Regulation of C2C12 myogenic terminal differentiation by MKK3/p38α pathway

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The signal transduction pathways connecting cell surface receptors to the activation of muscle-specific promoters and leading to myogenesis are still largely unknown. Recently, a contribution of the p38 mitogen-activated protein kinase (MAPK) pathway to this process was evoked through the use of pharmacological inhibitors. We used several mutants of the kinases composing this pathway to modulate the activity of the muscle-specific myosin light chain and myogenin promoters in C2C12 cells by transient transfections. In addition, we show for the first time, using a stable C2C12 cell line expressing a dominant-negative form of the p38 activator Dual-specificity MAPK kinase (MKK)3, that a functional p38 MAPK pathway is indeed required for terminal muscle cell differentiation. The most obvious phenotype of this cell line, besides the inhibition of the activation of p38, is its inability to undergo terminal differentiation. This phenotype is accompanied by a drastic inhibition of cell cycle and myogenesis markers such as p21, p27, MyoD, and troponin T, as well as a profound disorganization of the cytoskeleton.

mitogen-activated protein kinase; mitogen-activated protein kinase 3 dominant-negative mutant; muscle; signal- ing; cytoskeleton

During embryogenesis, skeletal muscle development depends on complex multifactorial processes. Under the influence of a number of extracellular signals, pluripotent mesodermal cells are programmed to give rise to muscle cell precursors. Such cells, called myoblasts, are able to proliferate and migrate, but they do not yet express muscle-specific genes. At precise steps during embryonic development, myoblasts begin to express early muscle-specific markers known as muscle regulatory factors (MRFs). The myf-5 gene is the first MRF activated on the eighth day of mouse embryonic development (3). Twelve hours later, transcription of the myogenin gene is turned on (33), followed by herculin (MRF-4, 9th day; Ref. 33) and MyoD (between the 10th and 11th days; Ref. 7). Expression of myf-5 is transient; it is turned off on the eleventh day. MRF-4, myogenin, and MyoD are expressed throughout the remainder of embryogenesis and in adult muscle tissue.

The myogenesis process is also marked by the inactivation of negative regulators of muscle differentiation such as the factor Id (inhibitor of differentiation), allowing the MRFs to play their role. Myoblasts then start expressing cell cycle inhibitors such as p21 (2) and p27 (11) and withdraw from the cell cycle, giving rise to differentiated muscle cells, the myocytes. At this time, these cells express late markers of differentiation, such as myosin heavy and light chains (MHC, MLC), muscle creatine kinase (MCK), and the acetylcholine receptor. Fusion of myocytes into multinucleated myotubes is the terminal step of muscle differentiation. In vitro, the major muscle differentiation steps can be reproduced with myoblastic cell lines such as the C2C12 murine myoblast cells used in this study. Once grown to confluence, these cells fuse to form myotubes when the serum concentration of the culture medium is switched from 10% to 2% over a period of 24–48 h.

The molecular mechanisms involved in the induction of myoblast differentiation and fusion are still unclear. However, recent studies based on the use of the kinase inhibitor SB-203580 implicated the stress-activated protein kinase p38 in the promotion of skeletal muscle differentiation in vitro (5, 36). The stress-activated protein kinases c-Jun NH2-terminal kinase (JNK) and p38 (20, 26) are part of the mitogen-activated protein kinase (MAPK) family, which includes extracellular signal-regulated kinase (ERK)1 and ERK2. ERKs are activated by mitogenic agents, whereas JNK and p38 are strongly activated by environmental stresses including ultraviolet light, septic, and osmotic shocks and also by proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 (9, 17). Dual-specificity MAPK kinase (MKK3, together with MKK4, MKK6, and MKK7, is one of the upstream

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activators of p38 (10). The stress kinases have been implicated in the phosphorylation of c-Jun, activating transcription factor (ATF)2, Elk-1, and myocyte-specific enhancer factor (MEF)2 transcription factors (15, 19, 37).

In this study, we demonstrate, using novel approaches, that terminal muscle cell differentiation is dependent on a functional p38 pathway. We show that modulating the p38 pathway with chemical or upstream kinase activators or inhibitors regulates the activity of the promoters of muscle-specific marker genes such as myogenin, MyoD, or MLC3F. In addition, we demonstrate that p38-induced myogenesis is dependent on MKK3 because a cell clone stably expressing a dominant-negative (DN) mutant of this kinase is unable to form myotubes in differentiation medium. Furthermore, this clone displays potently reduced MyoD, p21, and p27 protein expression as well as a profoundly disorganized cytoskeleton. We show that the regulation of several structural proteins that play a key role in muscle physiology, such as myosin, actin, and troponin T, is severely perturbed in a cell clone stably expressing a DN mutant of MKK3. Treatment with an inhibitor of p38, SB-203580, also reduced expression of differentiation markers. Together, these data allow us to conclude that p38α is required for C2C12 myogenesis.

MATERIALS AND METHODS

Materials. Cell culture reagents were purchased from Life Technology (Cergy Pontoise, France), SB-203580 and anisomycin were from Calbiochem (La Jolla, CA). Antibodies against p21 (goat polyclonal C-19), p27 (mouse monoclonal F-8), and MyoD (M-318) were purchased from Santa Cruz (Heidelberg, Germany). The mouse monoclonal anti-Flag M2 and anti-phosphorylated p38 MAPK (diphosphorylated p38, clone P38-TY) antibodies were from Sigma (Saint Quentin Fallavier, France). The anti-myogenin antibody (mouse monoclonal F5D) was purchased from Pharmingen (Le Pont de Claix, France). Anti-troponin T (C3T) and anti-MHC (MF-20) antibodies were purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). Fugene 6 transfection reagent was purchased from Roche Molecular Biochemicals (Meylan, France).

Plasmids. The reporter plasmid for the myogenin promoter (called pGSZ 1092) contains the β-galactosidase gene under the control of a 1.092-kb fragment of the mouse myogenin promoter (33). The reporter plasmid for MLC3F promoter (called p3F-nlacZ-E) contains the β-galactosidase gene under the control of the mouse MLC3F promoter and a 3’ enhancer from the MLC1/3F locus (22). pCMV5 DN and constitutively active (CA) MKK kinase (MEKK1) were constructed as previously described (25). pCMV5 wild type (WT) and DN p38 were described previously (10).

Cell culture. Mouse C2C12 myoblasts (American Type Culture Collection no. CRL-1772) were cultured at 37°C in an atmosphere of 5% CO₂ in growth medium (GM) consisting of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (penicillin, streptomycin). To induce differentiation, confluent cells were placed in DMEM complemented with 2% horse serum, 10 μg/ml insulin, and 5 μg/ml transferrin [referred to as differentiation medium (DM)].

Immunoblotting. Cells were treated as indicated, rinsed, and solubilized in ice-cold lysis buffer [in mM: 50 HEPES pH 7.4, 150 NaCl, 100 NaF, 10 EDTA, 10 Na₃PO₄, and 2 Na₃VO₄ with 1% Triton X-100 and supplemented with protease inhibitors: aprotinin (2 μg/ml), leupeptin (10 μM) and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF; 1 mM)]. Equal amounts of protein (25 μg) were separated by SDS-PAGE on 10% acrylamide gels. Proteins were transferred to Hybond-C Extra membrane (Amersham) and stained with amido black to verify an even transfer, and then the blots were incubated in blocking buffer [1× Tris-buffered saline (TBS), 0.1% Tween 20, 5% BSA] for 1 h at room temperature. The membranes were washed in washing buffer (1× TBS, 0.1% Tween 20) three times, for 5 min per wash, and probed with the primary antibody and then the secondary antibody for 1 h in 1× TBS, 0.1% Tween 20, 1% BSA. After each incubation, membranes were washed three times for 10 min in washing buffer. Proteins were then visualized by the Amersham ECL system.

Transient transfection. C2C12 cells, plated in 24-well dishes, were transfected with the Fugene 6 reagent with a total amount of 0.8 μg DNA per well: 0.2 μg of reporter plasmid and 0.6 μg of expression vector, either empty or coding for the indicated kinases. After 6 h, the medium was replaced with either GM or DM for another 48 h. To control for the variability in transfection efficiency, other cells were transfected in the same manner except that the reporter plasmid was replaced by a plasmid containing the β-galactosidase gene under the control of the cytomegalovirus (CMV) promoter (pCMVβGal). β-Galactosidase activity was then measured with a colorimetric assay. Cells were washed with ice-cold PBS and lysed in a 50-μl volume of 25 mM Tris-phosphate (pH 7.8) buffer containing 1% Triton X-100, 2 mM EDTA, and 2 mM DTT. Soluble extracts were harvested and placed in a microplate with 50 μl of a 2× concentrated β-galactosidase reaction buffer (200 mM sodium phosphate buffer pH 7.3, 2 mM MgCl₂, 100 mM β-mercaptoethanol, and 1.33 mg/ml o-nitrophenyl β-D-galactopyranoside). Microplates were incubated at 37°C, and optical density (OD) readings were measured at 405 nm every 30 min over 6 h.

Stable transfection. C2C12 cells were transfected (with Fugene 6) with a pcDNA3 construct expressing a Flag-tagged DN form of MKK3 (Ser189 and Thr193 mutated to Ala; Ref. 27). Resistant clones were selected with 800 μg/ml neomycin (Life Technology). Individual clones were assayed for the expression of Flag-DN MKK3 as well as the ability to form myotubes in DM. Control C2C12 cells were transfected with the pcDNA3 empty vector alone.

Immunocytochemistry. Cells grown on glass coverslips in 12-well plates were cultured in DM. At the indicated time, the cells were fixed for 10 min in 3% paraformaldehyde in PBS and then washed twice in PBS. Cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min and then washed twice in PBS. Blocking was performed in 3% BSA in PBS for 30 min, and the cells were then incubated with monoclonal anti-troponin T antibody for 12 h at 4°C in PBS-1% BSA. After three washes in PBS, the cells were incubated with a secondary fluorescein isothiocyanate (FITC)-conjugated antibody for 1 h at room temperature and then washed three times in PBS. The coverslips were mounted on fluorescent mounting medium (Dako, Trappes, France) and visualized on an Olympus BH2 epifluorescence microscope. Photographs were made with a Nikon Coolpix 990 digital camera.

F-actin staining. Control and DM MKK3 C2C12 cells were grown to confluence on glass coverslips. The medium was switched to DM, and the coverslips were processed each day

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for four subsequent days after addition of DM. Coverslips were rinsed once in sterile PBS, and the cells were permeabilized in 0.5% Triton X-100 in PBS and then fixed in 5% formaldehyde in PBS. The cells were stained with 5 μg/μl FITC-conjugated phalloidin (Sigma) for 30 min in the dark.

RESULTS

p38 Inhibitor SB-203580 blocks myogenin, MLC3F, and MyoD expression in C2C12 muscle cells. We first investigated the effect of p38 inhibition on the expression of two myogenic markers: the myogenin transcription factor and the MLC isoform 3F (MLC3F). For this purpose, we performed a reporter gene assay with two plasmids containing either the promoter of the myogenin or the MLC3F gene cloned upstream of the β-galactosidase gene. These two plasmids were separately and transiently transfected into C2C12 cells. Twelve hours after the transfection, cells were placed in DM for 24 h and either treated or not with the p38 inhibitor SB-203580. Induction of differentiation with DM resulted in a fourfold stimulation of myogenin and MLC3F promoter activity as reflected by the β-galactosidase activity, compared with the basal activity obtained in GM (Fig. 1A). We observed that SB-203580 treatment produced a significant inhibition (~2-fold) of the expression of these two markers. Western blot analysis showed that MyoD expression (Fig. 1B) was totally inhibited for at least 8 h after the induction of differentiation in cells treated with SB-203580. MyoD expression was reduced at 24 and 48 h after differentiation. These results therefore show that p38 is implicated in the control of myogenin, MLC3F, and MyoD expression during low-serum-induced myoblast differentiation.

Stress kinases MEKK1, MKK3, and p38 regulate myogenin and MLC3F expression. We next investigated the effect of the overexpression of WT and mutant forms of p38, MKK3 (an upstream activator of p38), and MEKK1 (which can act in both the JNK and p38 pathways) on myogenin and MLC3F promoter activity. Cells were transiently cotransfected with the reporter plasmids described above and the indicated expression vectors (Fig. 2). Results obtained from empty vector-transfected cells was assigned a value of 1.0 to enable comparison with separate transfections. Overexpression of WT p38 and a constitutively active mutant of MEKK1 induced an increase in myogenin (Fig. 2A) and MLC3F (Fig. 2B) promoter activity. This occurred in DM but also in growth conditions, although to a lesser extent. In contrast, overexpression of constitutively inactive mutants of p38, MKK3, and MEKK1 resulted in an inhibition of myogenin and MLC3F gene expression regardless of the medium used. Interestingly, the MLC3F promoter is more sensitive to the activation of the p38 pathway (more than twice as much as shown in Fig. 2 in DM) than the myogenin promoter, thereby suggesting a prominent role of this cascade in terminal differentiation. Considering that MEKK1 can phosphorylate and activate MKK3, these results strongly suggest that the MEKK1/MKK3/
p38 pathway is involved in the control of expression of muscle differentiation markers.

Expression of myogenin and MHC together with myotube formation are affected in C2C12 cells cultured with SB-203580. To demonstrate the role of p38 in differentiation, we first used SB-203580, a pharmacological inhibitor of p38. As shown in Fig. 3A, expression of myogenin and MHC were profoundly affected by SB-203580 during skeletal muscle cell differentiation. The pattern of expression of myogenin during differentiation was maintained, but the levels were seriously diminished. In contrast, expression of MHC, which peaks after 4 days in DM, was completely abolished after treatment with SB-203580.

This inhibition of muscle differentiation marker expression perfectly matched the inhibition of myotube formation observed after SB-203580 treatment (Fig. 3B). Indeed, after a 4-day treatment with 20 μM SB-203580, no myotube could be observed. These results show that inhibition of p38 with the specific inhibitor SB-203580 is sufficient to affect the expression of early and late myogenic markers as well as myotube formation.

p38 is transiently activated during myogenesis in control C2C12 cells but not in DN MKK3 cells that failed to form myotubes. To further demonstrate the involvement of the p38 pathway in C2C12 skeletal muscle cell differentiation, we established a C2C12 cell line expressing a Flag-tagged DN form of the p38 upstream activator MKK3 (34). In this mutant, Ser189 and Thr193 are mutated to Ala, resulting in a kinase that cannot be phosphorylated and therefore cannot be activated (DN MKK3). A control cell line was also established by stable transfection of the pcDNA3

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empty vector. Figure 4A shows the expression of Flag-DN MKK3, detected with an anti-Flag monoclonal antibody, in stably transfected C2C12 cells. This antibody cross-reacts nonspecifically with two other proteins present in both control and transfected C2C12 extracts. We then compared the levels of p38 activation, as reflected by its phosphorylation, in control vs. DN MKK3 cells (Fig. 4B). The p38 kinase was potently but transiently activated in control C2C12 cells on induction of differentiation despite a relatively high basal level (0 h time point). On the contrary, p38 was only very slightly activated in DN MKK3 C2C12 cells. This reflects the DN effect of the transfected MKK3 mutant on its direct downstream target, p38 (the same result was obtained by measuring p38 kinase activity; data not shown). Monitoring the expression levels of the Flag-tagged mutated MKK3 clearly revealed no variation throughout differentiation. Morphological analysis of this clone (Fig. 4C) showed that DN MKK3 cells failed to differentiate into myotubes even if they were cultured over 48 h in DM, whereas control cells fused into multinucleated myotubes under these same culture conditions. Three C2C12 clones expressing Flag-DN MKK3 were isolated, and all exhibited the same phenotype. Our attempts to isolate stable C2C12 cells expressing the other mutants used in Fig. 2 were unsuccessful, probably because of the deleterious effects of these proteins.

Expression of MyoD, p21, p27, troponin T, myogenin, MHC, and F-actin cytoskeleton are affected in stable DN MKK3 C2C12 cells. We next analyzed the differences in protein expression patterns of MyoD and of the cell cycle inhibitors p21 and p27 between control and DN MKK3-expressing cells. Both cell lines were induced to differentiate in DM and harvested at the indicated times for Western blot analysis (Fig. 5). The results showed a marked reduction in MyoD and myogenin expression for DN MKK3 cells compared with control C2C12 cells, although the time courses were
similar. Likewise, p21 expression was almost completely inhibited and p27 levels were markedly decreased. Thus stable expression of a DN form of M KK3 affects the expression of the muscle differentiation markers MyoD, myogenin, and cell cycle control proteins.

In addition, we monitored the expression of late differentiation markers such as troponin T or MHC throughout differentiation in control and DN M KK3-expressing cells. In the control cells, the expression of both markers was low after 2 days of differentiation, maximal at day 3, and slightly decreased at day 4. On the other hand, the expression of these markers was totally abolished in the DN M KK3-expressing cells. This additional result therefore points out a stronger inhibiting effect of DN M KK3 on late vs. early differentiation markers.

We finally looked at the expression of troponin T by immunocytochemistry during differentiation (Fig. 6). Our observations in control C2C12 cells show a steady increase of troponin T in myocytes and myotubes throughout differentiation, which reflects our above-described Western blotting results (Fig. 5). In DN M KK3-expressing cells, the expression of troponin T remained very low in most cells throughout the whole differentiation process. Nevertheless, very few DN M KK3 cells showed a strong accumulation of troponin T after 4 days of differentiation. Considering the unusual morphology of these cells, we tested whether they might have undergone apoptosis, but we have not been able to correlate the specific and intense troponin T staining with nucleus fragmentation (data not shown).

We finally compared the cytoskeletal rearrangements occurring during the induction of myogenesis in the control and DN M KK3 C2C12 cell lines. FITC-labeled phalloidin staining of the F-actin cytoskeleton in control and DN M KK3 C2C12 cells illustrated the dramatic differences between the stages of differentiation that occur in these two cell lines (data not shown). The control cells showed a more rapid phenotypic change in DM, with reorganization of the actin cytoskeleton and visible myotubes appearing after 2 days in DM. In contrast, a proportion of the mutant cells differentiate into myocytes but fail to undergo the major cellular reorganization necessary for myotube formation. The mutant cells do not fuse with one another to generate larger myotubes. Thus the expression of DN M KK3 in C2C12 cells has a greater effect on myotube fusion than myocyte formation.

DISCUSSION

In this report, we demonstrate that the induction of myoblast differentiation is controlled by the p38 kinase pathway. Our results provide evidence that stable ex-

![Fig. 6. Immunocytochemical analysis of troponin T in control vs. DN M KK3 cells during myogenic differentiation. Control and DN M KK3 cells were grown to near confluence on coverslips, and the medium was changed to DM. The coverslips were processed for troponin T staining (as described in MATERIALS AND METHODS) between days 0 and 4 after the onset of differentiation. Bar = 10 μm.](http://ajpcell.physiology.org/)
pression of a DN mutant of MKK3 or the use of an inhibitor of p38, SB-203580, is sufficient to profoundly perturb myoblast differentiation parameters and block terminal myotube formation. Moreover, our data show that inhibition of the p38 pathway affects the expression of early muscle differentiation markers including MyoD, p21, and p27, as well as the late differentiation markers, such as MLC3F, MHC, and troponin. Finally, we show that blocking the p38 pathway seriously perturbed cytoskeletal organization during the terminal stages of C2C12 myogenesis.

These results are in agreement with previous reports implicating the p38 kinase in promoting skeletal muscle differentiation (5, 24, 36). These studies were mainly based on the use of the p38 inhibitor SB-203580 (4, 6, 13). Indeed, we have also observed a potent inhibition of myotube formation in C2C12 cells induced to differentiate in the presence of SB-203580 (Fig. 3B). In addition, we observed a nearly 50% inhibition of both myogenin and MLC promoter activity and a drastic reduction of MyoD expression in the presence of this inhibitor (Fig. 1). However, it was recently shown that this compound, when used at the usual 10 μM concentration, also inhibits other kinases such as lymphocyte kinase (LCK, 70% inhibition; Ref. 30), Akt1/PKB (RAC α serine/threonine kinase, 38%), glycogen synthase kinase 3 (GSK-3, 34%), Rho-dependent protein kinase II (ROCK-II, 23%), and finally ERK2 (15%; Ref. 8). In addition, the potent inhibitory effects of SB-203580 on p38 are strictly restricted to the α- and β- but not to the γ- and δ-isofoms (8).

To circumvent some of these problems, and to confirm the involvement of p38 in skeletal muscle differentiation, we decided to employ several interfering mutants of the kinases composing the p38 signaling pathway. We first used these constructs in cotransfection experiments with the β-galactosidase reporter gene placed downstream of the myogenin or MLC3F promoter (Fig. 2). As a general rule, constructs that are expected to activate the p38 pathway (i.e., WT p38 or CA MEKK1) upregulated both myogenin and MLC3F promoter activity. Conversely, constructs expected to hinder this pathway (DN forms of p38, MKK3, and MEKK1) downregulated the activity of the myogenin and MLC3F promoters. We ultimately decided to isolate a C2C12 cell line stably expressing a mutant of the p38 activator MKK3, in which Ser189 and Thr192 were mutated to Ala (27). Apart from the expected inhibition of p38 phosphorylation, the most striking phenotype of this cell line was that it never underwent terminal differentiation, although a few myoblasts could be observed in DM. In addition, we recently showed (34) that inhibition of myogenin in the DN MKK3 cell line facilitates trans-differentiation of these cells into adipocytes. Inhibition of myogenin via the expression of DN MKK3 prevents the formation of differentiated myotubes, resulting in a population of pluripotent cells that are able to differentiate into other lineages. The inhibition of the later stages of myogenin correlates with the fact that the MLC3F promoter, which is known to be activated late in myogenin, appears to be more sensitive to the p38-activating or -inhibiting constructs. MKK3 preferentially activates the α-, γ-, and δ-isofoms of p38 (21). In addition, the phosphorylation of MEF2C, a member of the myocyte enhancer binding factor family known to act downstream of p38 in the induction of myogenesis (16), is mediated by the α-isofom of p38 (31, 32, 37). We and others have shown that SB-203580 blocks muscle cell terminal differentiation. Considering the restricted action of this compound on the α- and β- but not on the γ- and δ-isofoms of p38, we can conclude that p38α is absolutely required to complete myogenesis. These observations, together with the data presented here, show that p38α is very likely to be the isoform involved in the induction of myogenesis, although one cannot rule out the possible contribution of p38γ and p38δ. Our study, using stable expression of DN MKK3, clearly demonstrates the importance of this pathway in myogenesis.

The inability of the DN MKK3 C2C12 cell line to differentiate clearly correlates with the profound modification of several differentiation markers (Fig. 5), including MyoD, p21, and troponin T. Although the pattern of MyoD expression is comparable between control and DN MKK3 C2C12, its levels are clearly lower in the latter cell line. Regarding p21, its expression was barely detectable after 6 h in DM and then completely disappeared in the DN MKK3 cells. The expression of p21 is known to be a key event triggering cell cycle withdrawal, a prerequisite to myogenic differentiation (2). Moreover, a recent study described the decrease in Akt1 expression in C2C12 cells induced by treatment with p21 antisense oligonucleotides (12). Indeed, we have made a similar observation with the DN MKK3 cells: this cell line exhibits decreased amount of the Akt1 protein throughout differentiation (unpublished data). Our results suggest an interaction between the p38 and Akt pathways (unpublished observations). In addition to myoD and p21 we have also found that the expression of troponin T is significantly decreased in the DN MKK3 cells. Troponin is a marker of terminal differentiation in myoblastic cells (35). In the control C2C12 cells, troponin T staining was most pronounced in the large myotubes after 4 days of differentiation. The DN MKK3 cells expressed only negligible levels of troponin T. Only a few DN MKK3 cells, presenting an unusual shape, were still expressing troponin T after 4 days in DM. We ruled out the possibility that these cells might be undergoing apoptosis, but it is possible that they have lost the expression of DN MKK3 and thereby have reactivated troponin T expression. Thus the expression of both early and late markers of differentiation are dysregulated in C2C12 cells expressing DN MKK3.

To further identify the event leading to myogenesis inhibition in the DN MKK3 cell line, we decided to study its cytoskeletal reorganization during differentiation. A comparison of the F-actin staining pattern between the control and DN MKK3 cells suggests that the overexpression of DN MKK3 inhibits specific stages in the differentiation process, in particular the process of cell fusion that precedes myotube formation.
(data not shown). It is possible that p38 may also regulate the expression of cell fusion molecules necessary for myogenesis, such as melanin-α (14), or caveolin-1 (29).

We have been able to show that the mRNA expression of one such protein, vimentin, is dramatically delayed in DN MKK3 cells (data not shown). This protein is one of the major components of intermediate filaments that are known to participate, together with other cytoskeletal elements, in the mechanical stability of epidermal and muscle cells (18). Its mRNA has been shown to be downregulated in fully differentiated C2C12 myotubes as opposed to myoblasts (1). In addition, vimentin is thought to be involved in the early phase of myofiber regeneration when migration, fusion, and structural remodeling of myoblasts occur, recapitulating its role in myogenesis (28). In addition, several studies of the promoter region of the vimentin gene have shown the presence of AP-1 and MEF2 DNA-binding sites, both of which factors are known to activate, vimentin. We performed kinase assays with fusion constructs composed of glutathione S-transferase (GST)-vimentin or GST and the serine- and proline-rich NH2-terminal domain of vimentin (amino acid residues 1–125). However, we did not find p38 or JNK to be able to directly phosphorylate vimentin in vitro (data not shown).

However, this does not preclude an indirect interaction between p38 and vimentin, because the effects of p38 may be mediated through additional factor(s). Clearly, vimentin is another gene involved in myogenic differentiation whose expression is dysregulated in the DN MKK3 C2C12 cells. Further experiments are under way to identify other cell fusion molecules that may be downregulated or underexpressed in DN MKK3 cells.

In conclusion, the data presented here further demonstrate the crucial role played by the p38 MAPK in muscle cell myogenesis. In addition, we show that this role is dependent on a functional MKK3 activator and that it is likely to be exerted by the α-isofrom of p38. Finally, we show that p38 acts on the late stage of muscle differentiation known as myocyte fusion, possibly by affecting the expression of cytoskeletal elements including vimentin.

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