Involvement of Elk-1 in L6E9 skeletal muscle differentiation

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Abstract In L6E9 skeletal muscle cells ternary complex factor (TCF) Elk-1 expression increased with the onset of skeletal muscle differentiation, whereas its activation decreased as a function of differentiation. Its expression was predominantly restricted to cytoplasm and activated ones were predominantly restricted to the nucleus of the differentiated cells. Inhibition of ERK-1/-2 activities by PD098059 resulted into significant reduction in Elk-1 expression and phosphorylation during differentiation. In contrast, inhibition of p38 mitogen-activated protein kinase (MAPK) enhanced Elk-1 expression and activation, thereby mediating inhibition of skeletal muscle differentiation. Overexpression of inactive mutant Elk-1 enhanced differentiation. Data suggest that ERK-1/-2 and p38 MAPK activities modulate Elk-1 expression and activation to regulate skeletal muscle differentiation.

Key words: Elk-1; Myogenesis; ERK-1/-2; p38 Mitogen-activated protein kinase

1. Introduction

Mitogen-activated protein kinases (MAPK) are involved in various cellular processes such as cell growth, differentiation and apoptosis [1]. Much emphasis has been made on MAPK's involvement in various cellular processes however little is understood with regard to the role of transcription factors activated by them. Previously, we have reported that inhibition of ERK-1/-2 activity dramatically enhanced skeletal muscle differentiation; in contrast, inhibition of p38 MAP kinase completely prevented differentiation [2]. Moreover, ERK-1/-2 and p38 MAPK regulated each other's activities [2]. Recent evidence suggests that ternary complex factor (TCF) Elk-1 is a target of all three classes of MAP kinases ERK, JNK, and p38 MAPK [3]. Multiple MAP kinase sites are located in the C-terminal domains of Elk-1, which are phosphorylated by ERK, JNK, and p38 MAP kinases in vitro and in vivo [4]. This indicates that multiple MAP kinase pathways can converge on the TCFs. Elk-1 functions as a nuclear transcriptional activator via its association with serum response factor (SRF) and serum response element (SRE) present on the promoter of many immediate early genes such as c-fos, egr1, egr2, pip 92, and nurr77 [5]. The phosphorylation of TCFs, Elk and SAP-1 leads to enhanced DNA binding and TCF-mediated transcriptional activation [4]. No data yet indicate the role of Elk-1 in skeletal muscle differentiation in vivo.

In the present report we have extended our previous study in determining the activation profile of Elk-1 to understand the physiological significance in L6E9 skeletal muscle differentiation. Our data suggest a possible correlation between MAPK activity and activation of Elk-1 in regulating L6E9 skeletal muscle differentiation.

2. Materials and methods

2.1. Materials

Rat skeletal muscle cell line L6E9 was kindly provided by Dr. H. Blau, Stanford University School of Medicine, Stanford, USA and Dr. J. Dhawan, CCMB, India. PD098059 and SB203580 were purchased from Calbiochem, USA. Anti-phospho-Elk-1 antibody and anti-actin antibody were procured from Santa Cruz Biotechnology, USA. Anti-Elk-1 antibody was procured from Cell Signalling Technology, USA. Horse serum, Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL, USA. Fetal calf serum (FCS) was purchased from Biological Industries, Israel. Other reagents were obtained from Sigma, USA and Bio-Rad, USA. TransFast transfection reagent was obtained from Promega Corporation, USA. The pCMVElk-1 (S383/S389) expression plasmid was a generous gift from Dr. Ralf Janknecht of Mayo Clinic, Rochester, USA.

2.2. Cell culture

L6E9 rat skeletal muscle cells were proliferated in DMEM supplemented with 15% FCS and antibiotics for 2 days. Differentiation was initiated by shifting 70% confluent cells to DMEM supplemented with 2% horse serum for 4 days (fully differentiated) at 37°C in 5% CO_2 incubator. Differentiation medium was changed daily.

2.3. Cell lysis

Cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 μg/ml each of leupeptin and aprotinin at 4°C for 20 min. The lysates were centrifuged at 16000×*g* at 4°C for 10 min. The supernatants were collected and samples were frozen at −20°C until used.

2.4. Western immunoblotting

Proteins were resolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane (NC) and blocked for 1 h at room temperature in 5% bovine serum albumin (BSA) in TBST (10 mM Tris–HCl, pH 8.0,

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; EDTA, ethylenediamine tetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin

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150 mM NaCl, 0.05% Tween-20). The NC membrane was washed and incubated with primary antibodies (anti-Elk-1, anti-phospho-Elk-1 and anti-actin antibody) for overnight at 4°C. Proteins were detected by alkaline phosphatase-conjugated secondary antibody using 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) as a substrate or by chemiluminescence. Blots were stripped in stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS and 100 mM β-mercaptoethanol) at 50°C for 30 min and reprobed with anti-phospho-Elk-1 antibody.

2.5. Creatine kinase (CK) assay

The assay was performed according to standard protocol [6]. The change in absorbance was measured at 340 nm up to 5 min.

2.6. Cellular fractionation

Cells were fractionated according to Vandromme et al. [7]. Briefly, cells were lysed in hypotonic buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 1 mM EDTA, 1 mM PMSF, 4 μM leupeptin and aprotinin (2 μg/ml) and homogenized. Nuclei were collected by 10 min centrifugation at 2000×*g* and supernatant was taken as cytosol. Pellet was finally suspended in equal amount of Laemmli's sample buffer [8].

2.7. Treatment of cells by inhibitors

PD098059 and SB203580 were dissolved in dimethyl sulfoxide (DMSO) separately to the concentration of 10 mM each and were added directly to differentiation medium to a final concentration of 10 μM. Control was incubated with the same volumes of DMSO without inhibitors. The medium was replaced every 24 h with medium containing fresh inhibitors. At this concentration of the inhibitors there was no toxic effect observed as cells differentiated normally when the drug was withdrawn from the medium (data not shown).

2.8. Transient transfection

Exponentially growing L6E9 cells were transfected with 5 μg of Elk-1 (S383/S389) expression plasmid using TransFast transfection reagent according to manufacturer's instructions. 36 h after transfection cells were transferred in differentiation medium for 4 days. Degree of differentiation was determined by creatine kinase activity and overexpression was measured by Western immunoblot using anti-Elk-1 antibody.

2.9. Protein estimation

The protein concentration was estimated by Lowry's method [9] using BSA as a standard protein.

2.10. Densitometric analysis

Densitometric analysis of the Western immunoblots was performed by using a GS-670 Imaging Densitometer (Bio-Rad) and Molecular Analyst software (version 1.3, Bio-Rad). Control samples in each experiment were given an arbitrary value of 1.0 for calculating the relative value of the other samples.

3. Results

3.1. Increase in expression of Elk-1 during differentiation of L6E9 skeletal muscle cells

We determined the expression of Elk-1 as a function of L6E9 skeletal muscle differentiation by Western immunoblotting probed with anti-Elk-1 antibody. Fig. 1A, upper panel, shows that expression of Elk-1 was low in proliferating myoblasts, whereas it was significantly (350%) higher in differentiated myotubes as compared to proliferating cells (Fig. 1A, lower panel, lane 1 vs. lane 5). This increase of Elk-1 was specific because under the similar conditions levels of actin remained unchanged (Fig. 1B). Increase in Elk-1 expression with the onset of skeletal muscle differentiation suggests involvement of Elk-1 as a function of differentiation.

3.2. Decrease of phosphorylation of Elk-1 during differentiation of L6E9 skeletal muscle cells

We next evaluated the phosphorylation of Elk-1, which is

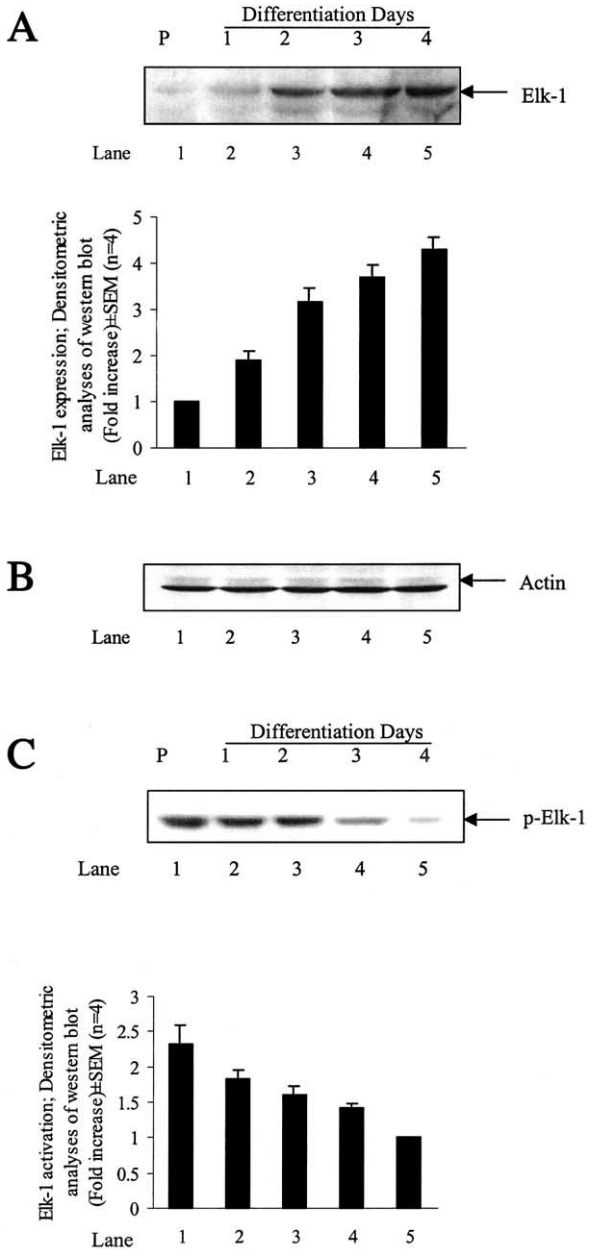
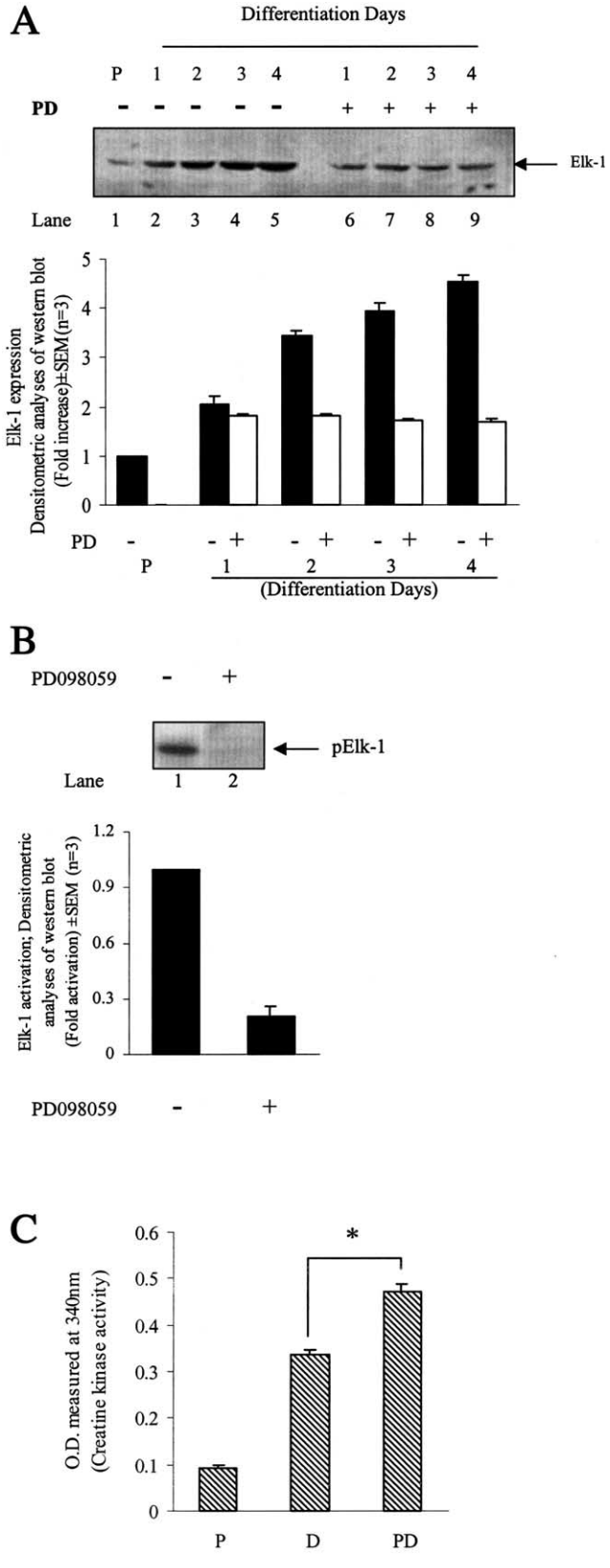


Fig. 1. Increase in Elk-1 expression during differentiation of L6E9 skeletal muscle cells. Cells were lysed at indicated intervals of time and were resolved on SDS–PAGE followed by Western immunoblotting with anti-Elk-1 antibody (A) or with anti-actin antibody (B). Elk-1 immunoblot was stripped and reprobed with anti-phospho-Elk-1 antibody (C) as mentioned in Section 2. P, proliferated cells. Upper panel: Western blot; lower panel: respective graphical representations. The experiments were carried out four times with similar results. One representative blot has been presented. The results are presented as mean ± S.E.M. for four independent experiments.

an indication of activation of the transcription factor, as a function of differentiation. Immunoblot of Elk-1 shown in Fig. 1A was stripped and reprobed with anti-phospho-Elk-1 antibody (Fig. 1C). Elk-1 was highly phosphorylated in proliferating myoblasts (Fig. 1C, upper panel, lane 1) whereas Elk-1 phosphorylation was decreased during differentiation (130% decrease in fully differentiated cells as compared to proliferated cells; Fig. 1C, lanes 2–5). Data indicate that acti-



vation of Elk-1 decreases with the differentiation. This suggests that decrease in Elk-1 phosphorylation is associated with skeletal muscle differentiation.

3.3. Inhibition of ERK-1/-2 activities by PD098059 causes decrease in Elk-1 expression and phosphorylation during L6E9 skeletal muscle differentiation

In our previous report [2] we have demonstrated inhibition of ERK-1/-2 and stimulation of p38 MAP kinase activity during the differentiation of L6E9 rat skeletal muscle cells. Complete inhibition of ERK-1/-2 activity by PD098059 dramatically enhanced differentiation, suggesting that ERK-1/-2 activation may be inhibitory to initiation and progression of differentiation [2]. In contrast, inhibition of p38 MAP kinase by SB203590 completely prevented differentiation; meaning p38 activation is required from the initiation till terminal differentiation of L6E9 cells [2]. To evaluate the interaction between ERK-1/-2 and Elk-1 as a function of differentiation, if any, we determined the expression and activation of Elk-1 under the condition where ERK-1/-2 activities were inhibited by PD098059. Fig. 2A, lanes 6–9, shows that expression of Elk-1 was decreased (60% decrease in fully differentiated cells, lane 9) when the cells were differentiated in presence of PD098059. Data suggest ERK-1/-2 activities regulate expression of transcription factor Elk-1. We next investigated Elk-1 phosphorylation in fully differentiated myotubes in absence or presence of PD098059. Fig. 2B indicates that phosphorylation of Elk-1, which was decreased in differentiated myotubes, was further decreased (80%) in presence of PD098059. Data suggest ERK-1/-2 activities regulate expression and activation of transcription factor Elk-1. In order to find out the involvement of Elk-1 in regulating differentiation, effect of PD098059 on creatine kinase activity was measured and the activity was significantly increased (28%) in myotubes differentiated in presence of PD098059 (Fig. 2C, lane PD) as compared to differentiated cells (Fig. 2C, lane D). Data suggest that ERK and Elk-1 activities negatively regulate skeletal muscle differentiation.

3.4. Increase in expression of Elk-1 is predominant in cytoplasm and not in nucleus during L6E9 skeletal muscle differentiation

Elk-1 is classically described as a nuclear target of activated ERK [10]; however, a recent report suggests that activated Elk-1 is present in both cytoplasm and nucleus of post mitotic neurons [11]. Therefore, to test this possibility we next investigated whether increase in Elk-1 expression during skeletal muscle differentiation was limited to cytoplasm or nucleus. Fig. 3A shows that Elk-1 expression was predominantly

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Fig. 2. Decreased Elk-1 expression and activation on treatment with PD098059 during differentiation of L6E9 skeletal muscle cells. Cells were differentiated in absence or presence of PD09859 and were lysed at indicated intervals of time. Samples were resolved on SDS–PAGE followed by Western immunoblotting with (A) anti-Elk-1 antibody, (B) anti-phospho-Elk-1 antibody. C: Creatine kinase activity was assayed as indicated in Section 2. P, proliferated cells; D, differentiated cells; PD, cells differentiated in presence of 10 μM PD098059. Upper panels: Western blots: lower panels: respective graphical representations. The experiments were carried out three times with similar results. One representative blot has been presented. The results are presented as mean ± S.E.M. for three independent experiments (*$P < 0.01$).

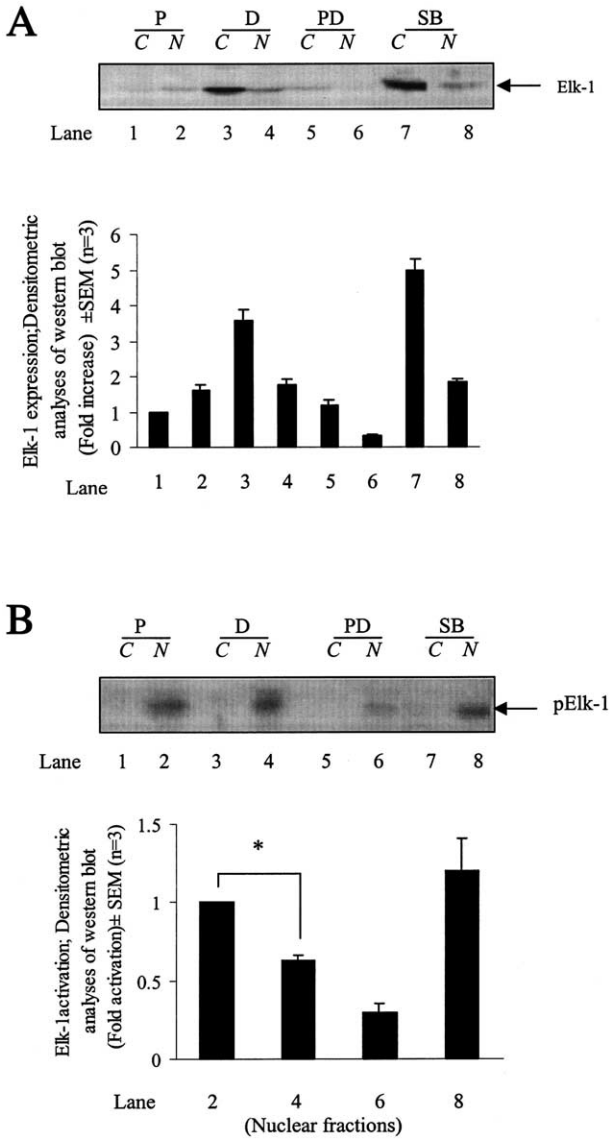


Fig. 3. Elk-1 is predominantly present in cytoplasm and not in nucleus of differentiated L6E9 cells. A: Cells were lysed and cytoplasmic (*C*) and nuclear (*N*) fractions were prepared as described in Section 2. Samples were resolved on SDS–PAGE followed by Western immunoblotting with anti-Elk-1 antibody. Lanes 1, 3, 5, and 7 represent cytoplasmic fractions of proliferated cells (P), differentiated cells (D), cells differentiated in presence of PD098059 (PD), cells differentiated in presence of SB203580 (SB). Lanes 2, 4, 6, and 8 represent nuclear fraction of above treated samples respectively. B: Above mentioned samples were resolved in SDS–PAGE followed by Western immunoblotting with anti-phospho-Elk-1 antibody. Graphical representation of only nuclear fractions is given in lower panel. Upper panels: Western blots; lower panels: respective graphical representations. The experiments were carried out three times with similar results. One representative blot has been presented. The results are presented as mean ± S.E.M. for three independent experiments ($*P < 0.001$).

present in cytoplasm and not in nucleus, in differentiated L6E9 skeletal muscle cells (lane 3 vs. lane 4). However, when the cells were differentiated in presence of PD098059, Elk-1 expression was abrogated in both cytoplasm and nucleus (Fig. 3A, lanes 5 and 6). Data indicate that although Elk-1 expression was increased with the skeletal muscle differentiation, its expression was limited to cytoplasm and not in nucleus in differentiating L6E9 cells (Fig. 3A). Elk-1 present in cytoplasm was not activated whereas Elk-1 present inside the nucleus was (Fig. 3B).

3.5. Inhibition of p38 MAPK by SB203580 increases expression and activation of Elk-1 during L6E9 skeletal muscle differentiation

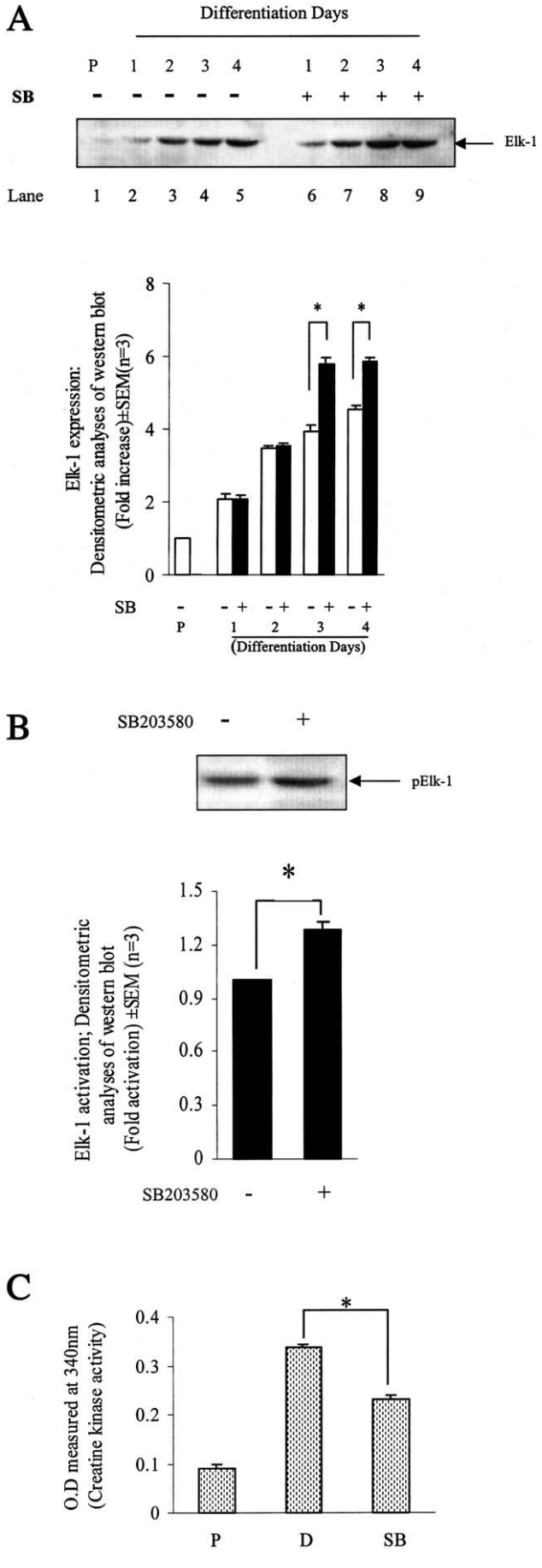
Since Elk-1 is thought to be a convergence point for all MAPK pathways [3,12], we determined the effect of p38 MAPK on Elk-1. Previously we have shown that inhibition of p38 MAPK by SB203580 inhibited skeletal muscle differentiation and activated ERK-1/-2 activities during differentiation [2]. Therefore, we determined Elk-1 expression and phosphorylation in the cells differentiated in absence and presence of SB203580. Fig. 4A shows that when the cells were differentiated in presence of SB203580, Elk-1 expression was significantly (22%) increased as a function of differentiation (Fig. 4A, lanes 6–9). Cellular fractionation studies showed that increased Elk-1 expression on treatment of SB203580 was limited to cytoplasm and not in the nucleus (Fig. 3A, lanes 7 and 8). Therefore, data suggest that induction of Elk-1 expression by inhibition of p38 MAPK might be involved in inhibition of skeletal muscle differentiation. We next investigated the effect of SB203580 treatment on activation of Elk-1 as a function of differentiation of L6E9 cells. Fig. 4B indicates that on inhibition of p38 MAPK, the Elk-1 was activated, however, under the same condition, creatine kinase activity was decreased (31%) (Fig. 4C, compare lane D and lane SB) as compared to differentiated cells. Data suggest that p38 MAPK-mediated Elk-1 regulation controls skeletal muscle differentiation.

3.6. Inactive Elk-1 (S383/S389) mutant induces skeletal muscle differentiation

It has been shown that phosphorylation of serines 383 and 389 of Elk-1 is critical for the transcriptional activation of Elk-1 [13]. Ser-383 and Ser-389 have previously been shown to be major ERK2 phosphorylated residues [14]. These two residues are major determinants of ERK-2 stimulated DNA binding and transcriptional activation by Elk-1 [14]. Mutation of either the phosphoacceptor sites (S383/S389) caused reduction in rate and extent of phosphorylation of each of these mutants [15]. We transiently transfected Elk-1 S383/S389 to evaluate the effect of inactive Elk-1 mutant on skeletal muscle differentiation. Transfected cells showed overexpression of inactive mutant Elk-1 protein (Fig. 5A) as compared to cells transfected with empty vector when checked by Western blot analysis by probing with anti-Elk-1 antibody. Transfected cells were differentiated and significant increase in differentiation was observed as determined by creatine kinase activity (35%) (Fig. 5B). Results suggest that overexpression of inactive Elk-1 enhances differentiation in L6E9 skeletal muscle cells.

4. Discussion

In our previous report we have shown that when the cells were differentiated in presence of PD098059, extensive myotube formation and increased creatine kinase activity were observed [2]. Under this condition Elk-1 activation was completely inhibited and expression was significantly decreased as shown in the present study (Fig. 2A and B). This indicates that ERK-1/-2 activities regulate expression as well as phos-



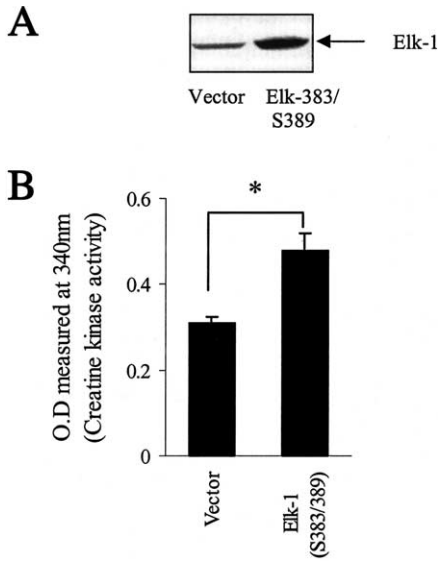


Fig. 5. Inactive Elk-1 (S383/S389) mutant induces skeletal muscle differentiation. Proliferating L6E9 cells were transfected with mutated Elk-1 (S383/S389) expression vector (pCMVElk-1 S383/S389) or empty vector (pCMV). A: Transfected proliferated cells were lysed and were resolved on SDS–PAGE followed by Western immunoblotting with anti-Elk-1 antibody. B: The transfected cells were incubated for 4 days in differentiation medium and finally processed for creatine kinase activity as mentioned in Section 2. Vector: pCMV-transfected cells; Elk-1 S383/S389: pCMVElk-1 S383/S389-transfected cells. The results are presented as mean ± S.E.M. for three independent experiments ($*P < 0.01$).

phorylation of Elk-1. Decrease in Elk-1 expression and phosphorylation, increase in CK activity, as observed in our current study, may be responsible for hyperdifferentiation observed due to PD098059 treatment as observed in our previous report [2]. In contrast, when the cells were differentiated in presence of SB203580, skeletal muscle differentiation was inhibited completely [2] and Elk-1 expression and phosphorylation were significantly increased (Fig. 4A and B). This suggests that inhibition of skeletal muscle differentiation due to p38 MAPK inhibition was mediated by increasing the expression as well as phosphorylation of Elk-1.

The increase in expression and decrease in phosphorylation of Elk-1 under low ERK activity during skeletal muscle differentiation were a paradox. Data observed from our cellular fractionation studies (Fig. 3A, lane 3 vs. lane 4) may be explained as ERK-1/-2 activities decrease with skeletal muscle

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Fig. 4. Increased Elk-1 expression and activation on treatment with SB203580 during differentiation of L6E9 skeletal muscle cells. A: Cells were differentiated in absence or presence of SB203580 and were lysed at indicated intervals of time. Samples were resolved on SDS–PAGE followed by Western immunoblotting with anti-Elk-1 antibody. B: Fully differentiated cells in presence or absence of SB203580 were lysed and resolved on SDS–PAGE and processed for immunoblotting with anti-phospho-Elk-1 antibody as mentioned in Section 2. C: Creatine kinase activity was assayed as indicated in Section 2. P, proliferated cells; D, differentiated cells; SB, cells differentiated in presence of 10 μM SB203580. Upper panels: respective Western blots; lower panels: respective graphical representations. The experiments were carried out three times with similar results. One representative blot has been presented. The results are presented as mean ± S.E.M. for three independent experiments ($*P < 0.01$).

differentiation [2], thereby less Elk-1 is activated (Fig. 1B), hence little translocation of Elk-1 into the nucleus takes place (Fig. 3A, lane 4). However, in proliferating cell expression of Elk-1 was very less (Fig. 1, lane 1) when compared to differentiated skeletal muscle cells (Fig. 1, lane 5), but Elk-1 was highly phosphorylated; therefore, most of the Elk-1 was present in the nucleus of the proliferating cells (Fig. 3B, lane 2). Transfection studies with inactive Elk-1 S383/S389 mutant revealed that overexpression of inactive Elk-1 enhanced skeletal muscle differentiation (Fig. 5B). This finding in conjunction with others, as mentioned above, strongly suggest that inactivation of Elk-1 is required for skeletal muscle differentiation. Recently, Elk-1 phosphorylation has been described to play an important role in nerve growth factor-induced PC12 neuronal differentiation [16]. Previously, activated Elk-1 was reported to be present in both the cytoplasmic and nuclear compartments of the neuronal cell types [11]. Our study on L6E9 skeletal muscle cells reveals presence of activated Elk-1 in the nucleus only. Recent reports suggest a link between Elk-1 activation and neuronal differentiation [11,16,17]. The involvement of Elk-1 phosphorylation has also been implicated in breast cancer cells [18]. However, to the best of our knowledge we are reporting for the first time the involvement of Elk-1 in skeletal muscle differentiation. In conclusion, our data suggest that MAPK converge at Elk-1 and modulation of Elk-1 expression and activation by MAPK may regulate skeletal muscle differentiation.

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