The p38α/β MAPK functions as a molecular switch to activate the quiescent satellite cell

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Somatic stem cells cycle slowly or remain quiescent until required for tissue repair and maintenance. Upon muscle injury, stem cells that lie between the muscle fiber and basal lamina (satellite cells) are activated, proliferate, and eventually differentiate to repair the damaged muscle. Satellite cells in healthy muscle are quiescent, do not express MyoD family transcription factors or cell cycle regulatory genes and are insulated from the surrounding environment. Here, we report that the p38α family of mitogen-activated protein kinases (MAPKs) reversibly regulates the quiescent state of the skeletal muscle satellite cell. Inhibition of p38α/β MAPKs (a) promotes exit from the cell cycle, (b) prevents differentiation, and (c) insulates the cell from most external stimuli allowing the satellite cell to maintain a quiescent state. Activation of satellite cells and p38α/β MAPKs occurs concomitantly, providing further support that these MAPKs function as a molecular switch for satellite cell activation.

Introduction

Maintenance and repair of skeletal muscle tissue is performed by a specialized somatic stem cell termed the satellite cell. Satellite cells, which comprise a small (1–6%) number of the total myonuclei, are located between the basal lamina and the muscle fiber and can remain quiescent for an average of 7 yr in adult humans (Schultz and McCormick, 1994; Seale and Rudnicki, 2000; Hawke and Garry, 2001). The quiescent satellite cell expresses few gene products and does not express members of the MyoD family (Cornelison and Wold, 1997; Cornelison et al., 2000). Activation of the satellite cell, a critical but poorly understood process, can be induced by HGF or TNFα injection in vivo (Tatsumi et al., 1998; Li, 2003). Activation of the satellite cell is accompanied by a general initiation of gene transcription including induction of MyoD and myf-5, an increase in cellular volume, and entry into the cell cycle (Cornelison and Wold, 1997; Cornelison et al., 2000). Quiescent satellite cells express c-met (the HGF receptor), FGF receptors 1 and 4, syndecan-3, and syndecan-4, all of which appear to be involved in satellite cell activation and proliferation (Tatsumi et al., 1998; Flanagan-Steed et al., 2000; Cornelison et al., 2001, 2004).

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Abbreviation used in this paper: ERK, extracellular signal-regulated kinase.
fusion without affecting expression of muscle-specific genes (Bennett and Tonks, 1997). Together, these results suggest that distinct subsets of MAPKs are likely to regulate proliferation and differentiation of skeletal myoblasts.

The p38α/β MAPKs, which are activated in response to growth factor stimulation (Morooka and Nishida, 1998; Iwasaki et al., 1999; Maher, 1999) have been reported to be required for late stages of myogenic differentiation of both C2C12 and L6 myoblasts (Cuenda and Cohen, 1999; Zetser et al., 1999). We found that active p38α/β (pp38α/β) MAPKs present in proliferating satellite cells and MM14 cells are localized to the nucleus, suggesting that these kinases may function before cell differentiation. Consistent with this, we observed that inhibition of p38α/β MAPK activity prevented activation and proliferation of satellite cells cultured on intact myofibers. We also show that p38α/β MAPKs are required for MyoD induction in satellite cells and for MM14 differentiation. Interestingly, inhibition of these MAPKs in either MM14 cells or satellite cells induces a reversible quiescent state whereby the cells are unresponsive to external stimuli, similar to that observed for normal adult satellite cells in uninjured muscle tissue.

**Results**

**Several MAPKs are expressed in proliferating and differentiated MM14 cells**

We have previously shown that ERK1/2 is required for proliferation but not differentiation in MM14 cells (Jones et al., 2001). To gain insight into which MAPKs may play additional roles in MM14 cell proliferation and differentiation, we performed RT-PCR for MAPKs on MM14 cells at various times after induction of differentiation. Neither erk1/2 nor erk3 MAPKs appear to change expression levels upon MM14 cell differentiation (Fig. 1 A). Both erk5 and p38α appear to decrease during differentiation, whereas p38γ showed an initial decrease followed by a marked increase at 72 h of differentiation. Interestingly, inhibition of p38α/β MAPKs in either MM14 cells or satellite cells induces a reversible quiescent state whereby the cells are unresponsive to external stimuli, similar to that observed for normal adult satellite cells in uninjured muscle tissue.
phosphorylated p38α/β (pp38α/β) was present in MM14 cells and found pp38α/β localized to the cell nucleus of proliferating cells (Fig. 1, B and C). Because MM14 cells are exquisitely sensitive to FGF removal, which is sufficient to trigger differentiation from the G1 phase of the cell cycle, we asked if removal of FGF would promote p38α/β activation. Upon removal of FGF in the presence of 15% horse serum, we found little if any detectable pp38α/β expression (Fig. 1 D). The apparent activation of p38α/β MAPKs by FGF-2 is not due to changes in p38α/β protein levels as indicated by reprobing the Western blot with an antibody that recognizes p38α/β (Fig. 1 D, bottom).

**Active p38α/β is required for proliferation of MM14 cells**

FGF-mediated activation of p38α/β MAPKs and their presence in the nucleus of proliferating myoblasts were unexpected, suggesting a role for these MAPKs in proliferating myoblasts. We asked what effect inhibition of p38α/β MAPKs would have on proliferating MM14 cells. If synchronized by mitotic shake-off MM14 cells require FGF within 4-6 h to proceed through the cell cycle (Clegg et al., 1987; Kudla et al., 1995) and thus we tested the effect of increasing doses of SB203580 on synchronized MM14 cells. Addition of SB203580 to proliferating cells prevented DNA synthesis in a dose-dependent manner, reducing DNA synthesis to control levels with 25 μM SB203580 (Fig. 2 A). To demonstrate that the SB203580-dependent inhibition of proliferation is specific for the p38α/β MAPKs, we attempted to rescue the SB203580 effects with an SB203580-resistant p38α (p38αRM; Eyers et al., 1999). In the presence of SB203580, MM14 cells expressing a control vector or wild-type p38α failed to proliferate (Fig. 2 B). In contrast, SB203580-treated cells expressing p38αRM proliferated, demonstrating rescue by the drug-resistant p38αRM (Fig. 2 C). Together, these data support the conclusion that p38α/β MAPKs are required for myoblast proliferation and that the phenotype of SB203580-treated cells is specifically due to loss of p38α/β MAPK activity.

**Active p38α/β is present in recently activated satellite cells**

A role for p38α/β MAPKs has not been demonstrated in proliferating myoblasts and thus might potentially reflect an artifact of the MM14 cell line. Therefore, we asked if p38α/β MAPKs were present in activated satellite cells cultured on intact myofibers. The p38α/β MAPKs appear present in freshly isolated myofibers at the time of muscle harvest. The protein appears localized in the cytoplasm of the myofibers adjacent to the myonuclei (Fig. 3 A, asterisk in myofiber panel). In addition, immunoreactive p38α/β was present in quiescent skeletal muscle satellite cells (Fig. 3 A, caret), readily identified by their syndecan-4 staining (Cornelison et al., 2001). In the satellite cells, the p38α/β MAPKs appear to localize to the cytoplasm (Fig. 3 A). If p38α/β MAPKs are required for satellite cell proliferation, pp38α/β should be present. When stained with an antibody that recognizes pp38α/β satellite cell nuclei on myofibers fixed at the time of harvest and on myofibers after 3 and 24 h of culture were pp38α/β positive (Fig. 3 B). When muscle tissue was removed, fixed, and myofibers teased from the fixed muscle tissue, 40% of the satellite cells were positive for pp38α/β (Fig. 3 B). The frequency of pp38α/β in muscle tissue immediately upon harvest suggests that p38α/β phosphorylation may rapidly occur upon satellite cell activation. Although we removed muscle tissue and fixed the tissue immediately, a minimum of 15–20 min required to dissect the muscle is essentially a massive injury and likely results in satellite cell activation. Thus, we expect isolation of muscle tissue to activate satellite cells. However, the induction of MyoD expression, regarded as an early marker for satellite cell activation, was not detected until after 3 h of myofiber culture (Fig. 3 B). The frequency of pp38α/β immunoreactivity in satellite cells is significantly higher than that of MyoD at 0.4 and 3 h of myofiber culture (Fig. 3 C), revealing that p38α/β MAPKs are activated before MyoD accumulation in satellite cells (Fig. 3, B and C) and that both events occur before satellite cell duplication.
Active p38α/β MAPKs are required for satellite cell activation and proliferation

Our data suggests that pp38α/β may play a role in the activated satellite cell. To elucidate the function(s) of p38α/β, we first tested for toxicity and verified that the p38α/β MAPK inhibitors would function on satellite cells in myofiber cultures. Addition of SB203580 (Davies et al., 2000) but not an inactive analogue, SB202474, at the time of myofiber isolation eliminated satellite cell immunoreactivity for pp38α/β MAPKs (Fig. 4 A), which were readily detectable by syndecan-4 staining (Fig. 4 A).

We assayed for induction of MyoD protein in fiber-associated satellite cells as a measure of satellite cell activation in the presence and absence of pp38α/β MAPK inhibitors. Addition of an inactive analogue, SB202474, had no detectable effect on MyoD protein induction in syndecan-4–positive satellite cells (see Fig. 6 A). Culturing intact myofibers in either active analogue (SB203580 or SB202190) did not detectably affect the satellite cell number at 24 h after isolation (Fig. 3 D, not depicted) but significantly diminished MyoD induction (Fig. 4 B). Quantitative analysis of these data show that treatment with either active analogue reduces the number of satellite cells that detectably express MyoD protein by 50% and 60% for SB202190 and SB203580, respectively, when compared with the SB202474 control (Fig. 4 C).

Proliferation of satellite cells on intact myofibers was also blocked by addition of SB203580. Intact myofibers treated for 48 h with SB203580 (Fig. 4 D) or 96 h (not depicted) possessed satellite cells as shown by syndecan-4 staining but these cells failed to incorporate BrdU (Fig. 4 D). When untreated myofibers are scored for the number of satellite cells per myofiber at 96 h in culture, a broad distribution is observed with an average of 12–14 satellite cells per fiber (Fig. 4 E). In contrast, fibers incubated for 96 h in SB203580 had a narrow distribution of satellite cells with an average of three to four satellite cells per fiber (Fig. 4 E), similar to the average number of satellite cells at the time of myofiber isolation, suggesting a role for p38α/β MAPKs in satellite cell activation and subsequent proliferation.

Active p38α/β MAPKs are necessary for MM14 and satellite cell differentiation

Published data has clearly demonstrated a requirement for p38α/β in myogenic differentiation, where p38α/β is reported to regulate MEF-2 activity and transcriptional activity of MyoD and myogenin (Zetser et al., 1999; Puri et al., 2000; Wu et al., 2000). A similar requirement for p38α/β activity in MM14 cell differentiation was revealed by treating MM14 cells with SB203580. MM14 cells grown in proliferation media are 5% MyHC positive after 36 h (Fig. 5 A) and 15% MyHC positive after 72 h (not depicted). In the presence of SB203580, there were no detectable MyHC-positive cells at 36 h (Fig. 5, A and B). When incubated for 36 h in differentiation media, 98% of the cells were MyHC-positive in the absence of the drug (Fig. 5, A and B) and only 20% were positive when SB203580 was included (Fig. 5, A and B). These data agree with those previously published and suggest that the p38α/β MAPKs are required for differentiation of the MM14 satellite cell-derived cell line.

Proliferation and differentiation are mutually exclusive events in skeletal muscle cells and thus, it is difficult to reconcile
a requirement for p38α/β MAPKs in both events. It is possible
that the p38α/β inhibitors are nonspecifically blocking myogen-
esis. Therefore, we tested the capacity of the p38α/β
inhibitors to rescue differentiation in SB203580-treated MM14
cells. MM14 cells maintained in the absence of FGF and in
DMSO for 48 h express MyHC, but cells maintained in
SB203580 fail to differentiate (Fig. 5 C). However, MM14 cells
expressing p38α/β induce MyHC expression even when main-
tained in SB203580 for 48 h (Fig. 5 C). Moreover, SB203580-
treated MM14 cells expressing p38α/β induce skeletal muscle-
specific gene expression whereas treated cells expressing p38α
fail to induce muscle gene expression when assayed by a mus-
cle-specific reporter assay (Fig. 5 D). Together, these data estab-
lish that inhibition of differentiation by SB203580 in MM14
cells is specific for p38α/β MAPKs and not due to nonspecific
activities even at high (20 μM) SB203580 concentrations.

Differentiation of primary satellite cells dispersed in
monolayer culture is also inhibited when cultured in 20 μM
SB203580 for 48 h (Fig. 5 E). The inhibitory effects of
SB203580 on satellite cell differentiation appears similar to
that seen for MM14 cells.

The requirement for p38α/β in
proliferation and differentiation
occurs in the G1 phase of the cell cycle

A requirement for p38α/β activity in proliferation and differen-
tiation of skeletal muscle MM14 cells appears counterintuitive
because these events are mutually exclusive. These results
could be explained if p38α/β functioned in the G1 phase of
proliferating cells to promote progression through the cell cy-
cle and in the G0 phase to promote differentiation, perhaps by
acting on distinct targets. To determine when in the cell cycle
p38α/β functions are required, we synchronized cells by mi-
totic shake-off at the M/G1 boundary and added SB203580 at
different times in the presence and absence of FGF-2 (Fig. 6
A). In untreated cultures, cells irreversibly withdraw from the
cell cycle if FGF-2 is removed for 4–6 h after cell synchroniza-
tion (see Fig. 7 A), indicated by loss of [3H]thymidine incor-
poration (Fig. 6 B). If synchronized cells are treated with
SB203580 at 2, 4, or 6 h after mitotic shake-off, the cells fail to
enter the cell cycle and do not incorporate [3H]thymidine (Fig.
6 B). However, if SB203580 is added after 6 h there is little ef-
fect on DNA synthesis, suggesting the requirement for p38α/β

Figure 4. p38α/β MAPK is required for satellite cell proliferation. (A) Intact myofibers were incubated upon removal with either an inactive p38α/β
MAPK inhibitor, SB202474, or SB203580 for 24 h. pp38α/β was identified by staining with anti-pp38α/β antibodies and satellite cells identified by
staining with syndecan-4. Nuclei were identified by staining with DAPI. (B) Mouse hindlimbs were removed and muscle fibers dissected and cultured in
either SB202474, SB203580, or SB202190 for 24 h. The myofibers were then fixed and stained for syndecan-4 (green), MyoD (red), and DAPI (blue).
(C) The number of MyoD-positive cells and syndecan-4–positive cells treated with each agent were scored and the number of MyoD-positive cells plotted
as a percentage of the total number of satellite cells. (D) Satellite cells on intact myofibers were incubated in BrdU-containing medium in DMSO (top) or
SB203580 for 48 h (bottom). The myofibers were probed with anti-syndecan-4 antibodies and anti-BrdU antibodies to identify satellite cells and DNA
synthesis, respectively. (E) Intact myofibers were cultured as in D for 96 h. The fibers were fixed and stained with anti-syndecan-4 antibodies to identify
satellite cells. The frequency of the number of satellite cells scored was plotted as a function of the average number of satellite cells per myofiber. Carets
denote syndecan-4–positive satellite cells in all panels.
in proliferation occurs in the G1 phase (Fig. 6 B). To determine the point in the cell cycle when differentiation of MM14 cells are sensitive to SB203580, cells were synchronized, plated in the absence of FGF-2 and SB203580 as indicated, fixed, and stained for MyHC (red). In DMSO-treated cells transfected and untransfected cells express MyHC whereas only p38αβ-transfected cells are MyHC-positive in the presence of SB203580. (D) MM14 cells cotransfected with LacZ, an α-cardiac actin promoter driving luciferase (the MSP reporter), and either pcDNA3, p38α, or p38αβ expression vectors were grown for 48 h with or without (20 μM) SB203580 in the presence or absence of FGF-2 and processed for luciferase assays. The extent of differentiation (RLU) in the presence and absence of FGF without SB203580 for each transfection was normalized to 1.0-fold to compare with transfected cells treated with SB203580. In p38αβ-expressing cells but not in control or p38α-transfected cells differentiation was rescued despite the presence of SB203580. (E) Dispersed primary satellite cell cultures were cultured for 48 h in differentiation conditions in the presence or absence of 20 μM SB203580. The cells were fixed, stained for syndecan-4 and MyHC and the number of MyHC-positive satellite cells scored. The number of MyHC-positive satellite cells was normalized to 100% in DMSO-treated cultures. All error bars indicate SEM for four independent experiments.

**P38αβ MAPKs signal to different substrates in proliferating or differentiated MM14 cells**

A requirement for p38αβ in both proliferation and differentiation could be explained if p38αβ MAPKs acted on different substrates in proliferating cells versus cells committed to terminal differentiation. To ask if these MAPKs signal differentially we analyzed p38αβ activity using the CHOP reporter assay specific for the ATF2 transcription factor (Xu and Cobb, 1997). Surprisingly, proliferating MM14 cells display CHOP reporter activity but this activity appears insensitive to FGF and insensitive to SB203580 (Fig. 7 A). At this time point the majority of cells are not committed to differentiation in the presence or absence of FGF-2. In contrast, if cells are cultured for 72 h in the absence of FGF-2, a large induction of CHOP is observed (Fig. 7 A). As expected, this activity is sensitive to SB203580, indicating that it is dependent on the p38 MAPKs. In proliferating cells, pp38αβ signals independently of ATF2 whereas in differentiated cells pp38αβ activates ATF2-dependent transcription denoting distinct differences in p38 MAPK signaling that appear dependent on the cellular phenotype.

An independent and less biased approach for identifying p38αβ MAPK substrates in proliferating and differentiated cells was performed by immunoprecipitation of cell extracts...
with an antibody that recognizes MAPK phosphorylation sites. Immunoprecipitation of MM14 cell extracts maintained in the presence of FGF-2 for 24 h (proliferating) or in the absence of FGF-2 for 48 h (differentiated) in the presence and absence of SB203580 reveal a number of potential p38 MAPK substrates unique to proliferating cells (Fig. 7 B, black arrowheads) and differentiated cells (Fig. 7 B, white arrowhead). These data provide further evidence that p38 MAPK functions differently in proliferating and differentiated cells. The majority of the bands present in the gel do not change when cells are treated with SB03580, indicating they are likely substrates of other MAPKs. The majority of proteins that change in intensity or mobility upon treatment with SB203580 are distinct for either the proliferating or differentiated cell populations. These data indicate that the majority of p38α/β substrates detected by this method are unique for each cellular phenotype (Fig. 7 B). Although we have not yet identified and verified these potential substrates, these data suggest that the functions performed by pp38α/β in proliferating and differentiated cells substantially differ.

Loss of p38α/β activity defines a reversible quiescent state for MM14 cells and satellite cells

We observed that MM14 cells treated with SB203580 exited the cell cycle but also failed to differentiate. This “quiescent state” closely resembles a similar quiescent state that occurs in the majority of the normal adult satellite cell population in vivo. Because the SB203580-treated cells exhibit characteristics similar to quiescent satellite cells we predicted that (a) cells will be unresponsive to changes in culture conditions while maintained in SB203580, and (b) that the SB203580-induced quiescence will be reversible. To test this hypothesis, MM14 cells were treated either with DMSO as a control or with SB203580 for 36 h in differentiation media (D = 5% horse se-
conditions was normalized to 100% (Fig. 8 A). Incorporation in control cultures maintained in DMSO under proliferating 3[H]thymidine. Incorporation of 3[H]thymidine into the DNA cells incubated an additional 24 h in proliferation media (P rum and no FGF-2). The drug was removed and the treated cells incubated an additional 24 h in proliferation media (P = 15% horse serum and 500 pM FGF-2) or in D media with 3[H]thymidine. Incorporation of 3[H]thymidine into DNA in control cultures maintained in DMSO under proliferating conditions was normalized to 100% (Fig. 8 A). Incorporation of 3[H]thymidine into DNA in cultures maintained in D conditions for 36 h was <5% of the proliferating control (Fig. 8 A) yet cells maintained for 36 h in D conditions with added SB203580 reentered the cell cycle upon removal of the inhibitor and restoration of P media (Fig. 8 A). These data indicate that SB203580-treated cells are indifferent to their environment because they show no effects of either P or D media during SB203580 treatment but proliferate or differentiate solely based on the conditions they are exposed to after SB203580 is removed. This effect is drug specific as cells cultured in its absence for 36 h in D conditions fail to reenter the cell cycle when switched to P media (Fig. 8 A).

When MM14 cells are maintained for 36 h in DMSO and D conditions, MyHC is induced in ≥95% of the cells (Fig. 8 B), whereas ≤5% of the cells are MyHC positive if maintained in P conditions for 36 h (Fig. 8 B). Because MM14 cells can undergo terminal differentiation from G1 without an intervening cell cycle when FGF is removed, we asked if cells maintained in SB203580 for 36 h were similarly capable of committing to terminal differentiation without an intervening S-phase. When cells treated with SB203580 for 36 h in D conditions were cultured for an additional 24 h in the absence of the drug, the majority of the cells differentiated (Fig. 8 B). In contrast, cells maintained in SB203580 and D conditions for 36 h and then switched to P conditions were <5% positive for MyHC (Fig. 8 B). These results are consistent with the idea that inhibition of p38 MAPKs maintains MM14 cells in a quiescent, nonresponsive state, and the cells are incapable of responding to either the presence or absence of FGF-2. Upon SB203580 removal, MM14 cells can either commit to S-phase or to terminal differentiation, depending on the environmental signals provided.

To test whether p38α/β MAPK activation was required for exit from a quiescent state in satellite cells and to test if inhibition of these MAPKs maintained satellite cell quiescence, we performed a series of experiments on satellite cells similar to those described for MM14 cells. Satellite cells on intact myofibers were cultured in the presence of SB203580 and BrdU to assess satellite cell proliferation. Intact myofibers cultured under normal conditions will induce DNA synthesis ~36 h after myofiber isolation (Fig. 8 C). If the fibers are cultured in the presence of SB203580, satellite cells fail to incorporate BrdU by 48 h reflecting a failure to activate and proliferate (Fig. 8 C). This is not due to loss of satellite cells because inclusion of SB203580 does not appear to decrease the number of satellite cells below that seen at initial harvest (Figs. 5 and 7). Removal of the p38 inhibitor at 24 h of culture rapidly reverses satellite cell quiescence, promoting DNA synthesis in 50 and 100% of the population within 24 and 48 h, respectively (Fig. 8 C).

**Discussion**

Skeletal muscle satellite cells are among the best understood adult somatic stem cell types and possess a tremendous capacity for regeneration of skeletal muscle tissue (Schultz and McCormick, 1994; Hawke and Garry, 2001; Seale et al., 2001). Skeletal muscle satellite cells are mitotically quiescent in adult...
uninjured skeletal muscle tissue and may remain quiescent for years in humans (Schultz et al., 1978; Schultz and McCormick, 1994). As such, satellite cells maintain a low metabolic profile and maintain gene expression for a small subset of growth-related genes that include FGF receptors 1 and 4 and c-met but no detectable expression of cell cycle–related genes or myogenic transcription factors (Cornelison et al., 2000). Satellite cell activation occurs rapidly after muscle injury; once activated, satellite cells induce expression of the myogenic regulatory factors, commit to DNA synthesis and begin proliferating in vivo (Schultz and Jaryszak, 1985; Schultz, 1996) and on intact myofibers (Bischoff, 1986; Yablokova-Reveni and Rivera, 1994; Cornelison and Wold, 1997). Removal of myofibers from intact skeletal muscle simulates an injury response, activating satellite cells with an accompanying rapid increase in cytoplasmic volume. Robust gene expression begins with the initiation of myogenic regulatory factor expression where MyoD is observed in 20–40% of the cells 3–6 h after isolation and 50–70% of the cells at 24 h. Proliferation is nearly synchronous for the first two divisions, occurring every 30–36 h (Bischoff, 1986; Cornelison and Wold, 1997).

Satellite cells within the muscle are both rare, comprising only 1–6% of the total muscle nuclei, and isolated; thus, little is known regarding the intracellular pathways regulating their activation, proliferation, and differentiation. We have been investigating the role(s) of MAPKs in skeletal muscle satellite cells in the MM14 satellite cell line, explanted myofibers and in dispersed cultures of satellite cells (Jones et al., 2001). For the p38 MAPK family, work performed on either myogenic cell lines or in fibroblasts (10T1/2) cells converted to muscle by ectopic MyoD expression shows that p38α/β MAPKs promote myogenesis, presumably via mechanisms that influence the transcriptional activation of the MyoD and MEF2 families (Cuenda and Cohen, 1999; Zetser et al., 1999; Puri et al., 2000; Wu et al., 2000; Xu et al., 2002). However, when examining the role of p38α/β MAPKs in the context of a developing limb it was observed that myogenesis was significantly enhanced when p38α/β MAPKs were inhibited, the opposite of what would be predicted for the role of p38α/β from studies involving myogenic cell lines (Weston et al., 2003). Explanations for these disparate observations have not yet been forthcoming.

The consequences of p38α/β inhibition are manifested as a failure to differentiate (a phenotype that is observed when p38α/β is inhibited in the MM14, C2C12, and L6/L8 cell lines) and a failure to proliferate in both MM14 cells and satellite cells, a novel observation. The inhibition of proliferation and the block to differentiation both occur when p38α/β activity is inhibited in the G1 phase of the cell cycle. Targets of the p38α/β MAPKs that mediate these events are unidentified. Although a significant body of data has shown that MEF2A and MEF2C are substrates of p38α and p38β2 (Ornatsky et al., 1999; Yang et al., 1999), satellite cells do not express detectable MEF2 transcripts until 96 h after myofiber isolation, suggesting that these are unlikely p38α/β targets for satellite cell activation or proliferation. Other known substrates of p38α/β include transcription factors (Max and ATF2), kinases (MAPKAP kinase 2 and 3), and phospholipase A2 (Lewis et al., 1998). In proliferating MM14 cells, manipulation of p38α/β activity by FGF removal or by SB203580 addition has no significant effect on ATF2-dependent transcription. However, in differentiated cells, ATF2-dependent transcription is SB203580 and FGF-2 sensitive. This suggests that p38α/β MAPKs act upon different substrates in proliferating and differentiating cells. A similar observation was noted for MM14 cells where differential activation of the ERK1/2 pathway occurred in cells committed to S-phase as compared with cells committed to terminal differentiation (Campbell et al., 1995). These reports provide supporting evidence for differences in signaling pathways that are dependent on the phenotypic state of skeletal muscle cells. When further analyzed for differences in substrate specificity, we found that the majority of p38α/β substrates were different in proliferating versus differentiated cells. Thus, the observation that p38α/β MAPKs are required for both proliferation and differentiation could be explained by differential substrate accessibility imposed by commitment of the cell to S-phase or to terminal differentiation. A better understanding of the roles of p38α/β MAPKs in these events will require identification of substrates specific for cells committed to proliferate and differentiate.

A requirement for p38α/β MAPKs in satellite cell proliferation appears similar to that observed in MM14 cells. However, pp38α/β is detected in the cell nucleus of satellite cells within 20 min of isolation, suggesting that these MAPKs play a role in signaling pathways that participate in activation of satellite cells. Consistent with this suggestion is the observation that inhibition of p38α/β activity in satellite cells prevents MyoD induction and proliferation, both markers of satellite cell activation. Although these data support a role for p38α/β MAPKs in satellite cell activation, the role of these kinases appears more complicated. Our data suggest that p38α/β is critical for balancing satellite cell activation and quiescence. Support for this hypothesis is the observation that both cultured satellite cells and MM14 cells fail to respond to changes in serum concentrations or FGF-2 when p38α/β is inhibited, indicating a general unresponsiveness to environmental stimuli. Importantly this unresponsive or quiescent state is reversible in

Figure 9. A model for the function of p38α/β MAPK in satellite cell activation. Upon injury to the muscle tissue, the p38α/β MAPK is immediately activated by as yet unidentified signals that may include signaling from either FGF receptor-1, FGF receptor-4, c-met, or the TNFα receptor. Once activated, p38α/β MAPK activity is maintained to allow satellite cell proliferation and reparation of damaged tissue. Activated p38α/β is also required for cell differentiation, and acts on distinct substrates to promote proliferation and differentiation. The quiescent state is maintained by inhibition of p38α/β MAPKs presumably via MAPK phosphatases.
both MM14 and satellite cells. When SB203580 is removed, MM14 cells can either reenter the cell cycle and proliferate or exit the cell cycle and terminally differentiate upon addition of P or D medium, respectively. Satellite cells on intact myofibers, which do not undergo differentiation reenter the cell cycle upon SB203580 removal. Because the behavior of MM14 and satellite cells in SB203580 resembles the quiescent state of satellite cells in adult uninjured skeletal muscle tissue, we propose that activation of p38α/β functions as an intracellular “molecular switch” for satellite cell activation (Fig. 9).

In vivo, mechanical stress activates satellite cells via an HGF and NO-dependent mechanism (Tatsumi et al., 2002); activation also occurs upon in vivo administration of TNFα (Li, 2003). It is noteworthy that p38α/β MAPKs are also activated by mechanical stress (Cowan and Storey, 2003; Kumar et al., 2003; Wretman et al., 2001), HGF (Recio and Merlino, 2002), TNFα (Geng et al., 1996; Roulston et al., 1998) and FGFs. Cellular stress responses often lead to an inflammatory response involving activation of p38α/β MAPKs in immune-responsive cells (Cowan and Storey, 2003; Kumar et al., 2003) and in skeletal muscle tissue (Wretman et al., 2001), where a severe stretch is capable of p38 activation. Our model (Fig. 9) for regulation of satellite cell activation proposes that these physiological events activating satellite cells is mediated via activation of p38α/β MAPKs. We further predict that the satellite cell quiescent state is maintained by inhibition of p38α/β MAPKs, presumably via a p38α/β MAPK phosphatase, perhaps MKP-1 (Bennett and Tonks, 1997).

We propose that activation of the skeletal muscle satellite cell, a well-studied adult somatic stem cell, is concomitant with the activation of p38α/β MAPKs and suggest that these MAPKs function as a molecular switch determining the activation state of the satellite cell. The universal response of the p38α/β MAPKs to stress suggests that similar mechanisms could be involved in the control of other somatic stem cell populations.

Materials and methods

Cell culture

MM14 cells were grown on gelatin-coated plates in growth media consisting of Ham’s F10C or F12C media supplemented with 15% horse serum as previously described (Clegg et al., 1987). FGF-2 was added in increasing concentrations (from 0.3 to 2.5 nM) every 12 h. Differentiation inducing culture media is comprised of Ham’s F10C or F12C media supplemented with 15% horse serum and no added FGF-2 unless otherwise noted. Primary myofibers were isolated and cultured as described previously (Cornelison et al., 2004).

Myofiber preparation, immunohistochemistry, and scoring

Myofibers with their associated satellite cells were prepared as described previously [Cornelison et al., 2004]. Briefly, muscle was dissected from adult mouse hindlimbs and digested with collagenase type I (Worthington) previously (Cornelison et al., 2004). In brief, muscle was dissected and digested with collagenase type I (Worthington) to yield single intact myofibers. BrdU is routinely added to cultures to facilitate cell cycle studies. Additional supplements to the medium included 50 μM SB203580 in DMSO or the DMSO carrier as a control. At the designated time points after harvest fibers were fixed and stained as described above.

Primary antibodies and dilutions used also included rabbit affinity purified polyclonal anti-p38 (C-20) (Santa Cruz Biotechnology, Inc.) at 1:50, mouse monoclonal anti–phospho-p38 (New England Biolabs, Inc.) at 1:50, mouse monoclonal anti–BrdU (BMB) at 1:10, mouse monoclonal anti–MyoD (Novacastra) at 1:10, and mouse monoclonal anti–myogenin (F5D; Cusella-DeAngelis et al., 1992), neat, chicken anti–mouse syndecan-4 (1:1,500). Secondary antibodies anti–rabbit, anti–mouse, and anti–chicken Alexa 488, anti–rabbit, anti–mouse Alexa 504, and anti–rat Cascade blue were purchased from Molecular Probes, Inc. and were used at 1:500 unless otherwise indicated. Counts of resident satellite cells per myofiber were done by counting DAPI-stained myofiber nuclei (which can be identified by their characteristic elongated shape) and DAPI-stained satellite cell nuclei coincident with syndecan-4–positive cell outlines. At least 20 myofibers containing at least 5,000 myonuclei total were counted per time point per condition. Counts of MyoD-positive satellite cells were done by counting MyoD-positive DAPI-stained nuclei within syndecan-4–positive cell outlines and comparing to counts of total DAPI-stained nuclei within syndecan-4–positive cell outlines.

Primary satellite cell cultures

Primary satellite cells were isolated and cultured as described previously (Cornelison et al., 2004) on gelatin-coupled coverslips, incubated in differentiation media for 48 h (with 2.5% horse serum), and 20 μM SB20350 or DMSO fixed and stained with anti–syndecan-4 and M20 antibodies at 1:1,500 and neat, respectively. Secondary antibodies were Alexa 488 and Alexa 504 at 1:500. Syndecan-4–positive cells were scored for MF-20 (MyHC) staining and plotted as a function of the syndecan-4–positive cells in the population.

Microscopy and image acquisition

All microscope images were obtained on a microscope (model E800; Nikon) using a 60× plan Apo lens at RT using a Cooke Sensicam digital camera and Intelligent Imaging Innovations Slidebook software to acquire images on a Macintosh computer. Fluorescence from labeled secondary antibodies was subtracted from all fluorescent images. Images were exported into Photoshop, if necessary the brightness and contrast was adjusted to just the entire image, the image cropped and individual color channels extracted without color correction adjustments or gamma adjustments.

Western analysis

MM14 cells were cultured on 100-mm plates under differentiating conditions (F10C supplemented with 1.5% horse serum) for 0, 6, 12, 24, and 72 h (five plates at a density of 250,000 cells per plate for each treatment). Total RNA was isolated as previously described (Chomczynski and Sacchi, 1987). 5 μg of total RNA was added to reverse transcriptase buffer (GIBCO BRL) containing 0.025 U oligo (dT)18 (GIBCO BRL), 0.01 M DTT (GIBCO BRL), 0.5 mM dNTP mix (GIBCO BRL), and 200 U of Superscript II reverse transcriptase (GIBCO BRL) and incubated for 50 min at 42°C. Nonreverse transcriptase controls were performed as described above with the exception of reverse transcriptase addition. PCR amplification was performed by adding increasing concentrations of cDNA in 2 μl (1:100, 1:10 dilution, undiluted) to PCR buffer containing 0.25 mM each dNTP, 1.5 mM MgCl2, 0.5 μM each of forward and reverse primers for ERK1/2, ERK3, ERK5, p38α/β, p38γ, MyoD, FGFR-1, 185 RNA, and SU of Taq polymerase (GIBCO BRL). Each reaction was amplified for 35 cycles using the following parameters: denaturation 94°C for 1 min, annealing at 50°C for 1 min, and elongation for 1 min. After amplification, each reaction was resolved on a 0.8% agarose gel containing ethidium bromide and visualized with a UV transilluminator.

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DNA synthesis assay

DNA synthesis was assayed by [³H]thymidine incorporation. In brief, MM14 cells were synchronized by mitotic shake-off and plated in 24-well plates at density 2,000 cells/well in the presence or absence of exogenous FGF. The cells were grown with the addition of 7.5 μM of DMSO or increasing concentrations of SB203580 for 8 h and then given 2 μCi of [³H]thymidine (Du Pont) in the presence or absence of exogenous FGF-2, and either 25 μM SB203580 or 500 μM FGF-2 was added at increasing time intervals. The cells were grown for a total of 14 h after plating. The amount of [³H]thymidine incorporated into DNA was determined by liquid scintillation counting as described previously.

For time course assays, MM14 cells were synchronized by mitotic shake-off and plated in growth media with 2 μCi of [³H]thymidine (Du Pont) in the presence or absence of exogenous FGF-2, and either 25 μM SB203580 or 500 μM FGF-2 was added at increasing time intervals. The cells were grown for a total of 14 h after plating. The amount of [³H]thymidine incorporated into DNA was determined by liquid scintillation counting as described previously. Thymidine incorporation for each sample was normalized to the thymidine incorporation of MM14 cells grown entirely in proliferating conditions.

Alternatively, MM14 cells were cultured in differentiation media and in the presence or absence of 25 μM SB203580 for 24 h. Cells were washed three times with PBS and media replaced with either growth or differentiation culture media with 2 μCi of [³H]thymidine (Du Pont). The cells were grown for 12 h and the amount of [³H]thymidine incorporated into DNA was determined by liquid scintillation counting. Thymidine incorporation for each sample was normalized to the thymidine incorporation of MM14 cells grown entirely in proliferating conditions.

Analysis of myosin heavy chain expression

MM14 cells were plated onto 6-well plates at a cell density of 8,000 cells/well in growth medium. After 6 h, either 2.5 μM of DMSO or SB203580 (50 μm) was added to the wells and cells were grown in the presence or absence of FGF-2 as described above. At either 36 h or 72 h after treatment, cells fixed and stained for myosin heavy chain expression as described previously (Kudla et al., 1995). MHC-positive cells were scored as a percentage of entire cell population. A minimum of 1,000 total cells was scored per plate. For time course assays, MM14 cells were synchronized by mitotic shake-off and plated in differentiation media and 25 μM SB203580 was added at increasing time intervals [0, 6, 12, 18, and 24 h] after plating. Cells were fixed 36 h after plating and stained for MHC expression. MHC-positive cells were scored as a percentage of entire cell population. A minimum of 1,000 total cells was scored per plate. Alternatively, MM14 cells were cultured in either proliferation media or differentiation media in the presence or absence of 25 μM SB203580. 36 h after plating, cells were either fixed and stained for MHC expression or wounded three times in PBS [to remove residual SB203580] and media replenished with either proliferation or differentiation media.

Immunoprecipitation

MM14 cells (3 x 10⁵ per plate, two plates each condition) in proliferation media were maintained in proliferation media or switched to differentiation media in the presence or absence of FGF-2 as described above. At either 36 h or 72 h after treatment, cells fixed and stained for myosin heavy chain expression as described previously. MHC-positive cells were scored as a percentage of entire cell population. A minimum of 1,000 total cells was scored per plate. For time course assays, MM14 cells were synchronized by mitotic shake-off and plated in differentiation media and 25 μM SB203580 was added at increasing time intervals [0, 6, 12, 18, and 24 h] after plating. Cells were fixed 36 h after plating and stained for MHC expression. MHC-positive cells were scored as a percentage of entire cell population. A minimum of 1,000 total cells was scored per plate. Alternatively, MM14 cells were cultured in either proliferation media or differentiation media in the presence or absence of 25 μM SB203580. 36 h after plating, cells were either fixed and stained for MHC expression or wounded three times in PBS [to remove residual SB203580] and media replenished with either proliferation or differentiation media.

Transient transfections

Clonal cell proliferation assay. MM14 cells were plated on 100-mm tissue culture plates at a density of 3,000 cells per plate and transfected as previously described. Calcium phosphate-DNA precipitates were made as previously described using 1 μg of a reporter construct containing the cytomegalovirus promoter driving the expression of β-galactosidase (CMV-LacZ) and 20 μg of either control DNA [pBSK+] or [Strategene] coMKK3, or dnMKK3 as described previously (Fedorov et al., 2002) or with Lipofectamine 2000 as per manufacturer’s instructions transfected with 1 μg of CMV-LacZ, p38M, or p38Δmut per well in a 6-well plate, transferred to 100-cm plates 12 h later and plated at clonal density, then fixed 40 h after plating and scored.

Muscle-specific reporter gene assay. MM14 cells were transfected and assayed as previously described (Fedorov et al., 2002) or plated at 30,000 cells/well (on a 6-well) 12 h before transfection using the Lipofectamine 2000 kit. For transfections, 2 μg of plasmid (either p38M, p38Δmut, or pDNA3) and 0.25 μg of LacZ and a muscle reporter (luciferase driven by the α-actin cardiac actin promoter) were added per well. All muscle reporter experiments used 20 μM SB203580 or an equivalent volume of DMSO in each well.

P38β/δ MAPK reporter assay

p38 MAPK activity was determined using PathDetect CHOP reporting system (Stratagene) (Xu and Cobb, 1997). For this assay, MM14 cells were plated on 6-well plates at a density of 10⁵ cells/well and cotransfected with 2.5 μg pFR-Luc reporter vector, 500 ng pFA-CHOP vector, and 1 μg CMV-LacZ vector per well. The cells were harvested and assayed for luciferase and β-galactosidase activities as described previously (Fedorov et al., 1998).

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