

Mutation of MyoD-Ser237 abolishes its up-regulation by c-Mos

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Abstract Recently we have shown that Mos could activate myogenic differentiation by promoting heterodimerisation of MyoD and E12 proteins. Here, we demonstrate that MyoD can be efficiently phosphorylated by *in vitro* kinase assay with purified Mos immunoprecipitated from transfected cells. Comparative two-dimensional tryptic phosphopeptide mapping combined with site-directed mutagenesis revealed that Mos phosphorylates MyoD on serine 237. Mutation of serine 237 to a non-phosphorylatable alanine (MyoD-Ala237) abolished the positive regulation of MyoD by Mos following overexpression in proliferating 10T1/2 cells. Taken together, our data show that direct phosphorylation of MyoD-Ser237 by Mos plays a positive role in increasing MyoD activity during myoblast proliferation. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: c-Mos; MyoD; Phosphorylation; Myogenesis

1. Introduction

Myogenic differentiation is under the control of a family of muscle-specific transcription factors (MRFs) whose MyoD is the prototypic molecule [1]. These MRFs are basic helix-loop-helix (bHLH) nuclear proteins [2–4] which bind to an E-box DNA consensus sequence (CANNTG) as heterodimers with the ubiquitous E proteins (E12 and E47) [5,6], to transactivate muscle genes and to efficiently convert non-muscle cells to a myogenic lineage [7,8].

Their overall activity is subject to negative and positive regulations in response to intracellular signaling pathways and to the expression of transcriptional accessory proteins. An additional mechanism through which the activities of MRFs are regulated is by differential phosphorylation. MRFs exist as phosphoproteins *in vivo* and their amino acid sequence contains several potential sites of specific phosphorylation, including consensus sites for cyclic AMP-dependent protein kinase (PKA) [9], protein kinase C [10,11], cyclin-dependent kinases [12–16] and casein kinase II (CKII) [17]. Furthermore, MRFs exhibit some specific features such as temporal expression during development, cell cycle progression, positive autoregulation loop or the targeting of different muscular genes [18–20].

For several years we have endeavoured to characterise mechanisms and factors that positively regulate MyoD activity. We recently reported that the proto-oncogene *c-mos* activated muscle differentiation [21]. We further demonstrated that the c-Mos protein interacts physically with unphosphory-

lated MyoD and that phosphorylation of the COOH domain of recombinant MyoD by Mos promotes the formation and DNA binding of MyoD–E12 heterodimers [22–23].

In this report we show by tryptic phosphopeptide mapping and mutational analysis that Mos phosphorylates MyoD on Ser237 *in vivo* and *in vitro*. We demonstrated that a non-phosphorylatable Ala237 mutant of MyoD is never up-regulated by Mos. These data show that direct Mos-dependent phosphorylation of MyoD on Ser237 is involved in positively regulating MyoD activity.

2. Materials and methods

2.1. Cell cultures, DNA transfection and luciferase assays

The 10T1/2 fibroblastic cells were maintained in growth medium supplemented with antibiotics (a mixture of penicillin and streptomycin (Life Technologies)) and 15% of foetal calf serum (FCS) in Dulbecco's modified Eagle's medium (DMEM). 10T1/2 cells were transfected by using the polyethylenimine essentially as described [24]. Briefly, 3×10^4 cells per well were plated onto 24-well plates. On the following day, cells were transfected with various combinations of plasmids as indicated in the figure captions. The total amount of DNA used for each plate was normalised with the respective empty expression vector. Luciferase activity was determined in aliquots of cell extracts from cells harvested 48 h after transfection in growth medium with a luminometer (Lumat LB9507-DLA Bertholet) and the Luciferase Assay System (Promega) as instructed by the manufacturer. One hundred ng of the pEGFP-C1 plasmid (Invitrogen) was included in transfections as an internal control for transfection efficiency. All luciferase activities were determined with equivalent quantities of proteins in triplicate and repeated at least twice.

2.2. Plasmids

pEMSV-MyoD-wt was a generous gift from the H. Weintraub laboratory. The pEMSV-MyoD-Ala200 mutant was obtained from M. Kitzmann. The pEMSV-MyoD-Ala237 mutant and pET-MyoD-Ala200 were generated by polymerase chain reaction with a 5' primer (5'-GAGGCGGTGCGCGAGGCCAGGCCAGGGAAG-3') and a 3' primer (3'-CTTCCCTGGCCTGGCCTCGCGCACCAGCCTC-5') using pEMSV-MyoD-wt as template and the QuickChange[®] site-directed mutagenesis kit (Stratagene, Ozyme) as instructed by the manufacturer. Expression vectors pCMV-HA-Mos^{wt} and pCMVHA-Mos^{KM} were generated by cloning three hemagglutinin epitope tags (3Tag HA) at the amino terminus of cDNA insert in pCDNA3 (Invitrogen). The reporter plasmid MCK-Luc (p1256-MCK), generously provided by S. Hauschka [25], contains the promoter-enhancer region from the mouse muscle creatine kinase. pET-MyoD-wt was obtained from Vandromme et al. [26].

2.3. Purified proteins

The production and purification of MyoD-wt protein has been described [22]. MyoD-Ala237 was purified by using the same protocol. The active kinase Mos^{wt} and the inactive kinase Mos^{KM} proteins were purified by immunoprecipitation from transfected cells. Whole cell extracts were prepared in ice-cold RIPA-EGTA buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 10 mM β -glycerophosphate, 0.1 mM Na₂VO₃, 1 mM NaF, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 1 mM PMSF and 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 1 μ g/ml pepstatin). Lysates were centrifuged at

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4°C for 10 min in a microfuge set at maximum speed and the supernatant was stored at –80°C in small aliquots. For immunoprecipitation, precleared cell lysates in IP buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 0.5% NP40, 0.5 mM Na-orthovanadate, 50 mM NaF, 80 mM β -glycerophosphate, 10 mM Na-pyrophosphate, 1 mM DTT, 1 mM EGTA and 10 μ g/ml leupeptin, 10 μ g/ml pepstatin and 10 μ g/ml aprotinin) were incubated with the anti-HA antibodies for 2–3 h at 4°C with gentle agitation. Immunocomplexes bound to protein G-Sepharose were collected by centrifugation and washed several times in IP buffer.

2.4. In vitro kinase assays

Immunoprecipitated proteins were washed three times in washing buffer (1% NP40, 0.15 M NaCl, 10 mM phosphate buffer (TPO₄, pH 7), 1 mM EDTA and 1 mM sodium pyrophosphate). In vitro Mos phosphorylation assays were done as described previously [19]. In brief, protein kinase reaction mixtures (100 μ l final) included 1 mM sodium pyrophosphate, 10 mM TPO₄, pH 7.0, 150 mM NaCl, 10 mM MnCl₂, 2 mM DTT, 25 μ M ATP, 50 μ Ci of (γ ³²P) ATP (3000 Ci mmol⁻¹) and 0.5 to 2 μ g of purified MyoD-wt or MyoD-Ala237 proteins. The reaction was incubated for 15 min at 30°C and was terminated by adding 50 μ l of 2 $\times$ sodium dodecyl sulphate (SDS) sample buffer. The mixture was boiled for 10 min and analysed by SDS–polyacrylamide gel electrophoresis (PAGE).

2.5. Phosphopeptide mapping

Bacterially purified MyoD-wt protein (pET-MyoD-wt) was in vitro phosphophorylated by Mos^{wt} immunoprecipitated from transfected cells as described above. The trypsin digest was analysed essentially as previously described [13].

2.6. Antibodies and Western blot analyses

For immunoblot analysis, total cell extracts or immunoprecipitates were solubilised in RIPA-EGTA buffer. Analyses were performed on 10% polyacrylamide gels with a 5% polyacrylamide stacking gel. Electrophoretic transfer of proteins from SDS–PAGE gels to nitrocellulose membranes were blocked with Tris–HCl 50 mM, pH 7.4, 150 mM NaCl and 0.05% Tween 20 containing 5% skimmed milk and incubated overnight at 4°C with primary antibodies: polyclonal Emos1 anti-Mos antibody diluted 1/1000 prepared in rabbit [27], and polyclonal C20 anti-MyoD diluted 1/500 were provided by Santa Cruz (Biotechnology, Santa Cruz, CA, USA). The monoclonal 12CA5 anti-HA antibody was provided by Boehringer (Mannheim, Germany). Membranes were washed and incubated for 1 h with a peroxidase-conjugated secondary antibody (Sigma) at a dilution of 1/10 000 with polyclonal antibodies and 1/4000 with monoclonal antibodies. After several washes, membranes were incubated with an enhanced chemiluminescence system (Amersham) according to the manufacturer's instructions. Exposure was done with Agfa Curix RP2 films.

3. Results

3.1. Mos-dependent phosphorylation of MyoD

We previously reported that a GST–Mos fusion protein activated by incubation in rabbit reticulocyte lysates was able to phosphorylate bacterially produced MyoD in an in vitro kinase reaction [18]. In the present work we used Mos protein from cell extracts. Wild-type (wt) and kinase-inactive Mos (KM) proteins were purified by immunoprecipitation from transfected 10T1/2 cells (Fig. 1A). Kinase activity was evidenced by autophosphorylation of the wild-type Mos^{wt} (Fig. 1B, lanes 1 and 2) but not of the kinase-inactive Mos^{KM} (Fig. 1B, lane 3). The immunoprecipitated Mos^{wt} but not the kinase-inactive Mos^{KM} was able to phosphorylate bacterially produced MyoD in an in vitro kinase reaction (Fig. 1B, lane 2).

To map the site(s) phosphorylated on MyoD, we used tryptic digestion of MyoD followed by 2D phosphotryptic peptide mapping. As illustrated in Fig. 1C, one phosphotryptic peptide was obtained after digestion of ³²P-labeled MyoD. To

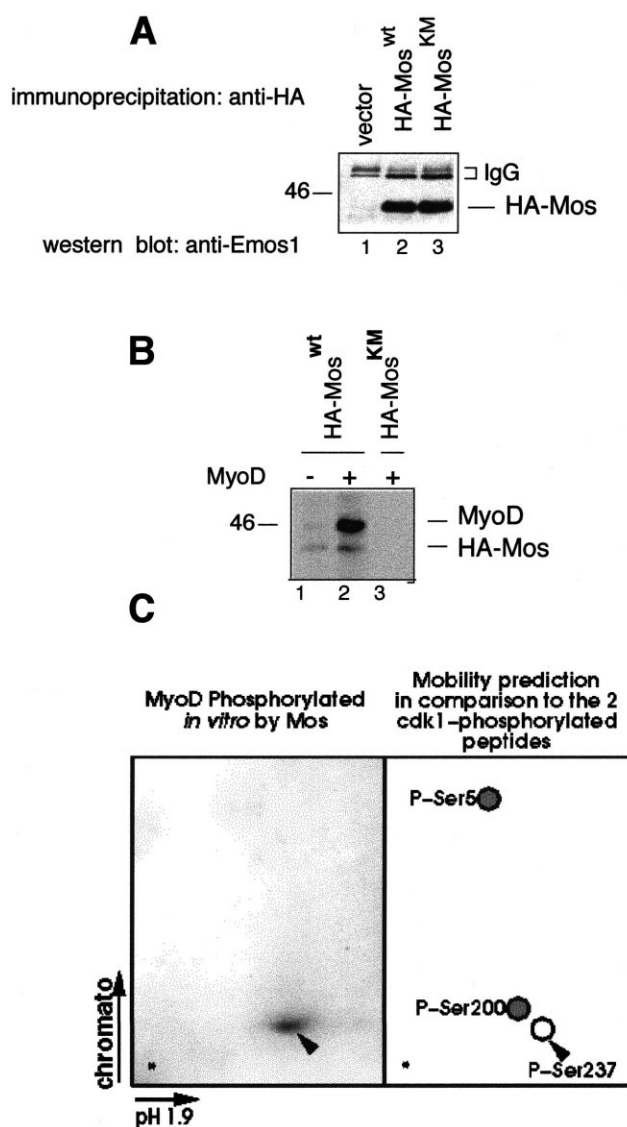
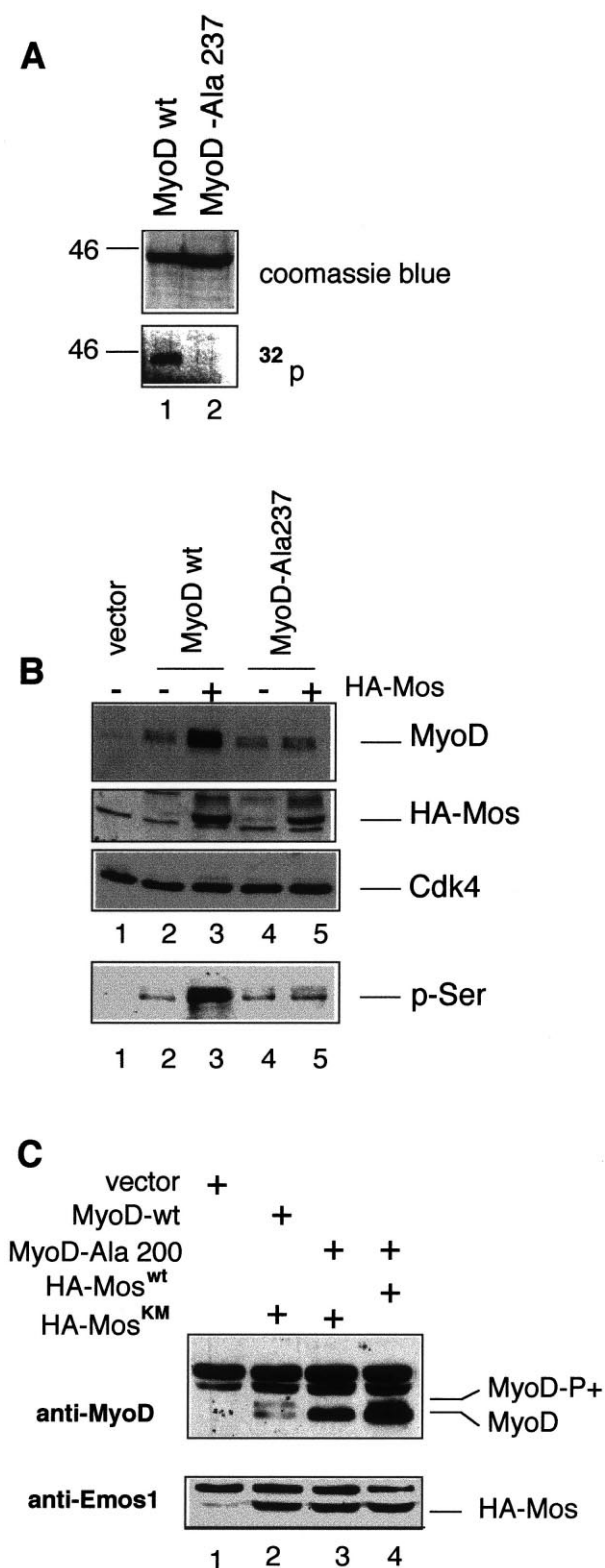


Fig. 1. Mos kinase from transfected cells phosphorylates MyoD efficiently. (A) 100 μ g of 10T1/2 cells transfected with an empty vector (lane 1), HA-epitope-tagged Mos^{wt} (lane 2) and HA-Mos^{KM} (lane 3) were immunoprecipitated with the 12CA5 monoclonal antibody, transferred onto nitrocellulose membrane and analysed by immunoblotting with the purified rabbit polyclonal anti-Emos 1 antibody. (B) Duplicate samples of immunoprecipitates were used in an in vitro kinase assay. Purified bacterially expressed MyoD [26] (1 μ g, lanes 2 and 3) were added to immunoprecipitates and were incubated in kinase buffer containing [γ -³²P]ATP. Lane 1 shows autophosphorylation of the Mos^{wt} protein. Proteins were separated by SDS–PAGE and the phosphorylated proteins were visualised by autoradiography. Exposure time was 4 h. (C) Phosphorylation sites on MyoD were analysed by tryptic digestion followed by 2D peptide mapping. The phosphopeptide map for in vitro phosphorylated MyoD was performed on bacterially produced MyoD phosphorylated in vitro by Mos. The only phosphopeptide found is pointed to by an arrow. Mobility prediction of MyoD tryptic phosphopeptide by using the Mobility 4 program [11] shows the pattern of migration for the two phosphopeptides that could be phosphorylated by Cdk1.

estimate which site is phosphorylated, we used the PhosPeptsort program previously used in the determination of MyoD-Ser200 as the major phosphorylation site of Cdk1 and Cdk2 [12,13]. According to the mobility prediction, the phospho-



peptide obtained after *in vitro* phosphorylation of MyoD by Mos would correspond to phosphorylation of Ser237. This result shows that Mos phosphorylates MyoD on one site which is predicted to contain Ser237 and agrees with previous data suggesting that Mos phosphorylates MyoD on serine residue(s) located in the COOH terminal region [22].

Fig. 2. MyoD serine 237 is the major phosphorylation site of Mos. (A) Purified bacterially produced MyoD-wt and/or MyoD-Ala237 proteins were incubated as in Fig. 1, in the presence of [γ - 32 P]ATP. After the phosphorylation reaction, the different proteins were separated by SDS-PAGE and visualised by autoradiography. The Coomassie blue stain of the protein substrates is also shown. (B) 10T1/2 cells were transfected with pEMSV-MyoD-wt (lanes 2 and 3) or pEMSV-MyoD-Ala237 (lanes 4 and 5) alone or in combination with pCMV-HA-Mos^{wt} (lanes 3 and 5). Forty-eight hours following transfection, cells maintained in DMEM supplemented with 15% FCS were harvested and Western blot analysis was performed. Ten μ g of whole cell extracts were solubilised in SDS loading buffer. After SDS-PAGE, HA-tagged Mos^{wt} proteins were detected by immunoblotting with the 12CA5 monoclonal antibody and MyoD proteins were detected with the affinity purified polyclonal C20 anti-MyoD antibody. Cdk4 protein expression from the same whole extracts used as internal control was visualised with the anti-Cdk4 (Santa Cruz). One hundred μ g of cell extracts were immunoprecipitated with the monoclonal anti-MyoD antibody (Clone 5.8A, Pharmingen) and resuspended in SDS containing samples. The immunoprecipitates were then subjected to SDS-PAGE and transferred to nitrocellulose membrane. Phosphorylated MyoD was detected by Western blot analysis using anti-phosphoserine antibody (Zymed). (C) In growing medium phosphorylation of the serine 200 by cyclin-Cdk complexes masks the phosphorylation shift of MyoD-Ala237 by the Mos kinase. 10T1/2 cells were transiently transfected with MyoD-wt (lane 2), MyoD-Ala200 (lanes 3 and 4) in combination with HA-Mos^{wt} (lane 4) or the kinase-inactive HA-Mos^{KM} (lanes 2 and 3). Twenty-five μ g of whole cell extracts from 10T1/2 expressing proteins were directly solubilised in SDS loading buffer. Proteins were separated by 10% SDS-PAGE. MyoD proteins were analysed by Western blotting for MyoD and Mos expression. Western blot showed that Mos^{wt} was able to phosphorylate the MyoD-Ala200 protein as evidenced by its reduced gel mobility.

3.2. Ser237 is the major site of Mos-dependent phosphorylation of MyoD

To determine precisely the site for *in vivo* Mos-dependent phosphorylation of MyoD, we mutated the serine 237 in MyoD. The mutant was generated by site-directed mutagenesis replacing the amino acid serine by a non-phosphorylatable alanine residue and named MyoD-Ala237. Bacterially produced wild-type MyoD and its MyoD-Ala237 mutant were subjected to phosphorylation by purified Mos kinase. Phosphorylated proteins were separated by SDS-PAGE and visualised by autoradiography. As expected, Mos kinase was able to phosphorylate MyoD-wt but could not phosphorylate MyoD-Ala237 (Fig. 2A). These data show that serine 237 is necessary for MyoD phosphorylation in this *in vitro* kinase assay. To extend to cell context, 10T1/2 cells were transfected with an expression vector encoding either MyoD-wt or MyoD-Ala237 in the absence or in the presence of Mos^{wt}. Two days after transfection, cells were lysed and whole cell extracts were subjected to 10% SDS-PAGE and MyoD was detected by Western blot analysis. As shown in Fig. 2B, MyoD-wt and MyoD-Ala237 migrated in SDS-PAGE as a doublet containing equivalent amounts of fast and slowly migrating forms (lanes 2 and 4) which probably represent different phosphorylation isoforms of MyoD as previously observed [28]. By contrast, when Mos^{wt} was co-transfected, a hyperphosphorylated form of MyoD-wt but not of MyoD-Ala237 accumulated as evidenced by using the polyclonal anti-P-Ser antibody (Fig. 2B, lane 3 and 5).

However, few hyperphosphorylated forms of MyoD can be detected when cells were transfected by the expression vector encoding MyoD-Ala237. In proliferating myoblasts serine 200

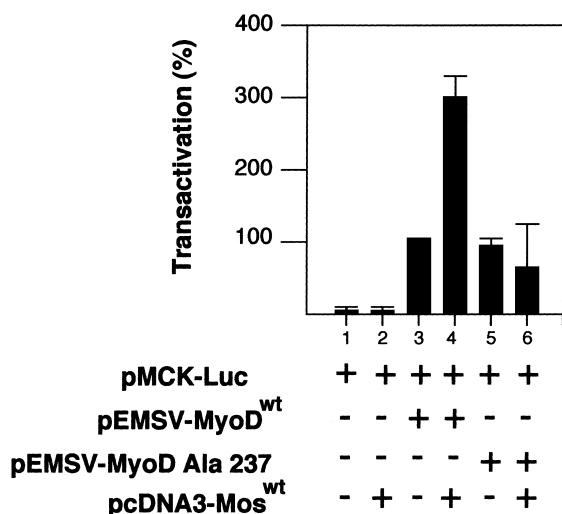


Fig. 3. Transactivating activity of MyoD-Ala237 is not enhanced when coexpressed with Mos. 10T1/2 cells were cotransfected with 0.5 μ g of MCK-luciferase reporter plasmid (lanes 1 to 6) together with 1 μ g of an expression vehicle encoding Mos^{wt} (lanes 2, 4 and 6), MyoD-wt (lanes 3 and 4) and MyoD-Ala237 (lanes 5 and 6). pEMSV expression vehicles without insert were included to normalise DNA in all transfections. Protein concentration was determined by the Bradford method. Luciferase activities were determined 48 h after transfection in high serum medium (15% FCS) and are expressed relative to that of each reporter plasmid transfected with pEMSV-MyoD, set as 100%.

is the major site of Cdk-dependent phosphorylation [12,13], so, in order to distinguish Cdk-dependent phosphorylation from Mos-dependent phosphorylation of MyoD, we used the MyoD-Ala200 mutant. 10T1/2 cells were transfected with MyoD-wt or MyoD-Ala200 with Mos^{wt} or the kinase-inactive Mos^{KM} expression vectors and whole cell extracts were subjected to Western blot analysis with the monoclonal antibody 12CA5 directed against HA-tagged Mos or the anti-MyoD antibody. When isolated from proliferating myoblasts ([28] and data not shown) or from cells cotransfected with MyoD and the kinase-inactive Mos^{KM} (Fig. 2C, lane 2), MyoD migrated in SDS-PAGE as a doublet containing fast and slow migrating forms while MyoD-Ala200 showed only a fast migrating form that was shown to be the hypophosphorylated species of MyoD [12,13]. However, we have previously shown that these two bands were resolved in a single fast migrating band after phosphatase treatment [28]. This result shows that MyoD is present under different phosphorylation states in myoblasts. In contrast, MyoD-Ala200 isolated from cells transfected with Mos^{wt} contained high levels of the slow migrating species (Fig. 2C, lane 4). In agreement with previous data, MyoD-Ala200 was expressed at higher levels than MyoD-wt, due to the stabilisation of the protein [13]. Altogether these data indicate that MyoD could be phosphorylated on Ser237 and that MyoD phosphorylation in the presence of Mos^{wt} was not due to Cdk-dependent phosphorylation.

3.3. Replacement of Ser237 to Ala237 in MyoD suppresses enhanced muscle gene-specific transacting activity by Mos

In an attempt to assess the consequence of Ser237 phosphorylation on MyoD activity, we tested abilities of MyoD-wt and MyoD-Ala237 to transactivate muscle-specific gene expression in the absence or presence of active Mos kinase

(Mos^{wt}). Plasmids expressing either MyoD-wt or MyoD-Ala237 alone or together with Mos^{wt} were transfected in 10T1/2 cells with a reporter plasmid containing the muscle creatine kinase promoter-enhancer driving the luciferase gene (MCK-Luc). Transfected cells were grown in proliferating medium for 48 h and transactivation of the reporter gene estimated by luciferase assays. No significant luciferase activity was observed when MCK-Luc was cotransfected with the empty expression plasmid (pEMSV) or the expression vector encoding the Mos kinase (Mos^{wt}) (Fig. 3, lanes 1 and 2). In addition, we have previously shown that Mos expression has no effect on the pEMSV promoter which drives MyoD expression [22]. As expected, luciferase activity was highly enhanced by MyoD-wt and MyoD-Ala237. Moreover, in the presence of Mos the level of MCK-luciferase activity was three-fold higher than that obtained with MyoD-wt alone. In contrast such an increase was not observed with the MyoD mutant Ala237 (Fig. 3, lanes 3 to 6). As previously shown, this accumulation of MyoD was not due to its metabolic stabilisation by Mos expression but to the activation of the positive autoregulation loop of MyoD [23]. Altogether our results clearly indicate that the increase in MyoD activity in response to Mos is due to a direct phosphorylation on MyoD protein and is not associated with hypothetical effects exerted by Mos on the obligate E partners: in vitro as well as in vivo we have never found a physical interaction between Mos and E-proteins nor a phosphorylation of the E-proteins by the Mos kinase [22,23]. In contrast, it has been recently shown that casein kinase II (CKII) increases the transcriptional activities of MyoD and MRF4, but independently of their direct phosphorylation [17].

Interestingly, sequence alignment revealed that serine at position 237 in MyoD is nearly identical in the transcription activation domains of myogenin (Ser204) and MRF4 (Ser189) but is missing in Myf-5, E12 and E47 proteins. This conservation suggests that this serine residue could be the Mos (or a related kinase) phosphorylation sites that play an important role in regulating the function of myogenic factors during the course of differentiation. In this context, it is noteworthy that, on the one hand, the phosphorylation of mos gene products (v-Mos/c-Mos) plays a key role in regulating its function (review in [29]) and, on the other hand, that PKA overexpression inhibits myogenesis by targeting molecules other than the MRFs [9]. Then, in vitro phosphorylation of v-Mos/c-Mos by PKA indicated that PKA inhibits v-Mos via phosphorylation on its Ser263, equivalent to residue ser232 in mouse c-Mos [30]. Thus, a set of phosphorylations implying c-Mos, PKA and MRF proteins could constitute one of the regulatory pathways of myogenic differentiation.

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