The p38α/β Mitogen-activated Protein Kinases Mediate Recruitment of CREB-binding Protein to Preserve Fast Myosin Heavy Chain IId/x Gene Activity in Myotubes*

Received for publication, September 25, 2006, and in revised form, December 13, 2006 Published, JBC Papers in Press, January 7, 2007, DOI 10.1074/jbc.M609076200

Joachim D. Meissner†1, Kin-Chow Chang†, Hans-Peter Kubis‡, Angel R. Nebreda*, Gerolf Gros†, and Renate J. Scheibe‡1

From the Departments of †Physiology and ‡Biochemistry, Hannover Medical School, D-30625 Hannover, Germany, the *Division of Animal Production & Public Health, University of Glasgow Veterinary School, G61 1QH Glasgow, United Kingdom, and the ‡CNIO (Spanish National Cancer Centre), 28029 Madrid, Spain

In skeletal muscle, the transformation of fast into slow fiber type is accompanied by shifts in fiber type-specific gene expression that includes down-regulation of the adult fast fiber myosin heavy chain IId/x (MyHCIIId/x) gene. Here, we report that the mitogen-activated protein kinases (MAPKs) p38α/β regulate MyHCIIId/x gene expression. Electrical stimulation of rabbit skeletal muscle cells with a slow fiber type activity pattern and treatment of C2C12 myotubes with Ca2+-ionophore inhibited p38α/β MAPKs and reduced fast fiber type MyHC protein expression and promoter activity. Pharmacological inhibition of p38α/β also down-regulated MyHCII gene expression. In controls, binding of the myocyte enhancer factor-2 (MEF-2) isoforms C and D as a heterodimer to a proximal consensus site within the MyHCIIId/x promoter and recruitment of a transcriptional coactivator, the CREB-binding protein CBP, were observed. Overexpression of wild type MEF-2C but not of a MEF-2C mutant that cannot be phosphorylated by p38 induced promoter activity. Mutation of the MEF-2-binding site decreased the inducing effect of overexpressed CBP. Inhibition of p38α/β MAPKs abolished CBP binding, whereas enforced induction of p38 by activated MAPK kinase 6 (MKK6EE) enhanced binding of CBP and increased promoter activity. Furthermore, knockdown of endogenous CBP by RNA interference eliminated promoter activation by MEF-2C or MKK6EE. In electrical stimulated and Ca2+-ionophore-treated myotubes, CBP was absent in complex formation at that site. Taken together, the data indicate that p38α/β MAPKs-mediated coactivator recruitment at a proximal MEF-2 site is important for MyHCIIId/x gene regulation in skeletal muscle.

Skeletal muscle fibers have been classified into fiber types based on their contraction speed, force development, fatigability, and metabolic functions (1). The characteristic fiber types are fast twitch glycolytic (type IID/X and IIB), fast twitch oxidative/glycolytic (type IIA), and slow twitch oxidative (type I/β). Fast fibers express the IID/X, IIB, or IIA isoform of the myosin heavy chain (MyHC), whereas slow fibers predominantly express the type I/β isoform. The functional, biochemical, and morphological differences between fiber types are a consequence of different fiber-specific gene expression patterns. Fibers are characterized by a remarkable plasticity and can be modulated by chronic low frequency electrical stimulation depending on the imposed activity pattern (2). Physiologically, this transformation process in fiber type occurs in response to altered demands, such as increases in muscle activity (3). Fiber type shifts are also observed during aging and disease. The importance of changes in intracellular Ca2+ as a trigger for fiber type transformation has clearly been demonstrated by a Ca2+-ionophore-induced fast-to-slow transformation in primary skeletal muscle cells (4, 5). The involvement of calcineurin, a Ca2+-calmodulin-regulated serine/threonine phosphatase, in transducing the Ca2+-signal into altered gene expression in skeletal muscle has been established (6, 7).

The mitogen-activated protein kinase (MAPK) p38 is pivotal for muscle differentiation based on studies with myogenic cell lines (8, 9). Unlike the prototypical activation program of p38 by stress and proinflammatory cytokines (10), an independent and persistent p38 activation occurs in differentiating muscle cells (11). Furthermore, the p38 MAPK has been implicated in adaptive processes in heart (12) as well as in skeletal muscle (13, 14). So far, little is known about a possible role for p38 in the regulation of gene expression in adult skeletal muscle cells. Four isoforms of p38 have been identified and characterized (10). Isoforms α and β are expressed ubiquitously, whereas p38γ is exclusively expressed in skeletal muscle. Isoform δ is not expressed in skeletal muscle. The kinases are activated through phosphorylation through the upstream dual specificity kinases MAPK kinase (MKK) 3 and 6 (15). Constitutively active MKK6

8 This work was supported by Deutsche Forschungsgemeinschaft Grants GR489/13 and GR489/20. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Physiology, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany, Tel.: 49-511-532-2753; Fax: 49-511-532-2938; E-mail: meissner.joachim@mh-hannover.de.

2 Present address: School of Sport, Health and Exercise Sciences, University of Wales, Bangor, UK.

The abbreviations used are: MyHC, myosin heavy chain; CBP, CREB-binding protein; CREB, cyclic AMP-responsive element binding protein; CsA, cyclosporin A; EMSA, electrophoretic mobility shift assay; MAPK, mitogen-activated protein kinase; MEF-2, myocyte enhancer factor 2; MKK, MAPK kinase; MKP-1, MAPK phophatase-1; DME, Dulbecco’s modified Eagle’s medium; DM, differentiation medium; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; siRNA, small interfering RNA; P-p38, phosphorylated p38.
*p38α/β MAPks Regulate MyHCIId/x Promoter Activity*

(MKK6EE) reportedly promotes expression of fast but not slow MyHC protein while enhancing differentiation of C2C12 muscle cells (16). However, in C2C12 myotubes fast MyHClId promoter activity was found to be unaffected by p38 inhibition (17).

The p38 MAPK pathway promotes skeletal muscle differentiation at least in part via muscle regulatory factors, recruitment of chromatin remodelling enzymes, and the transcription factor myocyte enhancer factor-2C (MEF-2C) (9, 18). The MEF-2 family is a key factor for controlling gene expression in myocytes (19). The four isoforms A, B, C, and D are highly expressed in skeletal muscle in distinct but overlapping patterns during differentiation and in the adult muscle. Binding sites of MEF-2 are important for expression of many muscle-specific genes (19). MEF-2 together with other transcription factor binding activities are thought to be linked to quantitative differences in MyHC isoform expression in mouse skeletal muscle (20). The transcriptional activity of the MEF-2A and C isoforms is stimulated by p38α and β2 through direct phosphorylation (21–24). Combinatorial action of MEF-2 through protein–protein interactions with other transcription factors, transcriptional coactivators, or corepressors (19, 25) are well known. For example, during muscle differentiation, MEF-2 interacts directly with muscle regulatory factors (26) and also with coactivator p300 (27).

The transactivation function of transcription factors is often mediated by coactivators with histone acetyltransferase activity like p300 and the cAMP-responsive element binding protein (CREB)-binding protein (CBP) (28). The histone acetyltransferase activity is important for chromatin remodeling to facilitate access of transcription factors and of the basal transcriptional machinery to DNA (28). In addition, histone acetyltransferases can modify transcription factors and serve as a scaffold for building of multicomponent transcriptional complexes (29). Their modulation of transcription factors alters gene expression. The interaction of p300 with MEF-2C in differentiating C2C12 cells and subsequent acetylation of the transcription factor resulted in enhanced DNA binding and transcriptional activity (30). Despite their high degree of homology, p300 and CBP are not completely redundant but play unique and distinct roles in gene regulation (28). They can differentially associate with other proteins and show differences in substrate specificity. Transcriptional coactivators are controlled by an array of various covalent modifications, suggesting the existence of a “coactivator code” that regulates their function in transcriptional gene regulation (31). For example, the function of both p300 and CBP can be regulated by phosphorylation (32), consistent with the emerging role of transcriptional coactivators as primary targets of physiological signals (33).

In the present study, we investigated the possibility that p38 MAPK regulates gene activities in differentiated skeletal muscle cells. We used myotubes of the C2C12 cell line and of a rabbit primary skeletal muscle cell culture that has been shown previously to develop exclusively adult MyHC isoforms (4, 5). We provide evidence that inhibition of p38α/β MAPKs resulted in the down-regulation of fast adult MyHClId/x gene activity. In turn, activated p38α/β MAPKs mediated MyHClId/x gene expression via recruitment of transcriptional coactivator CBP to a MEF-2C/D heterodimer complex at a proximal MEF-2-binding site in the promoter. Recruitment of CBP was inhibited by Ca2+-ionophore or electrical stimulation. Our data reveal a new role for p38α/β MAPKs in regulating MyHClId/x gene activity during fiber type transformation in skeletal muscle.

**MATERIALS AND METHODS**

**Cell Culture and Electrical Stimulation—Mouse C2C12 myoblasts (ATCC) were grown on Petri dishes in growth medium, consisting of complete Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum. At confluence, growth medium was replaced by differentiation medium (DM, DMEM plus 5% horse serum). COS-7 cells (ATCC) were grown in 10% DMEM supplemented with 10% fetal bovine serum. The mouse embryonic fibroblasts p38α/β cells, derived from p38α-based targeted inactivation of the mouse p38α gene (34), and wild type cells were grown in DMEM supplemented with 10% fetal bovine serum. All of the medium was replaced every 24 h. In some experiments, the cells were treated with 0.1 μM of Ca2+-ionophore A23187 (Sigma) in the presence or absence of 1 μM SB203580 (Sigma) or 0.1 μM Ca2+-ionophore in the presence or absence of different calcineurin inhibitors. To inhibit calcineurin, 500 ng/ml cyclosporin A (CsA) (Sigma), 80 ng/ml FK506 (Alexis Biochemicals), or 50 μM cell-permeable calcineurin autoinhibitory peptide (11R-CaN-AID) (Calbiochem), respectively, were used. In addition, some cultures were transiently transfected with an expression vector for constitutively active MKK6, pCDNA3-MKK6EE. Rabbit primary skeletal muscle cells were isolated, cultured for 14 days, and stimulated electrically with a slow fiber type activity pattern (45-min stimulation periods with 1 Hz for 15 min followed by a 30-min pause) for additional 4 or 6 days as described previously (6).

**Plasmid Construction—**To generate mutated MEF-2C T293A that cannot be phosphorylated by p38 in muscle cells (24), full-length MEF-2C (GenBank accession number NM025282) was mutated by changing nucleotide 849 A to G using the QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The MEF-2C expression plasmid pcDNA1-MEF-2C (35) was kindly provided by Dr. E. Olson. To generate MyHClId/x-2.8ΔMEF2, again the QuikChange II site-directed mutagenesis kit (Stratagene) was used.

**Transient Transfections and Luciferase Reporter Assays—**C2C12 myoblasts were transfected in growth medium at 50–60% confluence with 1.5 μg of promoter DNA (32), 0.35 μg of pCMV-Gal, 0.75 μg of expression plasmid or empty expression vector, and 2.5 μl/μg DNA of Lipofectamine 2000 transfection reagent (Invitrogen). After further 24 h, growth medium was replaced by DM (DMEM plus 5% horse serum), and myotubes were harvested 3 days after transfection. Mouse embryonic fibroblasts were transfected at 70% confluence with 1.5 μg of MyHC promoter DNA, 0.35 μg of pCMV-Gal, 0.75 μg of expression plasmid or empty expression vector, and 2.5 μl/μg DNA of Lipofectamine 2000 transfection reagent and harvested 2 days after transfection. COS-7 cells were transfected at 60–70% confluence with 1.0 μg of promoter DNA, 0.3 μg of...
pCMV-Gal, 0.5 μg of expression plasmid or empty expression vector, and 1.5 μl/μg DNA of Transfexat transfection reagent (Promega) and harvested 3 days after transfection. Treatment of cells (see above) started 1 day after transfection.

After lysis in 1× reporter lysis buffer (Promega), luciferase activity was determined using a Lumat Luminometer (Berthold Technologies). The luciferase assay reagent was composed of 200 mM Tricine, 10.7 mM (MgCO3)4Mg(OH)2 × 5H2O, 26.7 mM MgSO4 × 7H2O, 333 mM dithiothreitol, 5.3 mM ATP, 2.74 mM coenzyme A, and 470 μM-luciferin (Applichem). The cells were cotransfected with pCMV-Gal as an internal reference. The β-galactosidase activity was estimated in a standard assay (36).

RNA Interference Assays—C2C12 myoblasts transfected with MyHCId/x promoter DNA and MKK6EE or MEF-2C expression vector, or empty expression vector, respectively, were cotransfected after 1 day with 0.825 μg of a pool of double-stranded 20–25-nucleotide siRNA that specifically target mouse CBP (siRNA CBP) or a nonspecific double-stranded control siRNA (Santa Cruz Biotechnology, Inc.) and 8 μl/μg siRNA of siRNA transfection reagent (Santa Cruz Biotechnology, Inc.) according to the manufacturer’s instructions.

Immunofluorescence Studies—For immunofluorescence studies C2C12 myoblasts were seeded on glass coverslips and cultured for 6 days in DM. After 2 days in DM, the cells were treated with 0.1 μM of Ca2+-ionophore A23187, or 500 ng/ml CsA, or ionophore plus CsA for a period of 4 d. For immunofluorescence studies C2C12 myoblasts were seeded on glass coverslips and cultured for 7 days in DM as controls. After 3 days in DM, other cells were subjected to different treatments as described above for a period of 4 d. The cells were washed, fixed, and stained as described previously (37). For immunofluorescence studies anti-p38 (Santa Cruz Biotechnology, Inc.), anti-fast MyHC (Sigma), or anti-α-tubulin (Santa Cruz Biotechnology, Inc.) antibodies. Bound antibodies were detected with either anti-rabbit IgG or anti-mouse IgG conjugated to horseradish peroxidase (Promega). Signals were visualized by enhanced chemiluminescence detection.

RESULTS

p38 MAPK Inhibition by Electrical Stimulation and Ca2+-Ionophore Induces a Decrease in Fast Type MyHCII Protein Expression and MyHCId/x Promoter Activity—We investigated a possible role of p38 MAPK in regulating fiber type-specific gene expression. First, immunofluorescence microscopy of C2C12 cells grown in differentiation media for 7 days revealed multinuclear myotubes with a high expression of fast MyHCII (Fig. 1A) and minor expression of slow protein isoforms (data not shown and Ref. 40), representing a fast fiber type-like character of the cells. Following addition of 0.1 μM Ca2+-ionophore A23187 a decrease in fast adult MyHCII (Fig. 1, A and B) and an increase in slow MyHC proteins (data not shown and Ref. 40) were detected by immunofluorescence and Western blot analysis, indicating a fast-to-slow transformation. In our second cell system, primary rabbit skeletal muscle cells were grown in suspension culture for 2–3 weeks. These cultures have been shown previously to develop myotubes expressing predominantly fast adult MyHC isoforms (4). Electrical stimulation of primary skeletal muscle myotubes with a slow fiber type activity pattern for 6 days caused a reduction of fast adult MyHCII protein (Fig. 1, A and B). We further determined p38 MAPK activation in C2C12 myotubes and rabbit skeletal muscle cells by analyzing the level of phosphorylation of p38 MAPK. Immunofluorescence (Fig. 2A) and Western blot analysis (Fig. 2B) revealed P-p38 MAPK in unstimulated or untreated myotubes, indicating activation of p38 MAPK in these cells. Electrical stimulation of rabbit skeletal muscle cells using a slow fiber type pattern or treatment of C2C12 myotubes with Ca2+-ionophore inhibited p38 MAPK phosphorylation and therefore significantly decreased enzyme activation.

It has been shown in cardiac myocytes that activation of the protein phosphatase calcineurin with Ca2+-ionophore can enhance MAPK phophatase-1 (MKP-1) expression and decrease p38 activity (41). Furthermore, we have shown recently that the calcineurin has a small but significant effect on the activity of the MyHCId/x promoter (40). Therefore, the possibility of cross-talk between calcineurin and p38 was investigated by using calcineurin inhibitors CsA (500 ng/ml) (42), FK506 (80 ng/ml), or cell-permeable calcineurin autoinhibitory peptide (11R-CaN-AID, 50 μM) (43), respectively. The addition of CsA, or FK508, or 11R-CaN-AID, respectively, to controls did not affect the level of phosphorylated p38 and did not abolish the prominent decrease in the level of phosphorylated p38 in Ca2+-ionophore-treated cells (Fig. 2A). To conclude, Ca2+-ionophore treatment induces a decrease in p38 activation in a calcineurin-independent manner in C2C12 myotubes.
To establish a functional link between p38 MAPK inhibition and down-regulation of the MyHCII gene, we tested whether inhibition of p38 MAPK by addition of 1 μM of the pyridinyl imidazole compound SB203580 has an effect on MyHCII protein expression and the activity of a −2.8kb MyHCII/d/x promoter luciferase construct. SB203580, a pharmacological inhibitor specific for p38α/β MAPKs does not affect the phosphorylation status (Fig. 2A) but inhibits kinase activity by binding to its active site (44, 45). In C2C12 cells, SB203580 inhibited fast MyHCII protein expression (Fig. 1A and B) and the activity of the transiently transfected −2.8 kb MyHCII/d/x promoter (Fig. 3A). As detected with MyHCII protein levels (Fig. 1A), MyHCII/d/x promoter activity was also lowered by the addition of Ca²⁺-ionophore to C2C12 cells (Fig. 3A).

We next investigated whether p38 MAPK activation can be restored in Ca²⁺-ionophore-treated C2C12 myotubes by transient transfection of a constitutively active mutant of the direct upstream MAP kinase 6, M KK6EE. Immunofluorescence and Western blot analysis (Fig. 2, C and D) revealed a significant increase in p38 phosphorylation. In untreated C2C12 myotubes, p38 MAPK phosphorylation was slightly increased. In line with the effect of inhibition of p38α/β MAPKs, in myotubes overexpressing M KK6EE increased MyHCII/d/x promoter activity occurred in the absence of Ca²⁺-ionophore (Fig. 3A). In addition, the inhibitory effect on MyHCII protein expression (Fig. 1, compare A and C) and MyHCII/d/x promoter activity (Fig. 3A) by increased intracellular Ca²⁺ was clearly diminished without fully restoring the level of basal promoter activity. The data indicate that p38α/β MAPKs are necessary components for MyHCII/d/x promoter basal activity, and inhibition of the kinases is important but not sufficient for down-regulation of MyHCII/d/x gene expression during fiber type transformation.

Both p38α and β MAPKs and MEF-2 Regulate MyHCII/d/x Promoter Activity A possible candidate to mediate the p38α/β MAPKs signal is the MEF-2 transcription factor family isoform C, a direct substrate of the kinases (21–24). Forced expression of MEF-2C resulted in a transcriptional stimulation of MyHCII/d/x promoter activity in C2C12 cells (Fig. 3B) and fibroblast-like COS-7 cells (Fig. 3C). Inhibition of p38 MAPK with 1 μM SB203580 abolished the activating effect of MEF-2C on the MyHCII/d/x promoter activity in both cell lines, and coexpression of MEF-2C and M KK6EE synergistically activated the promoter. The inability of overexpressed M KK6EE alone to activate the promoter in COS-7 cells (Fig. 3C) could be explained by their lack of endogenous MEF-2 activity (46). Taken together, MEF-2C mediates the p38 MAPK signaling to the MyHCII/d/x promoter. It has been demonstrated previously that activation of MEF-2C transactivation function by p38 in muscle cells only requires the direct phosphorylation of one amino acid residue, threonine 293 (24). Compared with wild type MEF-2C, overexpression of mutated MEF-2C with a single amino acid substitution (T293A) nearly completely abolished the MEF-2C effect on the MyHCII/d/x promoter activity (Fig. 3D). The data demonstrate the importance of MEF-2C for the p38 effect on the MyHCII/d/x promoter.

We next investigated whether another MEF-2 isoform, MEF-2D, can affect MyHCII/d/x promoter activity. Ectopic expression of MEF-2D also transactivated the MyHCII/d/x promoter in C2C12 and COS-7 cells but, in line with the assumption that MEF-2D is not a target of p38α/β (22, 23), its coexpression with M KK6EE did not result in additional promoter activity (Fig. 3B and C). The small inhibitory effect of SB203580 on MEF-2D-mediated promoter induction might reflect the decrease of transcriptional activity of the endogenous MEF-2C by the compound in C2C12 myotubes. Thus, the effect of p38 MAPKα/β on MyHCII/d/x promoter activity is mainly mediated via MEF-
stressed promoter activity in p38α−/− cells, suggesting that p38β2 isoform to be involved to a small extent in that regulation process. As expected, MyHCIId/x promoter activity was also increased by MEF-2C in wild type MEF cells. Additional overexpression of p38α alone or coexpression with MKK6EE profoundly activates the MyHCIId/x promoter in p38α−/− cells to levels higher than in mouse embryonic fibroblast wild type cells with endogenous kinases only. Thus, p38α MAPK has a major and p38β a minor impact on maintenance of high MyHCIId/x promoter activity in fast fiber type myocytes.

The p38α/β MAPKs Mediated Recruitment of CBP to a MEF-2 Site Was Inhibited by Ca2+/Ionophore or Electrical Stimulation—In the search for MEF-2 binding sites within the MyHCIId/x promoter, we chose a short −500 bp upstream region because overexpression of MEF-2C and 2D can also efficiently activate a −500 bp promoter fragment that was also inhibited by treatment of cells with Ca2+/ionophore or SB203580 (data not shown). A putative MEF-2 consensus binding site at −233/−224 bp was identified. Mutating this putative MEF-2 site led to a pronounced reduction of the promoter activity (Fig. 3F), demonstrating the importance of the site for MyHCIId/x promoter basal activity. Furthermore, overexpression of MKK6EE had only a small activating effect on MyHCIId/x MEF2 promoter activity (Fig. 3, compare A and F), underlining the importance of the MEF-2 site for p38 MAPK-mediated activation of the promoter.

Using an oligonucleotide containing the MEF-2 site as a probe in EMSAs, nuclear extracts of untreated C2C12 myotubes showed two complex formations (Fig. 4A, lane 2), which were efficiently competed by 200-fold excess of unlabeled probe but not by the probe mutated within the MEF-2 core binding region, indicating specificity of complex formation (lanes 3 and 4). Specific antibodies against MEF-2C or 2D supershifted both complexes, whereas preimmune serum had no effect (lanes 8–10), demonstrating binding of MEF-2C and 2D as a heterodimer. Interestingly, the major complex formed with nuclear extracts

FIGURE 2. The p38 MAPK in C2C12 myotubes and primary rabbit skeletal muscle cells is inhibited by Ca2+/Ionophore and electrical stimulation. A and B, immunofluorescence (A) and Western blot analysis (B) of phosphorylated (activated) (P-p38) and total p38 MAPK (p38) in C2C12 myotubes and primary skeletal muscle myotubes as demonstrated by probing the blot with anti-P-p38 and reprobing with anti-p38 antibodies. The cells were grown in the presence or absence of p38α/β inhibitor SB203580, Ca2+/Ionophore, Ca2+/Ionophore plus SB203580, or Ca2+/Ionophore in the presence or absence of calcineurin inhibitors CsA, FK506, or cell-permeable calcineurin autoinhibitory peptide (11R-CaN-AID), respectively, for 4 days as indicated. C and D, immunofluorescence (C) and Western blot analysis (D) of phosphorylated (activated) and total p38 MAPK in C2C12 cells transfected with MKK6EE (+) or the empty expression vector (−) and cultured for 3 days in the presence or absence of Ca2+/Ionophore as indicated.

2C, whereas MEF-2D transactivates the MyHCIIId/x promoter independent of the kinases.

To verify the role of active p38 MAPK in maintaining high levels of MyHCIId/x promoter activity, we used a mouse embryonic fibroblast line derived from p38α knockout mice (34). Clearly, promoter activity was reduced in p38α-deficient mouse embryonic fibroblast cells compared with wild type cells (Fig. 3E, lanes 3 and 1). Expression of exogenous MEF-2C, which can be activated by p38 isoforms α and β2 (23), slightly

EMSAs, nuclear extracts of untreated C2C12 myotubes showed two complex formations (Fig. 4A, lane 2), which were efficiently competed by 200-fold excess of unlabeled probe but not by the probe mutated within the MEF-2 core binding region, indicating specificity of complex formation (lanes 3 and 4). Specific antibodies against MEF-2C or 2D supershifted both complexes, whereas preimmune serum had no effect (lanes 8–10), demonstrating binding of MEF-2C and 2D as a heterodimer. Interestingly, the major complex formed with nuclear extracts

FIGURE 2. The p38 MAPK in C2C12 myotubes and primary rabbit skeletal muscle cells is inhibited by Ca2+/Ionophore and electrical stimulation. A and B, immunofluorescence (A) and Western blot analysis (B) of phosphorylated (activated) (P-p38) and total p38 MAPK (p38) in C2C12 myotubes and primary skeletal muscle myotubes as demonstrated by probing the blot with anti-P-p38 and reprobing with anti-p38 antibodies. The cells were grown in the presence or absence of p38α/β inhibitor SB203580, Ca2+/Ionophore, Ca2+/Ionophore plus SB203580, or Ca2+/Ionophore in the presence or absence of calcineurin inhibitors CsA, FK506, or cell-permeable calcineurin autoinhibitory peptide (11R-CaN-AID), respectively, for 4 days as indicated. C and D, immunofluorescence (C) and Western blot analysis (D) of phosphorylated (activated) and total p38 MAPK in C2C12 cells transfected with MKK6EE (+) or the empty expression vector (−) and cultured for 3 days in the presence or absence of Ca2+/Ionophore as indicated.

2C, whereas MEF-2D transactivates the MyHCIIId/x promoter independent of the kinases.

To verify the role of active p38 MAPK in maintaining high levels of MyHCIIId/x promoter activity, we used a mouse embryonic fibroblast line derived from p38α knockout mice (34). Clearly, promoter activity was reduced in p38α-deficient mouse embryonic fibroblast cells compared with wild type cells (Fig. 3E, lanes 3 and 1). Expression of exogenous MEF-2C, which can be activated by p38 isoforms α and β2 (23), slightly

EMSAs, nuclear extracts of untreated C2C12 myotubes showed two complex formations (Fig. 4A, lane 2), which were efficiently competed by 200-fold excess of unlabeled probe but not by the probe mutated within the MEF-2 core binding region, indicating specificity of complex formation (lanes 3 and 4). Specific antibodies against MEF-2C or 2D supershifted both complexes, whereas preimmune serum had no effect (lanes 8–10), demonstrating binding of MEF-2C and 2D as a heterodimer. Interestingly, the major complex formed with nuclear extracts
p38α/β MAPks Regulate MyHClId/x Promoter Activity

A (x10^3) C2C12 cells

MyHCId/x-2.8-luc (RLU/β-Gal)

|          | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|----------|---|---|---|---|---|---|---|
| MKK6EE   | - | - | - | - | - | - | - |
| pcDNA    | - | - | + | + | + | + | + |
| Ionophore| - | - | + | + | + | + | + |
| SB203580 | - | - | + | - | + | + | + |

B (x10^3) C2C12 cells

MyHCId/x-2.8-luc (RLU/β-Gal)

|          | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|----------|---|---|---|---|---|---|---|---|---|
| MEF-2C   | - | - | - | - | + | + | + | + | + |
| MEF-2D   | - | + | + | + | + | + | + | + | - |
| MKK6EE   | - | - | + | + | + | + | + | + | + |
| pcDNA    | - | - | - | - | + | + | + | + | + |
| SB203580 | - | - | - | - | + | + | + | + | + |

C (x10^3) COS-7 cells

MyHCId/x-2.8-luc (RLU/β-Gal)

|          | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|----------|---|---|---|---|---|---|---|---|---|
| MEF-2C   | - | - | - | - | + | + | + | + | + |
| MEF-2D   | - | + | + | + | + | + | + | + | - |
| MKK6EE   | - | - | + | + | + | + | + | + | + |
| pcDNA    | - | - | - | - | + | + | + | + | + |
| SB203580 | - | - | - | - | + | + | + | + | + |

D (x10^3) C2C12 cells

MyHCId/x-2.8-luc (RLU/β-Gal)

|          | 1 | 2 | 3 |
|----------|---|---|---|
| MEF-2C   | - | + | - |
| MEF-2C(T223A) | + | - | - |
| pcDNA    | + | - | - |

E (x10^3) MEF-cells

MyHCId/x-2.8-luc (RLU/β-Gal)

|          | 1 | 2 | 3 | 4 | 5 | 6 |
|----------|---|---|---|---|---|---|
| p38α wt  | - | - | + | + | - | - |
| p38α -/- | - | + | + | + | - | - |
| MKK6EE   | - | - | - | - | + | + |
| pcDNA    | + | + | + | + | + | + |

F (x10^3)

Promoter Activity (RLU/β-Gal)

|          | 1 | 2 | 3 |
|----------|---|---|---|
| ilid/x-2.8wt | - | + | + |
| ilid/x-2.8 LMEF | + | - | - |
| MKK6EE   | - | - | + | + | + | + |
| pcDNA    | + | + | + | + | + | + |
of untreated myotubes (lane 2) migrated at a lower mobility than the second weaker band or bands obtained with in vitro translated MEF-2C or 2D (lanes 6 and 7), indicating an additional factor(s) bound in that complex. Because MEF-2 can directly recruit transcriptional coactivators (25, 27), we investigated involvement of coactivators in upper band complex formation and identified the coactivator CBP, a histone acetyltransferase important for chromatin remodelling, as part of the MEF-2-containing complex by its supershift with anti-CBP antibodies (lane 11).

In EMSAs with nuclear extracts from Ca\(^{2+}\)-ionophore-treated C2C12 myotubes, a single complex was obtained using the MEF-2 probe (Fig. 4B, lane 1) showing similar migration as the lower band of untreated cells. That complex was also efficiently competed by 200-fold excess of unlabeled probe but not by the mutated oligonucleotide (lanes 2 and 3). Furthermore, the complex was supershifted by anti-MEF-2C and -2D antibodies, whereas an anti-CBP antibody was ineffective (lanes 5–7), reflecting binding of the MEF-2/C/D heterodimer and Ca\(^{2+}\)-ionophore-induced absence of the coactivator. Similarly, treatment of C2C12 myotubes with SB203580 prevented binding of CBP to the MEF-2/C/D complex (Fig. 4A, lanes 13–15). Again, only one complex was found (lane 12). Enforced activation of the p38 MAPK pathway by MKK6EE led to the disappearance of the faster migrating band and increased CBP binding to the MEF-2 complex in untreated C2C12 cells (Fig. 4B, lanes 12 and 13, compare with Fig. 4A, lanes 2 and 11). Furthermore, an additional slower migrating complex (Fig. 4B, lane 8) and a supershift (lane 11) were found in Ca\(^{2+}\)-ionophore-treated cultures and indicated restored CBP binding. The data indicate that Ca\(^{2+}\)-ionophore-induced inhibition of p38 MAPK blocks recruitment of the coactivator CBP to a MEF-2/C/D heterodimer complex at the MyHCId/x promoter in C2C12 myotubes.

As shown in Fig. 4C, again two complexes were formed with nuclear extracts of unstimulated primary rabbit skeletal myotubes (lane 2) and efficiently competed by 200-fold excess of unlabeled MEF-2 probe but not by the probe mutated within the MEF-2 core binding region (lanes 3 and 4). Both complexes were again supershifted with anti-MEF-2C and -2D antibodies (lanes 6 and 7), whereas again only the slower migrating band was supershifted by an anti-CBP antibody (lane 8), indicating binding of coactivator CBP to a MEF-2/C/D heterodimer in unstimulated primary myotubes. Electrical stimulation for 4 days with a slow fiber type activity pattern (lanes 9–12) as well as inhibition of p38\(\alpha/\beta\) MAPKs by SB203580 (lanes 13–16) abolished CBP but not MEF-2/C/D binding to that MEF-2 site. Thus, inhibition of p38\(\alpha/\beta\) MAPKs activities and inducing a fast-to-slow transformation did not alter binding of the two MEF-2 isoforms to the −233/−224 bp MEF-2 site within the MyHCId/x regulatory region but abolished recruitment of transcriptional coactivator CBP not only in C2C12 myotubes but also in primary skeletal myotubes. Although our results do not exclude involvement of distal MEF-2 sites in regulating the MyHCId/x promoter independent of p38\(\alpha/\beta\) MAPK activities data presented here on complex formation at the −233/−224 bp MEF-2 site are in accordance with the view of binding of MEF-2 to target genes while regulating transcriptional activity by recruitment of either coactivators or corepressors (25).

To further examine the role of CBP for MyHCId/x promoter activity, we transiently transfected C2C12 cells with double-stranded mouse CBP siRNA in a RNA interference experiment. Immunoblotting revealed a significant decrease in CBP protein expression in myotubes transfected with specific CBP siRNA but not in cells transfected with nonspecific double-stranded control siRNA (Fig. 5A). The MyHCId/x promoter basal activity was clearly reduced only in C2C12 cells transfected with the specific CBP siRNA (Fig. 5B). In contrast, overexpression of CBP increased MyHCId/x promoter activity in a dose-dependent manner (Fig. 5D), underlining the importance of CBP for MyHCId/x promoter activation. Furthermore, the activating effect of MKK6EE on the MyHCId/x promoter was completely abolished by specific CBP siRNA (Fig. 5B), whereas the inducing effect of overexpressing MEF-2C was significantly but not completely abolished (Fig. 5C). Taking into account the very robust activation of the MyHCId/x promoter by MEF-2C, the latter effect might be correlated to the CBP protein knockdown, but not completely knock out as seen in transgenic animals, induced by the specific RNA interference. In addition, compared with the wild type promoter the activation of MyHCId/x \(\Delta\)MEF2 by CBP was clearly reduced (Fig. 5D). Taken together with the EMSA experiments, the data demonstrate that CBP is important for p38 MAPK-mediated maintenance of MyHCId/x promoter activity at the −233/−224 bp MEF-2 consensus binding site, and the CBP effect on the promoter is at least in part mediated via MEF-2C.

**DISCUSSION**

The possible role of p38 MAPK in regulating the adult fast MyHCId/x isoform was investigated in C2C12 and primary skeletal myotubes. Our finding that p38 MAPK is essential for the maintenance of high levels of basal MyHCId/x promoter activity extends the role of p38 MAPK in skeletal muscle cells. Previously, Delling et al. (16) have demonstrated that p38 MAPK activation by constitutively active MKK6 enhanced C2C12 cell differentiation and promoted fast but not slow muscle program of p38 MAPKs and MEF-2 regulate MyHCId/x promoter activity. A, luciferase activity measured in C2C12 cells transfected with the fast 2.8-kb MyHCId/x promoter reporter construct and cotransfected with constitutively active MKK6 (MKK6EE) or empty expression vector and grown in the presence or absence of p38\(\alpha/\beta\) inhibitor SB203580, Ca\(^{2+}\)-ionophore, or Ca\(^{2+}\)-ionophore plus SB203580 as indicated. B and C, C2C12 cells (B) or COS-7 cells (C) transfected with the −2.8-kb MyHCId/x promoter reporter construct and/or MEF-2C, MEF-2D, MKK6EE, or empty expression vector, cultured with or without SB203580 as indicated were assayed for luciferase activity. D, C2C12 cells transfected with the −2.8-kb MyHCId/x promoter reporter construct and cotransfected with wild type MEF-2C, mutant MEF-2C (T293A), or empty expression vector as indicated were assayed for luciferase activity. E, luciferase activity determined in p38\(\alpha/\beta\)-deficient (−/−) and wild type (wt) mouse embryonic fibroblast (MEF) cells transfected with −2.8-kb MyHCId/x promoter reporter construct and cotransfected with MEF-2C, MKK6EE, p38\(\alpha/\beta\), or empty expression vector as indicated. F, luciferase activity measured in C2C12 cells either transfected with the fast −2.8-kb MyHCId/x promoter reporter construct or MyHCId/x−2.8MEF2 and cotransfected with MKK6EE or empty expression vector as indicated. All of the luciferase activities were normalized to β-galactosidase levels. The results are expressed as relative light units/β-galactosidase (RLU/β-Gal). The values shown are the means and standard deviations of triplicate data points from one experiment representative of three independent experiments.
MyHC isoform protein expression. The effect of p38 MAPK inhibitor SB203580 on the fast MyHCII protein expression and MyHCII/d/x promoter activity indicates the involvement of the p38\(\alpha\) and p38\(\beta\) isoforms in myotubes. Furthermore, our data obtained with p38\(\alpha\)/myoblast cells suggest that p38\(\alpha\) MAPK has a major impact and p38\(\beta\) has a minor impact on maintenance of high MyHCII/d/x promoter activity. Together with DNA binding studies, these findings provide an additional role for p38 MAPK not only during myoblast differentiation (8, 9) but also in gene regulation of differentiated myotubes.

Immunofluorescence studies revealed a \(\text{Ca}^{2+}\)-ionophore- or electrical stimulation-induced down-regulation of p38 MAPK activity in the fast fiber type-like myotubes. In addition, we could show that \(\text{Ca}^{2+}\)-ionophore treatment also down-regulated MyHCII/d/x promoter activity. This was mainly mediated by inhibition of p38 MAPK activity, suggesting involvement of changes in the [\(\text{Ca}^{2+}\)] in that process. Interestingly, in the fast
extensor digitorum longus muscle of rat in vivo, the resting [Ca$^{2+}$], has been shown to be lower than in the slow soleus muscle and can be increased by electrical stimulation with a slow fiber type activity pattern (47). In addition, remarkable amounts of phosphorylated p38 MAPK have been found in unstimulated skeletal muscle in vivo, especially in fast type muscles (48–50). In rat fast extensor digitorum longus, p38 MAPK phosphorylation is much higher than in slow soleus muscle (50). Thus, our finding of an already high level of activated p38 MAPK in fast fiber type-like C2C12 myotubes and primary skeletal muscle cells and a low level of P-p38 MAPK in Ca$^{2+}$/ionophore-treated or electrical stimulated cells coincides with reports in vivo. Changes in [Ca$^{2+}$], in skeletal muscle cells often involve a signaling pathway dependent on calcineurin, a Ca$^{2+}$-calmodulin-regulated serine/threonine phosphatase (7, 51). In addition, an increase in [Ca$^{2+}$], by Ca$^{2+}$/ionophore A23187 has been shown to induce MKP-1 mRNA and protein in rat fibroblasts (52). MKPs are a family of dual specificity protein phosphatases with MKP-1 preferentially activating p38 MAPK and c-Jun N-terminal kinase at least in some cell lines (53). Prolonged but transient increases in [Ca$^{2+}$], have been shown to activate MKP-1 in a neuronal-like cell line (54). Interestingly, activation of endogenous calcineurin with Ca$^{2+}$/ionophore or constitutively active calcineurin could increase MKP-1 protein levels and decrease p38 MAPK activity in cardiac myocytes (41). Furthermore, the MKP-1 promoter was shown to be calcineurin-responsive. It was therefore tempting to speculate that possible cross-talk between calcineurin and p38 MAPK mediated the down-regulation of MyHCIId/x promoter activity during increased [Ca$^{2+}$]. However, by inhibiting calcineurin with different compounds, we could not find an alteration in Ca$^{2+}$/ionophore-induced down-regulation of activated p38 MAPK in C2C12 myotubes by immunofluorescence and Western blot (data not shown) analysis. In addition, CsA did not restore the basal activity of the MyHCIId/x promoter in Ca$^{2+}$/ionophore-treated C2C12 cells (40). Nevertheless, these findings do not exclude a possible role of MKP-1 or other MKPs in this process in a calcineurin-independent manner. Several kinases have been identified to activate MKK3/6 and therefore act as MAPK kinase kinase (15, 55). Thus, the activating effect of MKK6EE on the MyHCIId/x promoter in Ca$^{2+}$/ionophore-treated C2C12 cells might also be
explained by overriding the inhibitory action of Ca\(^{2+}\)-ionophore on an upstream kinase. In addition, MKPs can also inhibit upstream kinases. The molecular mechanism underlying the Ca\(^{2+}\)-ionophore-induced down-regulation of p38 MAPK activity, whether mediated by inhibition of an upstream kinase or by direct action on p38 via MKPs, remains to be determined.

Transient transfection assays with overexpressed MEF-2C or 2D revealed that the MyHCIId/x promoter basal activity is regulated by at least two isoforms of Myf-2 transcription factor family in C2C12 myotubes. Furthermore, in cotransfection experiments with MEF-2C carrying a mutation in the site sufficient in muscle cells for activation via direct phosphorylation by p38 (24), we could demonstrate that basal MyHCIId/x promoter activation by MEF-2C was mediated by p38 MAPK. Thus, p38 MAPK is capable of activating MEF-2C in muscle not only during myoblast differentiation (9) but also in myotubes. As expected, MEF-2D, which is not a substrate of p38 MAPK (22, 23), activated the MyHCIId/x promoter in a p38 MAPK-independent manner, indicating that p38 MAPK alone is not sufficient to mediate the basal activity of that promoter. Moreover, our findings with EMSAs revealed binding of MEF-2C and 2D to the proximal MEF-2 consensus site of the MyHCIId/x promoter primarily as a heterodimer. Interestingly, we could demonstrate that the MEF-2C/D heterodimer recruited transcriptional coactivator CBP at that site. Inhibition of p38 MAPK activity by treatment of cells with the pharmacological inhibitor SB203580 did not reduce DNA binding of MEF-2C and -2D to the proximal MEF-2 consensus site of the MyHCIId/x promoter. As expected, the MEF-2D, which is not a substrate of p38 MAPK (22, 23), activated the MyHCIId/x promoter in a p38 MAPK-independent manner, indicating that p38 MAPK alone is not sufficient to mediate the basal activity of that promoter. Moreover, our findings with EMSAs revealed binding of MEF-2C and 2D to the proximal MEF-2 consensus site of the MyHCIId/x promoter primarily as a heterodimer. Interestingly, we could demonstrate that the MEF-2C/D heterodimer recruited transcriptional coactivator CBP at that site. Inhibition of p38 MAPK activity by treatment of cells with the pharmacological inhibitor SB203580 did not reduce DNA binding of MEF-2C and -2D to the proximal MEF-2 site but completely inhibited recruitment of CBP. In addition, SB203580, Ca\(^{2+}\)-ionophore treatment and electrical stimulation eliminated recruitment of CBP to the proximal MEF-2 site, whereas again the MEF-2C and 2D isoforms remained bound primarily as heterodimers. The importance of CBP binding for high levels of fast adult MyHCIId/x promoter activity provides an explanation for the SB203580- and Ca\(^{2+}\)-ionophore-mediated down-regulation of the promoter. Ca\(^{2+}\)-ionophore treatment of C2C12 myotubes or electrical stimulation of primary skeletal muscle cells reduced the level of P-p38 MAPK, MyHCIId/x protein expression, and promoter activity. On the other hand, overexpression of constitutively active MKK6EE reestablished recruitment of the coactivator CBP and eliminated the effect of Ca\(^{2+}\)-ionophore on promoter activity. Taken together, our data demonstrate that inhibition of p38 MAPK activity is involved in down-regulation of MyHCIId/x promoter activity during increased [Ca\(^{2+}\)].

To conclude, we report that p38α/β MAPK-dependent recruitment of transcriptional coactivator CBP to a MEF-2C/D heterodimer complex at a proximal MEF-2-binding site located in the fast adult MyHCIId/x promoter region is essential for gene expression of MyHCIId/x at high levels in multinuclear myotubes. The data uncover a new role of p38α/β MAPKs in regulating gene activity in differentiated skeletal muscle cells during changes in [Ca\(^{2+}\)], capable of inducing transformation of the muscle fiber type.

Acknowledgments—We are grateful to Drs. E. Olson, M. Gaestel, R. Goodman, and T. Tamura for the generous gifts of plasmids (pcDNA1-MEF-2C/pcDNA1-MEF-2D1b, pcDNA3-p38α, pRec/RSV-mCBP-HA, and pcDNA3-MKK6EE, respectively) and Drs. M. Gaestel and W. H. Mueller for helpful discussions. We thank E.-A. Haller and W. Ziegel for technical assistance.

REFERENCES

1. Booth, F. W., and Baldwin, K. M. (1996) in Handbook of Physiology (Rowell, L.B., and Shepherd, I.T., eds) pp. 1075–1123, Oxford University Press, Oxford, UK
2. Pette, D., and Staron, R. S. (1997) Int. Rev. Cytol. 170, 143–223
3. Goldspink, G. (2002) Biochem. Soc. Trans. 30, 285–290
4. Kubis, H. P., Haller, E. A., Wetzel, P., and Gros, G. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 4205–4210
5. Meissner, J. D., Kubis, H. P., Scheibe, R. J., and Gros, G. (2000) J. Physiol. 523, 19–28
6. Meissner, J. D., Gros, G., Scheibe, R. J., Scholz, M., and Kubis, H. P. (2001) J. Physiol. 533, 215–226
7. Chin, E. R. (2004) Proc. Nutr. Soc. 63, 279–286
8. Cuenda, A., and Cohen, P. (1999) J. Biol. Chem. 274, 4341–4346
9. Zetser, A., Gredinger, E., and Bengal, E. (1999) J. Biol. Chem. 274, 5193–5200
10. Shi, Y., and Gaestel, M. (2002) Biol. Chem. 383, 1519–1536
11. Puri, P. L., Wu, Z., Zhang, P., Wood, L. D., Bhakta, K. S., Han, J., Feramisco, J. R., Karin, M., and Wang, J. Y. (2000) Genes Dev. 14, 574–584
12. Molkenstt, J. D. (2004) Cardiovasc. Res. 63, 467–475
