p38 MAPK phosphorylation sites within MRF4

Analysis of the MRF4 primary amino-acid sequence of five different mammalian species revealed two conserved MAPK phosphorylation consensus sites (SP/TP) (Figure 4A). Both sites corresponded to N-terminal Ser31 and Ser42 located within the transcription activation domain of rat MRF4. We examined whether these potential p38 phosphorylation sites were responsible for MRF4 phosphorylation by p38 *in vitro*. For this purpose, Ser31 and Ser42 were mutated to alanine in the GST-MRF4 fusion protein. Purified wild-type GST-MRF4, GST-MRF4 containing individual mutations and GST-MRF4 containing double mutations were incubated with MKK6-activated p38. As shown in Figure 4B, mutation of just one serine to alanine (S31/A or S42/A) reduced the phosphorylation level of MRF4 by p38, while double mutation of both serines (S31/42A) abolished p38-mediated phosphorylation of MRF4 *in vitro*. Furthermore, Figure 4C shows p38

phosphorylation of a GST-MRF4 fragment of 11 kDa (obtained after trypsin digestion), containing Ser31 and Ser42, but not of the corresponding fragment of double-mutated GST-MRF4-S31/42. Therefore, MRF4 contains two p38 phosphorylation sites, Ser31 and Ser42, within its N-terminal transactivation domain.

MRF4 is phosphorylated by MKK6-activated p38 *in vivo*

To determine whether the interaction of MRF4 with p38 also occurs *in vivo*, we constructed Myc-tagged versions of MRF4 since, to the best of our knowledge, no good anti-MRF4-specific antibody is currently available. Myc-MRF4, Myc-MRF4-S31/42A and p38 were coexpressed in 293T cells, Myc-tagged proteins were immunoprecipitated using the 9E10 antibody and the immunoprecipitates were probed with an anti-p38 antibody. As shown in Figure 5A, p38 was found associated to both wild-type and mutated MRF4, suggesting that the putative docking site for p38 is not abolished in MRF4-S31/42A. We next investigated whether MRF4 protein was phosphorylated by p38 *in vivo*. Accordingly, 293T cells were transfected with expression vectors for Myc-MRF4 or Myc-MRF4-S31/42A, with or without an expression vector for MKK6. Proteins were metabolically labeled with (32 P) orthophosphate and Myc-tagged MRF4 proteins were immunoprecipitated and resolved by SDS-PAGE, followed by autoradiography. As shown in Figure 5B, phosphorylation of MRF4 was increased by over-expression of MKK6, while MRF4-S31/42A remained unaffected. Phosphorylation of MRF4 by p38 was confirmed by treatment of cells with SB203580, which resulted in a significant reduction of wild-type MRF4 labeling in the presence of MKK6 (data not shown). Taken together, these results demonstrate that Ser31 and Ser42 are targets for phosphorylation by p38 *in vivo*.

Figure 4 Mutation of N-terminal Ser31 and Ser42 abolishes phosphorylation of MRF4 by p38. (A) Identification of SP residues in N-terminal MRF4 amino-acid sequences. Comparison of the N-terminal amino-acid sequences of MRF4 of five different species. The numbers above the sequence indicate the amino-acid position. Common amino-acid consensus sequences of p38 (SP) are in boldface. (B) Substitution of Ser31 and Ser42 by alanines abolishes MRF4 phosphorylation by MKK6-activated p38. Ser31 and Ser42 were either individually or doubly mutated to alanine on pGEX-MRF4 plasmid. The indicated GST-fusion proteins were incubated with activated p38 in the presence of γ -(32 P)ATP, separated by SDS-PAGE and detected by autoradiography. (C) p38 phosphorylation analysis of GST-MRF4 and GST-MRF4-S31/42A followed by trypsin digestion. Predicted positions of cleavage sites after trypsin digestion are indicated with arrows. GST-MRF4 and GST-MRF4-S31/42A were incubated with activated p38 in the presence of γ -(32 P)ATP, digested with trypsin, separated by SDS-PAGE and detected by autoradiography. Coomassie staining confirmed equal loading of the different MRF4 proteins (B, C).

Figure 5 p38 MAPK binds to and phosphorylates MRF4 *in vivo*. (A) p38 interacts with MRF4 and MRF4-S31/42A *in vivo*. 293T cells were transfected with pCS2-MRF4 or pCS2-MRF4-S31/42A, and with either an empty vector (–) or pcDNA3-HA-p38 and subjected to coimmunoprecipitation analysis. p38 was readily detected by Western blotting after immunoprecipitation of MRF4 using an anti-Myc (9E10) antibody. (B) Phosphorylation of MRF4 protein in 293T cells expressing activated p38. 293T cells were transfected with pCS2-MRF4 or pCS2-MRF4-S31/42A, together with either an empty vector (–) or pcDNA3-MKK6 and metabolically labeled with (32 P) orthophosphate. Myc-tagged proteins were immunoprecipitated from cell extracts, separated using SDS-PAGE and blotted. Phosphorylated proteins were visualized by autoradiography. (C) Phosphorylation of MRF4 protein by differentiation-induced p38 in C2C12 cells. C2C12 cells stably transfected with either empty vector or pCS2-MRF4 or pCS2-MRF4-S31/42A were cultured in DM for 2 days before labeling with (32 P) orthophosphate, in the presence or absence of SB203580 (right). Myc-tagged proteins were immunoprecipitated as in (B). The left panel shows immunoblotting with an anti-Myc antibody to confirm equal expression of Myc-tagged proteins. (D) C2C12 cells expressing Myc-tagged MRF4 were cultured for 1, 2 and 4 days in DM before labeling with (32 P) orthophosphate and analyzed as in (B). In (A), (B) and (D), the lower panel shows immunoblotting of the membrane with anti-Myc and/or anti-MKK6 antibodies to confirm an equal expression of transfected plasmids in all lanes.

MRF4 is phosphorylated at Ser31 and Ser42 by differentiation-induced p38 MAPK during C2C12 myogenesis

Our next aim was to investigate whether MRF4 is phosphorylated by p38 during C2C12 myoblast differentiation. Given the above-mentioned unavailability of a good specific anti-MRF4 antibody, and since MRF4 is expressed at low levels in C2C12 cells (see Figure 1C), we generated C2C12 cell lines stably expressing comparable levels of Myc-MRF4 and Myc-MRF4-S31/42A, respectively (Figure 5C, left). The different C2C12 cells were cultured for 48 h in DM with or without SB203580, and metabolically labeled with (32 P) orthophosphate. Only the wild-type form of MRF4, but not MRF4-S31/

Figure 6 Influence of MRF4 Ser31 and Ser42 phosphorylation by p38 on α -actin and TnI promoter activities. 10T1/2 cells were transiently transfected with p α -Actin-Luc (A) and pTnI-Luc (B), respectively, together with Myc-tagged MRF4 and MRF4-S31/42A, and with either an empty vector (–) or a MKK6 expression vector. Luciferase activities are expressed relative to the activity found for each MRF expression plasmid, which was given a value of 100. Error bars represent the standard error of the mean value. Ectopic expression of MRF4 and MRF4-S31/42A was comparable, as detected by Western blotting using an anti-Myc (9E10) antibody (data not shown).

42A, was phosphate labeled in C2C12 myocytes after 2 days in DM, and this labeling was abrogated in the presence of SB203580 (Figure 5C). Moreover, phosphorylation of MRF4 increased time-dependently during C2C12 differentiation in DM (Figure 5D). These results demonstrate that MRF4 protein is phosphorylated by p38 at Ser31 and Ser42 during C2C12 myoblast differentiation.

Ser31 and Ser42 are regulatory sites in MRF4-mediated muscle-specific gene transcription

As shown in Figure 2, activation of p38 inhibited the myogenic activity of MRF4. To determine the potential connection between this event and the phosphorylation of MRF4 at Ser31 and Ser42 by p38, we performed muscle-specific promoter/luciferase reporter assays, with different versions of MRF4. 10T1/2 fibroblasts were cotransfected with α -actin or TnI promoter/reporter plasmids, respectively, together with expression plasmids for MRF4 or MRF4-S31/42A, with or without MKK6. In the absence of MKK6, MRF4-S31/42A had a similar or even higher transcriptional activity than wild-type MRF4 on both promoters, suggesting that Ser31 and Ser42 are not involved in basal MRF4 myogenic activity (Figure 6A and B). Coexpression of MKK6, however, resulted in a 50% reduction of wild-type MRF4 transcriptional activity, but it had a negligible effect on the activity of double-mutated

MRF4 on both myogenic promoters (Figure 6A and B). These data indicate that phosphorylation of Ser31 and Ser42 by p38 can regulate MRF4-mediated muscle-specific transcription.

The N-terminal transactivation domain of MRF4 is responsible for the p38-mediated inhibition of transcription

All four MRFs contain at least an N-terminal transactivating (TA) domain flanking the bHLH domain, which is responsible for dimerization and DNA binding (revised in Olson, 1990). The TA domain of rat MRF4 was mapped between N-terminal amino acids 1 and 47 (Mak *et al*, 1992). Given that Ser31 and Ser42 are within this domain, we hypothesized that phosphorylation of MRF4 by p38 would be affecting its transactivating potential rather than dimerization or DNA binding. To confirm this hypothesis, we constructed DNA plasmids containing the GAL4 DNA-binding domain fused to the N-terminal domain of either wild-type MRF4 or double-mutated MRF4, and we transfected them in 10T1/2 cells, in the absence or presence of MKK6, using the GAL4-Luc plasmid as a reporter. As shown in Figure 7A, MKK6 overexpression resulted in a decrease of GAL4-MRF4-dependent luciferase activity, but did not affect significantly the activity dependent on GAL4-MRF4-S31/42A, in 10T1/2 cells. As a control, overexpression of MKK6 was shown to stimulate GAL4-MyoD-dependent luciferase expression (Figure 7B). This suggested

Figure 7 The N-terminal transactivation domain of MRF4 mediates p38-induced transcriptional repression. 10T1/2 cells were transiently transfected with pGAL4-MRF4, pGAL4-MRF4-S31/42A (A) and pGAL4-MyoD (B) together with an empty vector (–) or an MKK6 expression vector. pGAL4-DBD was transfected as a control for basal activity. Luciferase activities are expressed relative to the activity obtained after pGAL4-MRF4 and pGAL4-MyoD transfection, which were given a value of 100. Ectopic expression of GAL4-MRFs was comparable, as detected by Western blotting using an anti-GAL4 antibody (data not shown). Error bars represent the standard error of the mean value.

that the N-terminus of MRF4 was responsible and sufficient for the inhibition of MRF4 transcriptional activity by p38. Altogether, these results demonstrate that the inhibition of MRF4 transcriptional activity by p38 is conferred by phosphorylation of Ser31 and Ser42 located within the N-terminal TA domain.

Inhibition of MRF4 activity by activated p38 affects the endogenous expression of muscle-specific genes

MRFs have the unique property of activating muscle-specific genes in nonmuscle cells (Braun *et al*, 1989; Edmonson and Olson, 1989; Rhodes and Konieczny, 1989). Since ectopic expression of MKK6 could inhibit the activity of MRF4 on the α -actin promoter (see Figure 2), we investigated whether the overexpression of MKK6 could affect the endogenous expression of muscle-specific genes, such as α -actin and desmin genes, in 10T1/2 fibroblast cells expressing MRF4. For this purpose, 10T1/2 cells were transfected with expression plasmids for Myc-MRF4 or Myc-MRF4-S31/42A, or Myc-MyoD (used as control), with or without MKK6, and immunocytochemical analyses were performed using antibodies against α -actin, desmin and Myc (for MRF4 and MyoD), respectively. Cells double positive for Myc and α -actin, and Myc and desmin were counted and representative areas of each transfection plate are shown (Figure 8E). Ratios of α -actin/Myc and desmin/Myc represented the number of transfected cells expressing α -actin or desmin. As shown in Figure 8B and D, cotransfection of MKK6 and Myc-MyoD in 10T1/2 fibroblasts resulted in a higher number of α -actin- and desmin-positive cells, suggesting a positive effect of MKK6 on these two MyoD regulated genes. However, the number of α -actin- or desmin-positive cells upon transfection of Myc-MRF4 in 10T1/2 cells was reduced in the presence of MKK6 (Figure 8A and 8C). In contrast, the number of α -actin- or desmin-positive cells due to Myc-MRF4-S31/42A transfection was not decreased by overexpression of MKK6 (Figure 8A and 8C), suggesting that activated p38 plays an inhibitory role on MRF4-dependent muscle-specific gene expression *in vivo*. Taken together, these results indicate that p38 MAPK regulates differently MyoD and MRF4 transcriptional activities during myogenic differentiation. Moreover, the repressive action of p38 on MRF4 myogenic activity appears to be direct.

Myoblast fusion and differentiation are advanced in MRF4-S31/42A-expressing cells

If wild-type MRF4 phosphorylation by p38 contributes negatively to myogenic differentiation, it is conceivable that constitutive overexpression of the nonphosphorylatable mutant of MRF4 should be sufficient for advancing or even inducing myogenesis. For this purpose, we generated 10T1/2 cell lines stably expressing wild-type MRF4 and MRF4-S31/42A, in comparable levels (Figure 9A). Both cell lines were cultured in DM and myoblast differentiation was analyzed. As shown in Figure 9B, the number of cells expressing the differentiation marker myosin heavy chain (MHC) at 1 and 3 days of differentiation was higher in 10T1/2-MRF4-S31/42A cells. Next, we analyzed desmin and α -actin mRNA levels in both cell types after 1 and 3 days in DM. As shown in Figure 9C, after 1 day in DM, desmin expression was detected in 10T1/2-MRF4-S31/42A cells but not in 10T1/2-MRF4 cells, while α -actin expression levels remained unchanged. However, after 3 days in DM, the expression of both genes

Figure 8 Influence of MRF4 Ser31 and Ser42 phosphorylation by p38 on endogenous α -Actin and Desmin expression. 10T1/2 cells were transiently transfected with Myc-tagged versions of MRF4, MRF4-S31/42A (A, C) and MyoD (B, D) with either an empty vector (–) or an MKK6 expression vector. After 2 days in DM, cells were double-stained with anti- α -Actin and anti-Myc antibodies (A and B) and anti-Desmin and anti-Myc antibodies (C and D), respectively. Representative areas of each transfection plate are shown (E). The number of positive cells for Actin or Desmin (in red) was normalized for the number of Myc-tagged positive cells corresponding to MRF4, MRF4-S31/42 and MyoD transfected cells (in green), respectively. The number of positive cells for Actin or Desmin normalized for Myc-tagged positive cells upon MRF4 or MyoD (or empty vector) transfection is considered to be 100%. Error bars represent the standard error of the mean value.

was higher in cells bearing MRF4-S31/42A than in those cells bearing wild-type MRF4. Altogether, these results indicate that myoblast differentiation was accelerated in by the non-phosphorylatable MRF4 mutant and confirm the promyogenic role of the S31/42A mutated version of MRF4 under normal myogenesis-promoting conditions.

Selective repression of muscle-specific genes by p38 MAPK: p38 inhibition induces α -actin and desmin expression at later stages during myogenesis

The results described so far strongly suggest that desmin and α -actin expression is regulated by the phosphorylation state of MRF4 in a p38-dependent manner. To investigate whether the expression of these genes was modulated during the course of myogenic differentiation, C2C12 cells were cultured in DM for 7 days, and different parameters were analyzed. As shown in Figure 10A, the expression of desmin and α -actin was high in C2C12 myocytes during the first 3 days of

Figure 9 Effect of overexpression of MRF4 and MRF4-S31/42A in 10T1/2 cells. (A) 10T1/2-MRF4 and 10T1/2-MRF4-S31/42A cells express comparable levels of MRF4. 10T1/2 cells were stably transfected with either empty vector or expression vectors of wild-type MRF4 or MRF4 mutated at Ser31 and Ser42, respectively. Total RNA from cells grown in GM was analyzed for MRF4 expression by Northern blotting. (B) Myogenic differentiation in 10T1/2-MRF4 and 10T1/2-MRF4-S31/42A cells was analyzed by MHC expression. Cells were cultured for 1 and 3 days in DM, and MHC induction was visualized by immunofluorescence analysis. Views of representative fields are shown. Magnifications are $\times 400$. (C) Cells were cultured as in (B) for 1 and 3 days in DM, and the expression of desmin and α -actin mRNA (and GAPDH, used as control) was analyzed by RT-PCR/Southern.

Figure 10 p38 inhibition induces α -actin and desmin expression at later stages during myogenesis. (A) Analysis of desmin, α -actin, MCK and MRF4 expression during myogenesis. C2C12 cells were cultured for 1, 3, 5 and 7 days in DM, and the expression of desmin, α -actin, MCK and MRF4 (and GAPDH, used as control) mRNA was analyzed by RT-PCR followed by Southern blotting using specific cDNA probes. p38 activation was analyzed using anti-P-p38 and anti-p38 antibodies, respectively. (B) Desmin and α -actin, but not MCK, mRNA levels are induced by SB203580 cell treatment at late stages of myogenic differentiation. C2C12 cells were cultured for 5 days in DM, and then treated for 5 and 15 additional hours with SB203580. The expression of the indicated mRNAs was analyzed as in (A).

differentiation (days 1 and 3), but then decreased in a time-dependent manner (days 5 and 7). The decrease in desmin and α -actin expression during myogenesis was inversely correlated with the induction of MRF4 expression as well as with the increase in p38 phosphorylation (Figure 10A). In contrast to α -actin and desmin, MCK expression increased time-dependently during myogenic differentiation. These results indicate that the induction of α -actin and desmin expression at late stages of myogenesis might be specifically mediated by p38-dependent modification of MRF4. If this were the case, inhibition of p38 activity should raise the level of these two transcripts, but not the level of MCK. To test this hypothesis, C2C12 cells cultured for 5 days in DM were treated for 5 and 15 additional hours with SB203580. As shown in Figure 10B, inhibition of p38 resulted in the upregulation of the otherwise low levels of desmin and α -actin mRNAs, reinforcing the idea that p38 activity is repressive for the expression of these genes at late stages of myogenesis. In contrast, the expression of MCK remained unaffected by SB203580 treatment, suggesting that repression of myogenesis by p38 at late stages of differentiation is selective.

Altogether, these results strongly support a mechanism through which p38 selectively inhibits the expression of a specific subset of genes during late stages of muscle differentiation via MRF4 phosphorylation.

Discussion

In this study, we provide evidence that the transcriptional activity of MRF4 is subjected to regulation by p38 MAPK during myoblast differentiation. Through a series of *in vitro* binding and phosphorylation assays using recombinant proteins, MRF4 was identified as a substrate for p38. Moreover, MRF4 could be phosphorylated during C2C12 myoblast differentiation in a p38-dependent manner. We have identified two potential sites (Ser31 and Ser42) that can be phosphorylated by this enzyme *in vitro* and *in vivo*. To test the potential involvement of these phosphoacceptor sites in MRF4 function, we constructed mutants that can no longer be phosphorylated by p38 and determined their biological activity in 10T1/2 fibroblasts. Using this approach we could demonstrate that phosphorylation of MRF4 by p38 causes decreased transcriptional activity of MRF4, resulting in downregulation of muscle-specific gene expression; in contrast, nonphosphorylatable MRF4 mutants presented increased transcriptional capacity and were able to induce the fusion and differentiation index of 10T1/2 cells. Importantly, activation of p38 did not inhibit MyoD-mediated muscle-specific gene expression in 10T1/2 cells. Therefore, we postulate that p38 can modulate specifically muscle gene expression through its ability to regulate differently the activity of critical MRFs. Furthermore, we showed that inhibition of p38 activity at late stages of C2C12 cell differentiation resulted in increased expression of α -actin and desmin genes, but not in MCK, suggesting that MRF4-mediated repression of myogenesis by p38 at late stages of differentiation is selective.

Different groups have reported that p38 is involved in the initiation of myogenic differentiation by increasing the activity of MEF2 and MyoD, through direct and indirect mechanisms, respectively (Zhang *et al*, 1990; Han *et al*, 1997; Zetser *et al*, 1999; Zhao *et al*, 1999; Wu *et al*, 2000). We propose here

that p38 may also contribute to the termination of myogenic differentiation by decreasing the activity of the late MRF, MRF4. Previous studies had shown that the MRF4 N-terminal domain is crucial for negative regulation in chick primary myotube cultures, since N-terminal MRF4 was capable of conferring repressive activity onto the MyoD or Myogenin C-terminus, in promoter/reporter transfection experiments (Moss *et al*, 1996). Ser31 and Ser42 are conserved in MRF4 proteins from mouse, human, rat, chicken and frog. The transactivation domain of rat MRF4 is located between residues 1 and 47 (Mak *et al*, 1992), and therefore includes the p38 phosphorylation sites (SP) Ser31 and Ser42. Because both phosphorylation sites lie outside of the bHLH region, we hypothesized that they were involved neither in dimerization nor in DNA binding but would rather be affecting the transactivating activity of MRF4. Accordingly, overexpression of MKK6-activated p38 resulted in reduced GAL4-luciferase expression in response to GAL4 fused to wild-type N-terminal MRF4, while GAL4 fused to mutated MRF4 (which was no longer a substrate for p38) was refractory to this effect. Importantly, myoblast fusion and differentiation were advanced in 10T1/2 fibroblast stably transfected with the non-phosphorylatable form of MRF4 with respect to cells transfected with wild-type MRF4 in low serum differentiation-promoting conditions. We propose that phosphorylation may alter the efficiency with which the transcription activation domain of MRF4 interacts with other components of the transcriptional machinery to activate target muscle genes. One mechanism through which this might occur would be a phosphorylation-induced conformational change in MRF4. Several examples of negative control of transactivation by phosphorylation have been reported such as repression of the transcription factor ADR1 by PKA (Cherry *et al*, 1989) and phosphorylation-dependent *trans*-repression of the c-fos promoter by c-Fos itself (Ofir *et al*, 1990). Interestingly, studies by (Zhou and Olson, 1994) showed that phosphorylation of Myogenin at Ser43 by an unidentified kinase diminished its transcriptional activity, similarly to the effect observed by phosphorylation of MRF4 by p38 (this study). Myogenin phosphorylation at Ser43 occurs also in an SP consensus sequence, a position corresponding to Ser42 in MRF4, suggesting that Myogenin may also be a potential target of p38. Further studies need to be performed to assess whether p38 MAPK could be the kinase phosphorylating Myogenin at Ser43. If this were the case, phosphorylation of MRFs by p38 might constitute a general mechanism for modulating MRF transactivating activity.

MRF4 is predominantly expressed in adult skeletal muscle. It has been proposed that MRF4 may have a functional role distinct from MyoD, Myf5 or Myogenin in regulating muscle genes that are not expressed in adult muscles (Moss *et al*, 1996). In line with this, cardiac α -actin is activated early during the development of embryonic skeletal and cardiac muscle (Vandekerckhove *et al*, 1986). The gene product remains highly expressed in adult cardiac muscle, yet is dramatically reduced in skeletal muscle. As reported by Moss *et al* (1996), using transient transfection assays, cardiac α -actin promoter was activated by endogenous MRFs but was inhibited in the presence of coexpressed MRF4, in differentiating myoblast cultures. It is tempting to suggest that p38 activation could have been modifying MRF4 activity in those assays. Here, we propose that the N-terminus of MRF4 is a

mediator of repressive activity. In particular, MRF4 phosphorylation by p38 at Ser31 and Ser42 resulted in specific reduction of muscle gene expression, whereas nonphosphorylatable MRF4 advanced the onset of myoblast fusion and differentiation, revealing novel regulatory roles for p38 and MRF4. Specifically, we demonstrated that the expression of desmin and α -actin genes decreases at late stages of myogenesis in an inverse correlation with the increase in MRF4 expression and the activation of p38 MAPK. Since specific p38 inhibition at late myogenesis resulted in re-induction and restoration of α -actin and desmin, but not MCK, gene expression, we propose that p38 mediates selective gene repression at late myogenic stages by modifying the phosphorylation state of MRF4. This conclusion may seem paradoxical since MRF4 is predominantly expressed in skeletal muscle, and is thus supposed to maintain muscle-specific gene transcription in adult tissue. Nevertheless, it is important to note that p38 activity, in spite of its well-accepted role during *in vitro* myogenesis, is undetectable in normal, differentiated, resting adult muscle (Aronson *et al*, 1998). Therefore, MRF4 transcriptional activity may not be reduced by p38 in adult muscle; then, its considered 'weak' activity (as derived from *in vitro* studies) may be sufficient for maintaining E-box-mediated gene transcription in terminally differentiated muscle. Additionally, the activation of the human actin promoter in skeletal muscle was shown to require the integrity of DNA-binding sites for SRF, Sp1 and MRFs (Biesiada *et al*, 1999). If this cooperative transcriptional mechanism were functioning in adult skeletal muscle, it is tempting to suggest that MRF4 activity might be significantly enhanced to sustain muscle-specific transcription in terminally differentiated adult muscle. Interestingly, skeletal muscle regeneration was found to be significantly impaired in transgenic muscle with advanced expression of MRF4, suggesting that altered timing of MRF4 expression exerts a 'negative' effect on the *de novo* myogenesis during regeneration (Pavlati *et al*, 2003), in line with this study's main conclusion. Altogether, we hypothesize that the relative ratios of MRFs and their posttranslational modifications may have fundamental roles in selecting specific muscle genes for activation and/or repression. Decreasing MRF4 activity relative to that of other myogenic factors during development such that certain genes are repressed while others remain activated may ultimately contribute to the restriction of the muscle cell phenotype.

In summary, we have shown that the MRF4 transcription factor integrates the p38 MAPK signaling pathway and muscle gene expression *in vivo*, serving to silence certain genes that need to be repressed at terminal differentiation stages. These studies demonstrate for the first time that the activity of a myogenic transcription factor belonging to the MRF family, MRF4, is regulated by direct MAPK phosphorylation. Moreover, an unexpected repressive function of p38 MAPK during late myogenic differentiation is shown.

Materials and methods

Cell culture and reagents

The C2C12, 10T1/2 and 293T cell lines were obtained from the American Type Culture Collection and cultured in GM: DMEM containing 10% FBS. To induce differentiation, GM was replaced by

DM: DMEM supplemented with 2% HS. SB203580 (Calbiochem) was used at a final concentration of 10 μ M.

Plasmid constructs

In this paper, the following plasmids were used (details are provided as Supplementary data): pEMSV-MRF4, pcDNA3-MKK6(b)E, pcDNA3-HA-p38, p4RE-tk-Luc, p α -Actin-Luc and pTnl-Luc reporter plasmids; pGEX-MRF4, pGAL4-MRF4, pGAL4-MyoD and pGAL4-Luc. pEMSV-MRF4-S31/42A, pGEX-MRF4-S31/42A and pGAL4-MRF4-S31/42A (containing the indicated substitutions of serines by alanines) plasmids were generated in our laboratory by oligonucleotide-directed mutagenesis using the Quickchange site-directed mutagenesis kit (Stratagene). Expression plasmids for myc-tagged MRF4, MRF4-S31/42A and MyoD (pCS2-MRF4, pCS2-MRF4-S31/42A and pCS2-MyoD, respectively) were generated by standard PCR techniques.

Transfections

10T1/2 and 293T cells were transiently transfected with the different plasmids by the standard calcium phosphate precipitation method. Details of the procedure are described in the Supplementary data. For the generation of stably transfected C2C12 and 10T1/2 cell lines, cells were transfected with pcDNA3-Neo and either pcDNA3-MKK6 or empty pcDNA3, and either pCS2-MRF4, pCS2-MRF4-S31/42A or empty pCS2 expression vectors for C2C12 cells and either pEMSV-MRF4, pEMSV-MRF4-S31/42A or empty pEMSV expression vectors for 10T1/2 cells. Pools of G418 resistant colonies were isolated after 15 days of selection with 400 μ g/ml G418 in culture medium.

Immunostaining

Immunostaining was performed as described in the Supplementary data. Antibodies' dilutions: anti-Desmin, 1:500 (clone DE-1-10; Sigma); anti-Actin, 1:10 (muscle-specific clone MSA06; Neomarkers); anti-Myc, 1:50 (sc-789; Santa Cruz Biotechnology); and anti-MHC (clone MFZO), 1:50 (Developmental Studies Hybridoma Bank).

RNA analysis

In all, 10 μ g of total RNA was subjected to Northern analysis, as previously described (Munoz-Canoves *et al*, 1997). For reverse transcription-polymerase chain reaction (RT-PCR) analysis, 5 μ g total RNA was reverse transcribed using the first-strand complementary DNA synthesis kit (Pharmacia). DNA primers and details of the procedure are described in the Supplementary data. PCR products were run on agarose gels, transferred to nylon membranes and hybridized with the corresponding (32 P)DNA probes, in order to compare the amount of specific product generated in each sample.

Western blot analysis

The cell lysates or immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membranes. Antibody dilutions: anti-p38 MAPK, 1:1000 (sc-535; Santa Cruz Biotechnology); antiphosphop38 MAPK Thr180/Tyr182, 1:1000 (Cell Signaling); anti-Myogenin (clone F5D), 1:100; and anti-Myc (clone 9E10), 1:100 (Developmental Studies Hybridoma Bank).

Purification of GST-fusion proteins and pull-down assays

pGEX, pGEX-MRF4, pGEX-MRF4-S31A, pGEX-MRF4-S42A, pGEX-MRF4-S31/42A and pGEX-ATF2 fusion proteins were expressed in *Escherichia coli* BL21 (DE3) and purified using glutathione-sepharose beads (Amersham Pharmacia Biotech). Details of the procedure are described in the Supplementary data.

Immunoprecipitation and Western blotting

Cells were chilled on ice, washed twice with ice-cold PBS and lysed in ice-cold IP buffer. Soluble lysates were prepared as described in the Supplementary data. Immunoprecipitated proteins were resolved by 15% SDS-PAGE, and immunoblot analysis was performed as described above, incubating the membrane with anti-p38 antibody overnight at 4°C.

In vitro protein kinase assays

Activated p38 was incubated with purified GST-fusion proteins. Phosphorylation reactions were performed as described in the Supplementary data. For trypsin digestion assay, sequencing

grade modified trypsin (Promega) was used as instructed by the manufacturer.

In vivo protein phosphorylation assays

293T cells transiently transfected with different myc-tagged expression plasmids were labeled 36 h after transfection. Stably transfected C2C12 cells were kept in DM for 1, 2 and 4 days and then labeled with 1 mCi (32 P) orthophosphate per ml, in the absence or presence of 10 μ M SB203580. Details of the procedure are described in the Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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