Stress-activated Protein Kinase-2/p38 and a Rapamycin-sensitive Pathway Are Required for C2C12 Myogenesis*

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The differentiation of C2C12 myoblasts to myotubes was found to be accompanied by a strong activation of p70 S6 kinase and the mitogen-activated protein kinase (MAPK) family member SAPK2/p38, without significant activation of p42 MAPK and only slight activation of SAPK1/JNK and protein kinase Bo. Consistent with these findings, SB 203580 (a specific inhibitor of SAPK2/p38) or rapamycin (which blocks the activation of p70 S6 kinase) prevented the formation of multinucleated myotubes, as well as the expression of muscle-specific proteins that included SAPK3 (another MAPK family member). PD 098059 (which prevents the activation of p42 MAPK) had no effect on myotube formation. Surprisingly, the slow activation of p70 S6 kinase during differentiation was not only prevented by rapamycin but also by SB 203580, and the activation of MAPKAP kinase-2 (an in vivo substrate of SAPK2/p38) was not only prevented by SB 203580 but also by rapamycin. In contrast, the acute activation of p70 S6 kinase in C2C12 myoblasts induced by phorbol esters was unaffected by SB 203580 and the acute activation of MAPKAP kinase-2 induced by anisomycin was unaffected by rapamycin. These results show for the first time that SAPK2/p38 plays an essential role in C2C12 cell differentiation.

The differentiation of skeletal muscle starts when myoblasts withdraw from the cell division cycle and is characterized morphologically by the alignment, elongation, and fusion of multinucleated myoblasts into multinucleated myotubes. Myogenesis is largely controlled by the myogenic basic helix-loop-helix family of transcription factors (MyoD, myogenin, myf5, and MRF4), and the myocyte enhancer factor-2 (1, 2), which control the expression of many muscle-specific genes, such as the myosin heavy chain and creatine kinase. Cell division is prevented during muscle differentiation by the induction of cyclin-dependent protein kinase inhibitors (3, 4).

Skeletal muscle differentiation is negatively influenced either by treating myoblasts with serum or growth factors (e.g. basic fibroblast growth factor-2, transforming growth factor β1) or by oncogenes, such as c-myc, c-jun, c-fos, Ha-ras, and E1a (1, 5, 6), and differentiation can be induced by switching the cells to medium containing low concentrations of serum. Some differentiation inhibitors activate the classical mitogen-activated protein kinase (MAPK)1 pathway, which has been implicated in regulating proliferation and differentiation in a variety of cells (7, 8). For this reason, the role of the MAPK pathway in regulating skeletal muscle differentiation has been studied extensively, but the results have been controversial. For example, the compound PD 098059 (which prevents the activation of MAPK by inhibiting the activation of MAPK kinase-1 (9)) was reported to increase the rate of myotube formation in the presence of insulin-like growth factor-1 (IGF-I) in L6 (10) and 23A2 cells (11), suggesting a negative role for MAPK in this process. In contrast, another laboratory reported that MAPK plays a positive role in myogenesis (12). They reported that MAPK activity is induced concomitantly with muscle differentiation in either C2 myoblasts or muscle cells derived from MyoD-expressing 10T1/2 fibroblasts. They also reported that PD 098059 partially inhibited the fusion of myoblasts to multinucleated myotubes and the induction of MyoD expression that normally takes place during terminal differentiation, without affecting the expression of certain muscle-specific proteins. Another group reported that MAPK has a negative role at the early stages of myogenesis because myoblasts overexpressing MAPK phosphatase-1 (MKP1) showed enhanced production of muscle-specific genes. However, overexpression of MKP1 inhibited myotube formation at later times, and it was suggested that the MAPK pathway plays positive, as well as negative roles in muscle differentiation (13). Other investigators reported that PD 098059 did not affect differentiation of C2 myoblasts, implying that the MAPK pathway is not required for this process (14). They also reported that wortmannin, an inhibitor of phosphatidylinositol 3-kinase, inhibits the IGF-I-dependent differentiation of C2 myoblasts.

MKP1 is not specific for MAPK but can also inactivate other MAPK family members in vitro, such as SAPK2a/p38α (15) and SAPK1/JNK (16). SAPK2a/p38α and SAPK1/JNK lie in distinct signal transduction pathways and, in many cells, become activated in response to cellular stresses (such as the protein synthesis inhibitor anisomycin), lipopolysaccharide, or proinflammatory cytokines (reviewed in Ref. 17). The effects of MKP1 on muscle differentiation (13) could therefore be explained by the inhibition of other MAPK cascades. For this reason, we have investigated the potential role of SAPK2/p38 in skeletal muscle differentiation by studying the effect of SB 203580, a specific inhibitor of both SAPK2a/p38α and its close relative SAPK2b/p38β (collectively referred to as SAPK2/p38) (18–21). We demonstrate that the SAPK2/p38 pathway is activated during C2C12 cell differentiation and that SB 203580 (but not PD 098059) blocks myotube formation and the expression of several muscle-specific genes; MKP1, MAPK phosphatase-1; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein, IGF-1, insulin-like growth factor-1; GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-acetate; MAPKAP-K2, MAPK activated protein kinase-2; FCS, fetal calf serum; PKB, protein kinase B; PI 3-kinase, phosphatidylinositol 3-kinase.

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MKP1, MAPK phosphatase-1; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein, IGF-1, insulin-like growth factor-1; GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-acetate; MAPKAP-K2, MAPK activated protein kinase-2; FCS, fetal calf serum; PKB, protein kinase B; PI 3-kinase, phosphatidylinositol 3-kinase.
Myoblasts were cultured in 20% FCS until 80–90% confluency, p42 MAPK (A), lysed at the times indicated, and MAPKAP-K2 (B), p70 S6 kinase (E), or PKBα (E) activities were measured. Cells in the differentiation medium for 0 or 6 days were stimulated for 15 min with 300 ng/ml PMA or 100 ng/ml IGF-1 or for 30 min with 10 μg/ml anisomycin before lysis. The results are presented as mean ± S.E. for three experiments. In panel C, PD 098059 (50 μM) was added 1 h before PMA.

**FIG. 1.** Activity of different kinases during C2C12 differentiation. Myoblasts were cultured in 20% FCS until 80–90% confluency, changed to differentiation medium containing 5% horse serum, and differentiation medium was added without or with 10 μM SB 203580 (19) or 100 nM rapamycin (24), 10 μM LY 294002 (10), or 50 μM PD 098059 (9). All the inhibitors were dissolved in dimethyl sulfoxide (Me2SO) to give concentrations of 1–50 μM, and 1 μl was added per 1 ml of culture medium. The equivalent volume of Me2SO was added to control cultures in every experiment. The medium was replaced with fresh medium (with or without inhibitors) every 24 h.

At various times, C2C12 cells were lysed in 50 mM Tris acetate, pH 7.5, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% (w/v) Triton X-100, 0.01% (w/v) Brij-35, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and leupeptin (5 μg/ml). The extracts were centrifuged for 5 min at 13,000 g of GST-c-Jun-(1–194) bound to GSH-agarose. The supernatant (term lymphate) was removed and either used immediately or quick frozen in liquid nitrogen and stored at −80 °C.

**Immunoprecipitation and Assay of Protein Kinases—**MAPKAP-K2 (22), p70 S6 kinase (25), protein kinase Be (PKBα) (26), and p42 MAPK (27) were immunoprecipitated from 0.1 mg of C2C12 lysates as described previously for other cell lines. After immunoprecipitation, MAPKAP-K2 was assayed using the peptide KKLNRILSVA (28), p70 S6 kinase using the specific peptide substrate KKKRRTRTV (29), PKBα using Crosstide (26), and p42 MAPK using MBP (27). To assay SAPK1/JNK in C2C12 extracts, 10 μg of GST-c-Jun (1–194) bound to GSH-agarose beads was incubated with cell lysate (150 μg) for 3 h at 4 °C to allow SAPK1/JNK to bind to its substrate GST-c-Jun (1–194). The beads were washed, and SAPK1/JNK activity was assayed as described previously (30). One unit of protein kinase is that amount which catalyzes the phosphorylation of 1 nmol of peptide or protein substrate in 1 min.
was gradually activated during differentiation (half-time 1 day), being maximally activated after 3 days as shown by the failure of PMA to increase activity further (Fig. 1B). These experiments also indicated that the expression of MAPKAP-K2 and p70 S6 kinase do not alter during differentiation.

In contrast to MAPKAP-K2 and p70 S6 kinase, p42 MAPK of the classical MAPK cascade was not activated at all during differentiation but was activated 6-fold in response to PMA (Fig. 1C). SAPK1/JNK was not activated significantly up to the first 3 days of differentiation, at which stage multinucleated myotubes predominated, but was slightly activated (1.8-fold) at 6 days when the myotubes were fully formed (Fig. 1D). The stimulation of myoblasts (day zero) or myotubes (day six) with anisomycin caused a four-fold activation of SAPK1/JNK (Fig. 1D). PKBo, which is activated in vivo via a phosphatidylinositol 3-kinase-dependent pathway, was slightly activated up to 6 days, but activation was only 5–10% of that which occurred after stimulation for a few minutes with IGF-1 (Fig. 1E).

Effect of Inhibitors of Different Protein Kinase Cascades on the Differentiation of C2C12 Cells—To further investigate the mechanisms involved in differentiation, we studied the effects of several inhibitors of particular signal transduction pathways on C2C12 cell morphology during differentiation. During the first 24 h of differentiation, the myoblasts elongated and aligned with each other while, in the following 48 h, multinucleated contractile myotubes were formed (Fig. 2B). When 10 μM SB 203580 was added to the differentiation medium, cells remained quiescent, and a delay in the elongation and alignment of myoblasts was observed after 3 days (Fig. 2C). Furthermore, even after 6 days in differentiation medium (data not shown), SB 203580 prevented the fusion of C2C12 myoblasts into multinucleated myotubes. Myotube formation was also largely (but not completely) blocked when 100 nM rapamycin or 10 μM LY 294002 (an inhibitor of PI 3-kinase) was present in the differentiation medium (Figs. 2, D and E). Consistent with the MAPK pathway not becoming activated during differentiation, 50 μM PD 098059 did not affect the rate of myotube formation (Fig. 2F) although the PMA-induced activation of MAPK was strongly suppressed by PD 098059 (Fig. 1C).

We next studied the effects of inhibitors on the expression or activity of several proteins whose levels rise during muscle differentiation (Fig. 3). SB 203580, rapamycin, and LY 294002 severely inhibited the induction of creatine kinase, myogenin, and myosin heavy chain after 3 days. In contrast, the induction of p21 was only partially suppressed by SB 203580 or rapamycin but was prevented by LY 294002. PD 098059 did not inhibit the induction of any of the four differentiation markers studied but actually appeared to increase creatine kinase activity. The level of p42 MAPK, which is not a muscle-specific protein, did not change during C2C12 differentiation and was not affected by any of the inhibitors (Fig. 3).

Effect of Rapamycin and SB 203580 on the Activation of SAPK2/p38 and p70 S6 Kinase and on the Expression of Creatine Kinase in C2C12 Cells—The lack of myotube formation in the presence of 10 μM SB 203580 or 100 nM rapamycin and the strong activation of MAPKAP-K2 and p70 S6 kinase during the conversion of C2C12 myoblasts to myotubes indicated that SAPK2/p38 and a rapamycin-sensitive pathway are required for differentiation. As expected from previous work in other cell
types, SB 203580 inhibited the activation of MAPKAP-K2, and rapamycin inhibited the activation of p70 S6 kinase (Fig. 4). Unexpectedly however, rapamycin also prevented the activation of MAPKAP-K2, whereas SB 203580 prevented the activation of p70 S6 kinase during the differentiation process (Fig. 4). In contrast, rapamycin had no effect on the acute activation of MAPKAP-K2 induced by anisomycin (Fig. 5A), and p70 S6 kinase (Fig. 5B), and creatine kinase (C) activities were assayed. The results are presented as averages (mean ± S.E.) for three separate experiments.

Expression of SAPK3 during C2C12 Differentiation—SAPK3 (also termed ERK6 (31) and p38γ (32)) is a MAPK family member that, when overexpressed in cell lines, can be activated by the same cellular stresses and proinflammatory cytokines as SAPK2/p38 (20). Moreover, SAPK3 mRNA expression is induced during the differentiation of C2C12 cells, and overexpression of SAPK3 in C2C12 cells enhances the fusion rate of the myoblasts (31). In the present study, we confirmed that the level of SAPK3 increases during C2C12 myotube formation (Fig. 7A) and also showed that induction is prevented by either SB 203580 or rapamycin (Fig. 7B) but not by PD 098059 (data not shown).

DISCUSSION

Previous work suggested both positive and negative roles for the classical MAPK cascade in the differentiation of myoblast cell lines to multinucleated myotubes (see the Introduction), but in this study, we failed to find any evidence for a role for p42 MAPK in C2C12 myogenesis. The differentiation of C2C12 cells was not accompanied by any activation or inhibition of p70 S6 kinase during the differentiation process (Fig. 4). In contrast, rapamycin had no effect on the acute activation of MAPKAP-K2 induced by anisomycin (Fig. 5A), and SB 203580 had no effect on the acute activation of p70 S6 kinase by PMA, in myotubes (Fig. 5B). Consistent with the effects of SB 203580 and rapamycin on the activation of MAPKAP-K2 and p70 S6 kinase, respectively, these inhibitors also prevented the induction of creatine kinase activity (Fig. 4C). The activation of MAPKAP-K2 and p70 S6 kinase, and the induction of creatine kinase during C2C12 differentiation, were also suppressed by LY 294002 (Fig. 4).

The elevation in creatine kinase activity (Fig. 4C) during differentiation paralleled the rise in the activation of MAPKAP-K2 (Fig. 4A) and p70 S6 kinase (Fig. 4B). The activation of MAPKAP-K2 and the induction of creatine kinase were both blocked by SB 203580 with IC50 values of <1 μM (Fig. 6A). The conversion of C2C12 myoblasts to myotubes was suppressed by SB 203580 at a similar concentration (Fig. 6B).
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FIG. 6. Inhibition of C2C12 cell differentiation at different SB 203580 concentrations. A, effect of SB 203580 on MAPKAP-K2 activation and creatine kinase activity during C2C12 differentiation. MAPKAP-K2 (open circles) and creatine kinase (closed circles) were assayed in the lysates of C2C12 cells that had been incubated for 3 days in differentiation medium containing the indicated concentrations of SB 203580. B, effect of SB 203580 on C2C12 cell morphology during differentiation. C2C12 cells were incubated for 3 days in differentiation medium containing the indicated concentrations of SB 203580.

FIG. 7. SAPK3 is induced during muscle differentiation. A, C2C12 myoblasts were differentiated for the times indicated. Cell lysates (30 μg of protein) were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose, and the samples were immunoblotted with anti-SAPK3 or anti-p42 MAPK antibodies. B, effect of rapamycin (Ra) or SB 203580 (SB) on the expression of SAPK3 and p42 MAPK. C2C12 myoblasts were differentiated for 3 days in the absence or presence of 10 μM SB203580 or 0.1 μM rapamycin. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted as in panel A.

muM LY 294002 could therefore be explained by the inhibition of mTOR, rather than the inhibition of PI 3-kinase. This would be consistent with the slight activation of PI 3-kinase during differentiation, which is indicated by the minute activation of PKB (Fig. 1E). However, LY 294002 completely prevents the induction of p21, whereas rapamycin only has a partial effect (Fig. 3). LY 294002 must therefore suppress the induction of p21, at least in part, by a rapamycin-insensitive pathway.

SAPK2/p38 is not inhibited by rapamycin (data not shown), and rapamycin does not prevent the acute activation of SAPK2/p38 by anisomycin (Fig. 5). It was therefore surprising that the slow (but strong) activation of SAPK2/p38 during C2C12 differentiation is suppressed by rapamycin (Fig. 4). This suggests that a rapamycin-sensitive pathway may induce the expression of a factor that triggers the activation of SAPK2/p38. Another unexpected observation was that the slow (but strong) activation of p70 S6 kinase during differentiation is not only blocked by rapamycin but also by SB 203580. SB 203580 does not inhibit mTOR because phorbol ester-induced activation of p70 S6 kinase (which is blocked by rapamycin) (Fig. 5) is unaffected by SB 203580. This suggests that the activation of SAPK2/p38 induces the expression of a factor that triggers the activation of mTOR and p70 S6 kinase. An intriguing possibility is that SAPK2/p38 and mTOR are both required to induce the same factor(s), which then activates both pathways creating a positive feedback loop that drives differentiation to completion. Whatever the mechanism, our results strongly suggest that SAPK2/p38 and mTOR both play essential roles in the differentiation of C2C12 myoblasts to myotubes.

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Myotube formation and muscle-specific gene expression is also blocked by rapamycin in C2C12 (Figs. 2 and 3) or L6A1 myoblasts (10). Rapamycin interacts with FK506-binding protein (FKBP), and the rapamycin-FKBP complex then inhibits the protein kinase termed mammalian target of rapamycin (mTOR) (33). mTOR phosphorylates 4EBP1, which may stimulate protein synthesis by triggering its dissociation from eIF4E (34). mTOR is also required for the activation of p70 S6 kinase (35), but whether it phosphorylates p70 S6 kinase directly or suppresses the activity of a p70 S6 kinase phosphatase is unclear.

LY 294002 is best known as an inhibitor of PI 3-kinase (24), but it also inhibits other members of the PI 3-kinase superfamily. This includes mTOR, which is inhibited by LY 294002 with an IC50 of 5 μM (36). The blockade of C2C12 myogenesis by 10 μM LY 294002 could therefore be explained by the inhibition of mTOR, rather than the inhibition of PI 3-kinase. This would be consistent with the slight activation of PI 3-kinase during differentiation, which is indicated by the minute activation of PKB (Fig. 1E). However, LY 294002 completely prevents the induction of p21, whereas rapamycin only has a partial effect (Fig. 3). LY 294002 must therefore suppress the induction of p21, at least in part, by a rapamycin-insensitive pathway.

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