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Nuclear import of the myogenic factor MyoD requires cAMP-dependent protein kinase activity but not the direct phosphorylation of MyoD

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SUMMARY

MyoD is a nuclear phosphoprotein that belongs to the family of myogenic regulatory factors and acts in the transcriptional activation of muscle-specific genes. We have investigated the role of cAMP-dependent protein kinase (A-kinase) in modulating the nuclear locale of MyoD. Purified MyoD protein microinjected into the cytoplasm of rat embryo fibroblasts is rapidly translocated into the nucleus. Inhibition of A-kinase activity through injection of the specific inhibitory peptide PKI prevents this nuclear localisation. This inhibition of nuclear location is specifically reversed by injection of purified A-kinase catalytic subunit, showing the requirement for A-kinase in the nuclear import of MyoD. Site-directed mutagenesis of all the putative sites for A-kinase-dependent phosphorylation on MyoD, substituting serine or threonine residues for the non-phosphorylatable amino acid alanine, had no effect on nuclear import of mutated MyoD. These data exclude the possibility that the effect of A-kinase on the nuclear translocation of MyoD is mediated by direct phosphorylation of MyoD and imply that A-kinase operates through phosphorylation of components involved in the nuclear transport of MyoD.

Key words: MyoD, A-kinase, nuclear localisation

INTRODUCTION

MyoD is a nuclear phosphoprotein expressed exclusively in skeletal muscle cells, which belongs to the family of muscle-specific helix-loop-helix (HLH) proteins. These include Myf5, myogenin and MRF4/Myf6/Herculin. These proteins act as transcriptional activators of muscle-specific gene expression during muscle differentiation (Emerson, 1990; Olson, 1990; Weintraub et al., 1991), through binding to the E-box consensus sequence after heterodimerisation with the ubiquitous HLH proteins E12 or E47 (Brennan and Olson, 1990; Lassar et al., 1991). MyoD expression is constitutive in both myoblasts and myotubes, in contrast to myogenin and MRF4, which are induced throughout differentiation. Although MyoD activates its own transcription by an autoregulatory loop in myoblasts (Thayer et al., 1989), it does not appear to initiate the expression of other muscle-specific genes (such as myogenin and MRF4) until cells are committed to differentiate. Thus, a stringent regulation must then exist to prevent transactivation of muscle-specific genes by MyoD in proliferative myoblasts. Since in vitro muscle differentiation is triggered by serum withdrawal from the culture medium, it appears that differentiation is tightly controlled through a repressive mechanism involving serum and exogenous growth factors such as FGF and TGF-β that prevent myoblasts from undergoing muscle differentiation (Spizz et al., 1986; Kelvin et al., 1989; Salminen et al., 1991; Florini et al., 1991). Various reports have shown that myogenic HLH proteins serve as ultimate targets in the signal transduction pathways that repress myogenesis, either through inhibition of their expression (Vaidya et al., 1989; Lassar et al., 1989) or shut-off of their activities (Bengal et al., 1992; Benezra et al., 1990; Edmonson et al., 1991; Martin et al., 1992; and for a review, see Olson, 1992). Post-translational modification of myogenic HLH proteins, in particular phosphorylation, may constitute an important mechanism by which mitogens regulate differentiation. Indeed, a recent report of L. Li et al. (1992b) identified such a mechanism in the case of the inhibitory effect of FGF on myogenesis, establishing a direct link between the PKC signal transduction pathway and myogenic factors. Protein kinase C has long been implicated in the signal transduction cascade that represses myogenesis (Adamo et al., 1989; Cohen et al., 1977; Choi et al., 1991). However, the role of the cAMP-dependant protein kinase (A-kinase) in muscle cell differentiation has remained more speculative. Recently, it has been shown that overexpression of the catalytic subunit of A-kinase prevented activation of the myogenic programme and transactivation of an E-box reported gene by myogenic HLH proteins in non-muscle cells (L. Li et al., 1992a; Winter et al., 1993). These results are in agreement with data showing that agents that promote an elevated intracellular level of cAMP have a negative effect on myogenesis (Hu and Olson, 1988; Salminen

et al., 1991; Winter et al., 1993). This inhibitory effect, at least for myogenin, did not require direct phosphorylation (L. Li et al., 1992a). Moreover, phosphorylation of MyoD and Myf5 by A-kinase in vitro had no effect on their ability to bind DNA (Winter et al., 1993). However, such an inhibitory effect of cAMP on muscle differentiation has been explained by others as a result of the inhibition of cell proliferation, thereby preventing cells reaching an appropriate density to initiate differentiation (Zalin, 1973; Epstein et al., 1975). In contrast, agents that raise intracellular cAMP concentration have also been reported to stimulate myoblast differentiation (Zalin, 1973, 1977; Betz et al., 1979; Curtis et al., 1981). Moreover, it has been described that a transient increase in cAMP preceded fusion of cultured chick (Zalin, 1974; Zalin and Montague, 1977) and rat myoblasts (Ball and Samwal, 1980).

Here, we report that the nuclear import of MyoD is positively regulated by the cAMP-dependent protein kinase (A-kinase). However, because directed mutation of the three A-kinase sites (substitution of Ser/Thr by Ala) in the MyoD protein did not alter its nuclear localisation, we conclude that A-kinase regulation of MyoD nuclear import is not mediated by direct phosphorylation of MyoD but rather through the phosphorylation of factors involved in its active transport such as nuclear localisation sequence (NLS)-binding proteins, known to participate in the transport of other nuclear proteins.

MATERIALS AND METHODS

Production of mouse MyoD in bacteria

Mouse full-length MyoD was produced by amplifying the coding sequence by the polymerase chain reaction using *Taq* polymerase. The 5′ primer sequence 5′-TGGCGCATATGGAGCTTCTATCGCCG-3′ spanned 18 nucleotides from the ATG and included a *Nde*I restriction site for subsequent cloning. The sequence 5′-CGCGCGGGATC-CAAGCACCTGATAAATCGC-3′ was used as 3′ primer and included the *Bam*HI site present in the non-coding sequence of MyoD cDNA. After digestion with *Nde*I and *Bam*HI, the restriction fragment was purified by agarose gel and then ligated into the T7 expression vector cut by the same restriction enzymes. The resulting plasmid was used to transform *Escherichia coli* BL21 (DE3) bacteria for expression as previously described (Studier et al., 1990). Four hours of IPTG induction (1 mM final concentration) led to a good expression of MyoD in a fully soluble form. The MyoDΔ1.2.3 mutant, which lacks the three A-kinase sites, was produced with the same protocol.

Mutagenesis

The MyoD cDNA was mutagenised in the Moloney sarcoma virus LTR expression vector pEMSV-scribe (a generous gift from Dr H. Weintraub, Seattle, Washington; Davis et al., 1987). MyoD mutant was obtained by oligonucleotide-directed mutagenesis using the Promega in vitro mutagenesis kit (COGER, Paris, France). Generally, the manufacturer's instructions were followed except that the mutagenesis was carried out directly in pEMSV-scribe expression vector. Oligonucleotides were 29-30 nucleotides in length with, 13-14 nucleotides of exact homology with MyoD in the region flanking the substitution. Mutant clones were screened with the oligonucleotide used for mutagenesis, which has been labelled with T4 polynucleotide kinase using [γ-^{32}P]ATP (3000 Ci/mmole; Amersham, les Ulis, France). Selected clones were used for preparative plasmid isolation and then sequenced using the sequenase 2.0 kit (USB) and [α-^{35}S]dATP (3000 Ci/mmole, Amersham). The resulting triple mutant, which lacks the three A-kinase sites following substitution of serine or threonine by alanine, was designated as MyoDΔ1.2.3.

Antibodies, production and characterisation

Bacterially produced MyoD was used to immunise New Zealand rabbits (0.4-0.5 mg per injection). MyoD was prepared as followed: IPTG-induced bacteria were pelleted, lysed in Laemmli buffer (40 mM Tris-HCl, pH 6.8, 5 mM DTT, 1% SDS, 7.5% glycerol, 0.01 % bromophenol blue), and proteins were separated on 12.5% SDS-polyacrylamide gels. The MyoD band was excised and then electroeluted in buffer containing 25 mM Tris-HCl, 190 mM glycine and 0.1% SDS. The resulting antiserum was purified using partially purified MyoD bound to CNBr-activated Sepharose 4B (Pharmacia, St Quentin, Yvelines, France), following the manufacturer's instructions. Affinity-purified anti-MyoD antibodies give a specific nuclear staining in both C2 myoblasts and myotubes, and do not react with L6 muscle cells in both myoblasts and differentiated myotubes (data not shown). They also give no staining in normal fibroblast cell lines such as C3H10T1/2 or REF52 fibroblasts but give a bright nuclear staining when these same cells were injected with MyoD expression vector or MyoD protein (see below). The specificity of anti-MyoD antibodies was further confirmed by immunoblotting a C2 cell extract in which they revealed two bands of relative molecular mass of 45 kDa and 48 kDa, which were not detected in L6 or REF52 cell extracts (data not shown).

Purification of native MyoD proteins.

MyoDwt and MyoDΔ1.2.3 were purified as follows: IPTG-induced bacteria were lysed by freezing and thawing in buffer A (10 mM Hepes, pH 7.2, 1 mM DTT, 1 mM EDTA, 1 mM PMSF) and sonicated for 10 minutes to break the DNA. After clearing by centrifugation (12,000 ***g***, 15 minutes), bacterial extract was submitted to a 20% ammonium sulphate precipitation, which preferentially precipitates MyoD. After centrifugation (12,000 ***g***, 20 minutes), the pellet was resuspended in buffer B (12 mM Hepes (pH 7.9), 4 mM Tris-HCl (pH 7.9), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 12% glycerol) and dialysed overnight against buffer B. The dialysed solution was loaded on an E-box affinity chromatography column pre-equilibrated with buffer B. The synthetic double-stranded oligodeoxyribonucleotide used for affinity purification was derived from the R site of the MCK enhancer (Weintraub et al., 1990; Blackwell and Weintraub, 1990) and had the following sequence: 5′-GATCCCCCCAACAGCT-GTCGCCTGA-3′. After loading, the column was washed with buffer B and finally eluted with buffer C (20 mM Tris-HCl (pH 6.8), 1 M NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 12% glycerol). Fractions containing MyoD were collected, dialysed against buffer D containing 40 mM Hepes, 150 mM NaCl, 3 mM DTT and if necessary concentrated to a final concentration of 0.5-1 mg/ml. After this step, the MyoD protein was suitable for microinjection experiments, and kept at −20°C until use.

In vitro phosphorylation of purified MyoD protein by A-kinase

In vitro phosphorylation of bacterially produced fusion proteins was done in 60 µl of kinase buffer containing 20 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 10 µM ATP plus 5 µCi of [γ-^{32}P]ATP (3000 Ci/ mmole; Amersham, les Ulis, France), and 0.5 µg of purified protein. The reaction was started by adding catalytic subunit of A-kinase (a kind gift from Dr John Scott, Portland, Oregon) at a final concentration of 45 nM, and incubated at 30°C for 30 minutes. Proteins were separated on a 12.5% acrylamide gel. After fixation in 10% (v/v) acetic acid, 10% methanol (v/v), the gel was dried before autoradiography.

Microinjection and immunofluorescence

Rat embryonic fibroblasts (REF52) were cultured on glass coverslips, in Dulbecco's modified Eagle's medium (DMEM), supplemented with 8% calf serum as previously described (Fernandez et al., 1991).

For microinjection studies, REF52 cells were microinjected with purified MyoD protein (0.5-1 mg/ml) or pEMSV-MyoD expression

vector (0.5 mg/ml), together with mouse inert IgG antibodies (1 mg/ml) to serve subsequently as markers in identifying injected cells. After microinjection, cells were returned to the incubator for the indicated times. Cells were then fixed with formalin as described before (Vandromme et al., 1992) and MyoD localisation was analysed by immunofluorescence using affinity-purified anti-MyoD antibodies (dilution 1/20) followed by reaction with biotinylated anti-rabbit antibodies (Amersham, les Ulis, France) and by subsequent reaction with Texas Red-Streptavidin (Amersham) to visualise MyoD staining together with fluorescein-conjugated anti-mouse antibodies (Cappel, Organon Technica, Fresnes, France) to visualise injected cells. MyoD proteins were injected at a concentration of 0.5-1 mg/ml, inhibitory PKI peptide, PKi(6-24), and non-inhibitory PKI, PKi(15-24), (Fernandez et al., 1991) were injected at 2.10^{-5} M and catalytic subunit of A-kinase was at 0.5 mg/ml. When cells were injected with PKI before injection of MyoD proteins, the mouse marker IgGs were included and injected with the PKI solution (not with the MyoD proteins) to ensure that all the cells positive for the microinjection marker and for MyoD had been first injected with PKI.

RESULTS

Microinjection of PKI abolishes the nuclear localisation of microinjected MyoD in REF52 cells

We recently reported that nuclear import of MyoD is an active process that involves two nuclear localisation sequences present inside the basic-helix 1 region of MyoD (Vandromme et al., unpublished data). Since this region also contains three putative sites for phosphorylation by A-kinase (see Fig. 2, below), we questioned whether the nuclear import of MyoD was regulated by A-kinase. This protein kinase has been implicated in the regulation of nuclear compartmentalisation of other transcription factors such as c-fos and c-rel-related proteins (Roux et al., 1990; Mosialos et al., 1991). The role of A-kinase in the nuclear localisation of MyoD was investigated by microinjecting purified bacterially expressed MyoD (see Materials and Methods) into the cytoplasm of non-muscle cells (rat embryonic fibroblasts: REF52). The intracellular localisation of the injected protein was then analysed by indirect immunofluorescence using rabbit anti-MyoD antibodies. We chose to use microinjection of a fibroblast cell line to examine the regulation of nuclear import for the following reasons: firstly, these cells are devoid of endogenous MyoD proteins, eliminating the possibility that endogenous MyoD proteins would cross-contaminate the nuclear signal for injected MyoD. Although these cells do not synthesise MyoD or undergo differentiation under normal conditions, they still present an environment in which MyoD can act physiologically, since overexpression of MyoD is sufficient to induce expression of differentiation markers in a pathway mimicking myoblast differentiation (Davis et al., 1987; Weintraub et al., 1989). Secondly, we have encountered several problems in attempting to analyse the regulation of endogenous MyoD localisation in various myoblast cell lines. In particular, the levels of MyoD nuclear staining in a typical myoblast population are extremely heterogeneous, making it difficult to assess changes in nuclear locale as a direct function of microinjection. Purified MyoD protein was injected in REF52 cells, alone or 5 minutes after prior injection of a specific inhibitor of A-kinase, PKIm, a peptide derived from the thermostable protein kinase inhibitor that is a potent inhibitor of A-kinase in vivo (Fernandez et al., 1991). Injected cells were fixed 30 minutes following the injection, a time sufficient for MyoD to acquire its nuclear locale. In all cases, an inert mouse antibody was added to the solution of inhibitor peptide (or to MyoD protein, when only MyoD was injected) to serve for subsequent identification of injected cells. As shown in Fig. 1, MyoD injected alone was predominantly nuclear 30 minutes after its cytoplasmic injection in REF52 fibroblasts (Fig. 1A and B). In contrast, MyoD was found essentially in the cytoplasm if cells were first injected with PKI, showing a requirement for A-kinase activity in the nuclear import of MyoD (Fig. 1C and D). This effect was specific for A-kinase, since injection of an inactive form of PKI, in which the essential sequence for inhibition had been altered, had no effect on the localisation of injected MyoD, which showed a similar nuclear distribution as when MyoD was injected alone (Fig. 1E and F). Moreover, the inhibition of MyoD nuclear import was reversed when A-kinase was injected together with MyoD into cells first injected with the PKI inhibitor (Fig. 1G and H), further proving that this effect on MyoD nuclear transport is specific for A-kinase inhibition. We further questioned whether the nuclear retention of MyoD in the nucleus would also be a process that requires A-kinase activity, by injecting PKI into REF52 cells 15-20 minutes after injection of MyoD protein. In this case, the injected MyoD protein remained localised in the nucleus, indicating that only the transport and not the retention of MyoD in the nucleus was affected by inhibition of A-kinase activity (data not shown). To ensure that the cytoplasmic retention of MyoD in cells first injected with PKI was not a consequence of indirect effects of A-kinase inhibition on cell metabolism, MyoD was injected into cycloheximide-treated cells. Such treatment did not affect the nuclear import of the protein, showing that this process only involved post-translational regulation (data not shown).

Taken together these results show that A-kinase activity is required for the nuclear import of microinjected MyoD into non-muscle cells.

Mutagenesis of A-kinase phosphorylation sites in MyoD does not inhibit nuclear import

Since inhibition of A-kinase blocked nuclear import of MyoD, we investigated whether this effect was mediated by direct phosphorylation of MyoD. For this purpose, we performed site-directed mutagenesis to substitute the phosphate acceptor amino acid (serine or threonine) of the three optimal putative consensus phosphorylation sites for A-kinase (R/K-R/K-X-S/T; Feramisco et al., 1980, and see sequence in Fig. 2A) with a non-phosphorylatable residue, alanine.

The full-length MyoD protein mutated for the three A-kinase sites (MyoDΔ1.2.3) was produced and purified as described for the wild-type protein (MyoDwt; see Materials and Methods). As a control, in vitro phosphorylation of the two proteins by A-kinase was performed and showed that, in contrast to the wild-type MyoDwt, which is strongly phosphorylated by A-kinase (Fig. 2B, lane 1), MyoDΔ1.2.3 was no longer phosphorylated (Fig. 2B, lane 4). This result excludes the possibility that the mutated MyoDΔ1.2.3. protein contains any additional sites for phosphorylation by A-kinase. To test the influence of the lack of phosphorylation sites for A-kinase in the nuclear localisation of MyoD, wild-type and mutant cDNAs, kept in the original pEMSV expression vector during the mutagenesis procedure, were microinjected into growing

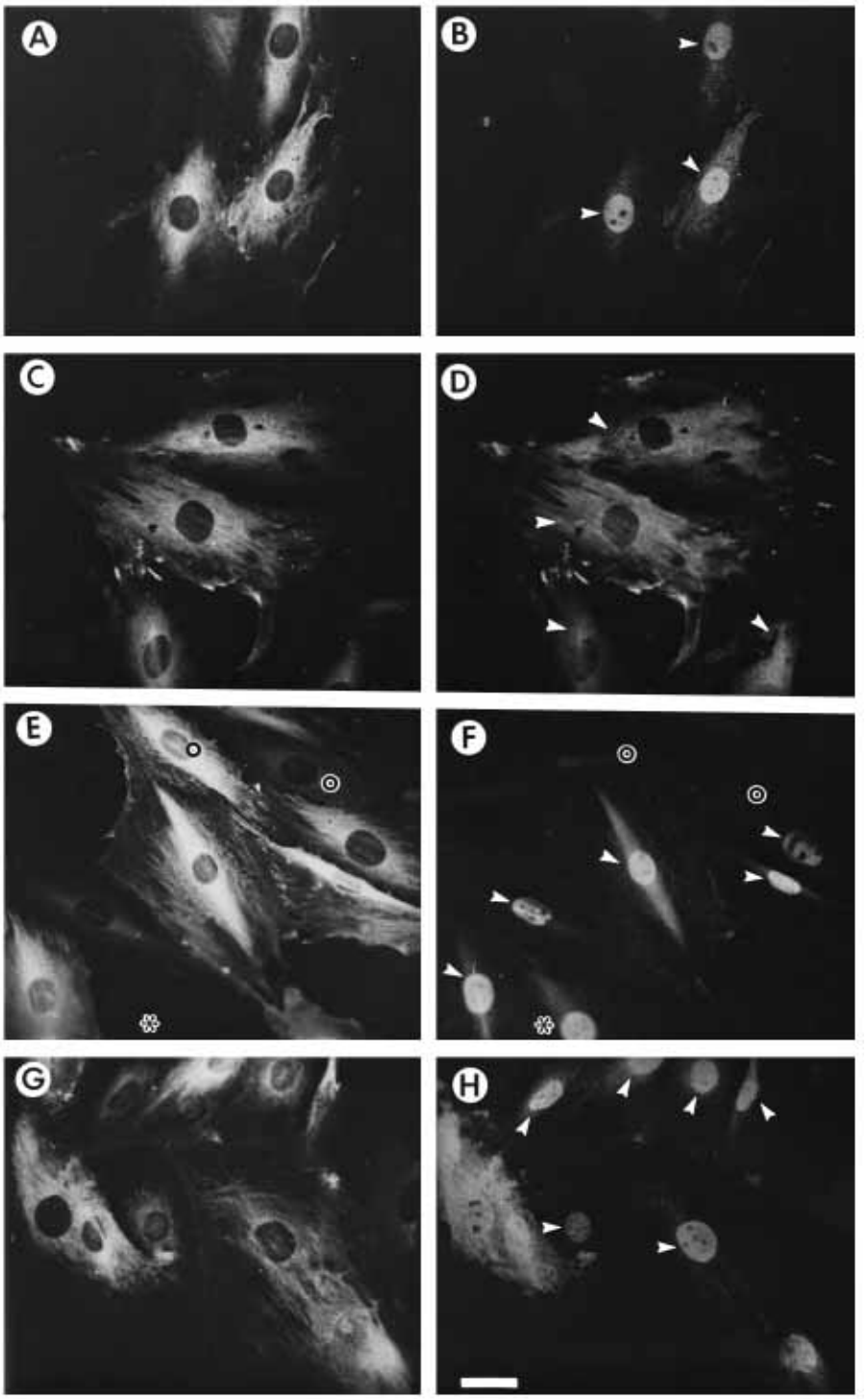

Fig. 1. Inhibition of A-kinase prevents the nuclear import of MyoD. REF52 fibroblasts were microinjected into the cytoplasm with: purified MyoD protein only (A-B); MyoD protein either after prior injection of PKI, the specific inhibitor peptide of A-kinase (C-D) or after prior injection of a non-inhibitory (shorter) PKI peptide (E-F); MyoD protein and A-kinase after prior injection of the inhibitory PKI peptide (G-H). Cells were fixed after 30 minutes and stained for MyoD as described in Materials and Methods. Mouse IgGs were included with PKI peptides in the first set of microinjections (when cells were double injected (C-H)) or with the MyoD protein when only MyoD was injected (A-B) to act as a marker of injected cells. These mouse IgGs are visualised by reaction with fluorescein anti-mouse antibodies in (A,C,E and G). Staining for the distribution of injected MyoD proteins is shown in (B,D,F and H). The injected cells are marked by arrowheads. In (E and F) an asterisk marks a cell that was injected with MyoD only, whereas two open circles mark cells that were injected with the non-inhibitory PKI peptide and mouse markers only. Bar, 10 μm.

REF52 cells, and MyoD expression was investigated by indirect immunofluorescence 12 hours after injection. We observed that both mutant and wild-type-expressed MyoD proteins were able to enter the nucleus (Fig. 3). These results show that the absence of A-kinase sites on MyoD does not appear to impair its nuclear transport. However, plasmid injection does not allow us to examine or control for the kinetics of nuclear import, and thus for a possible effect of MyoD phosphorylation by A-kinase on the rate of its nuclear transport. Therefore, each purified protein, MyoDwt and MyoDΔ1.2.3, was microinjected into the cytoplasm of REF52 fibroblasts and cells were fixed 15 minutes and 30 minutes after the injection. As shown in Fig. 4, no significant differences have been observed in the nuclear import kinetics of MyoDΔ1.2.3 versus MyoDwt. The two proteins acquired a nuclear localisation within 15 minutes (Fig. 4A and C) after their cytoplasmic injection into REF52 cells and they were both essentially nuclear after 30 minutes (Fig. 4B and D).

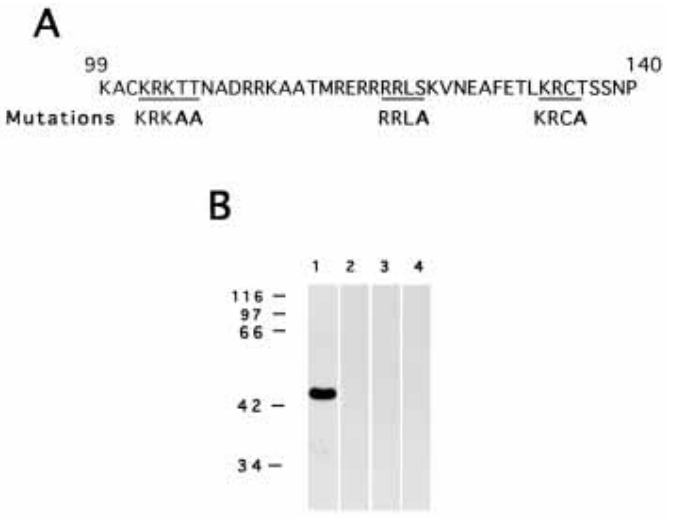

Fig. 2. Mutation of A-kinase putative phosphorylation sites on MyoD protein and in vitro phosphorylation by A-kinase. (A) Partial amino acid sequence of mouse MyoD protein underlining the 3 putative A-kinase phosphorylation sites on MyoD proteins that have been mutated to replace the potential serine and threonine by alanine as indicated underneath the sequence. (B) In vitro phosphorylation of purified wild-type and mutated MyoDΔ1.2.3 proteins by A-kinase: in vitro phosphorylation reactions were performed as described in Materials and Methods using 0.5 mg of either wild-type protein (lanes 1 and 2), or A-kinase site mutated MyoD (lane 4). Lane 1 shows incubation of wild-type MyoD with A-kinase only; lane 2 shows wild-type MyoD incubated with A-kinase in the presence of PKI; lane 3 shows incubation of A-kinase alone; lane 4 shows incubation of mutated MyoDΔ1.2.3 with A-kinase.

Moreover, coinjection of the PKI inhibitor with MyoDΔ1.2.3 also resulted in its cytoplasmic retention as observed for the wild-type protein (data not shown). Taken together, these results exclude the possibility that the requirement for A-kinase activity in the nuclear import of MyoD is mediated by direct phosphorylation of MyoD and suggest a mechanism involving the phosphorylation of co-factors that would be implicated in the process of nuclear transport of MyoD.

DISCUSSION

In the present study we have shown that the nuclear import of MyoD proteins requires the activity of the cAMP-dependent proteins kinase (A-kinase). This role of A-kinase, however, does not involve the direct phosphorylation of MyoD, since mutations of all three putative A-kinase sites on MyoD did not impede its nuclear transport. The conclusion that the nuclear import of MyoD is modulated and dependent on A-kinase activity is consistent with previous reports addressing the functions of A-kinase in transcriptional control. A-kinase directly phosphorylates (at least in vitro) and modulates the in vivo activity of the CREB family of transcriptional activators (Gonzalez and Montminy, 1989). In addition A-kinase has also been implicated in the regulation of the subcellular localisation of a variety of other transcriptional factors such as c-rel protein, r-FNIL6 and c-fos (for a review, see Whiteside and Goodbourn, 1993). Phosphorylation by A-kinase has been proposed to stimulate the translocation of c-rel protein from the cytoplasm where it is sequestered as an inactive form, to the nucleus where it becomes activated (Mosialos et al., 1991). In PC12 cells, cAMP has been shown to stimulate the C/EBP-

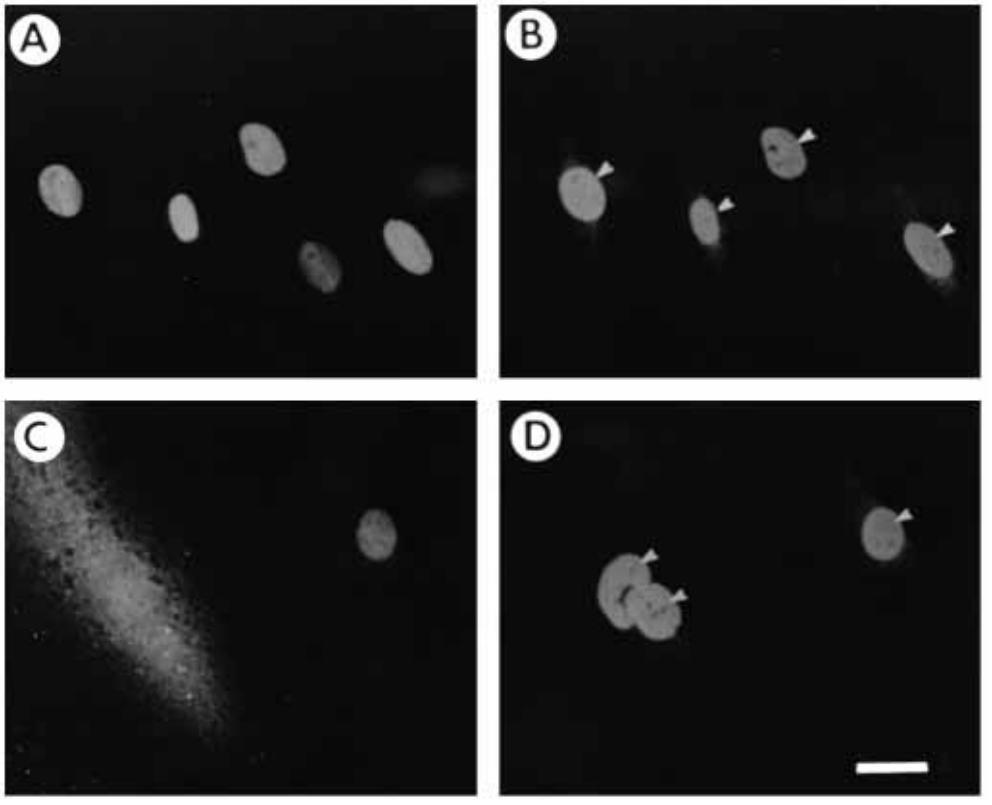

Fig. 3. Expression of wild-type and mutated MyoD protein after injection of their coding expression vector. REF52 cells were microinjected in the nucleus with the pEMSV-wild-type or mutant MyoD expression vector, together with mouse IgGs. Expression was allowed for 12-15 hours before fixation of the cells and staining for the distribution of expressed MyoD protein (B and D, arrowheads) and for microinjected cells through visualisation of injected mouse IgGs as before (A and C). (A and B) Injection of wild-type MyoD expression vector; (C and D) injection of mutated MyoDΔ1.2.3 expression vector.

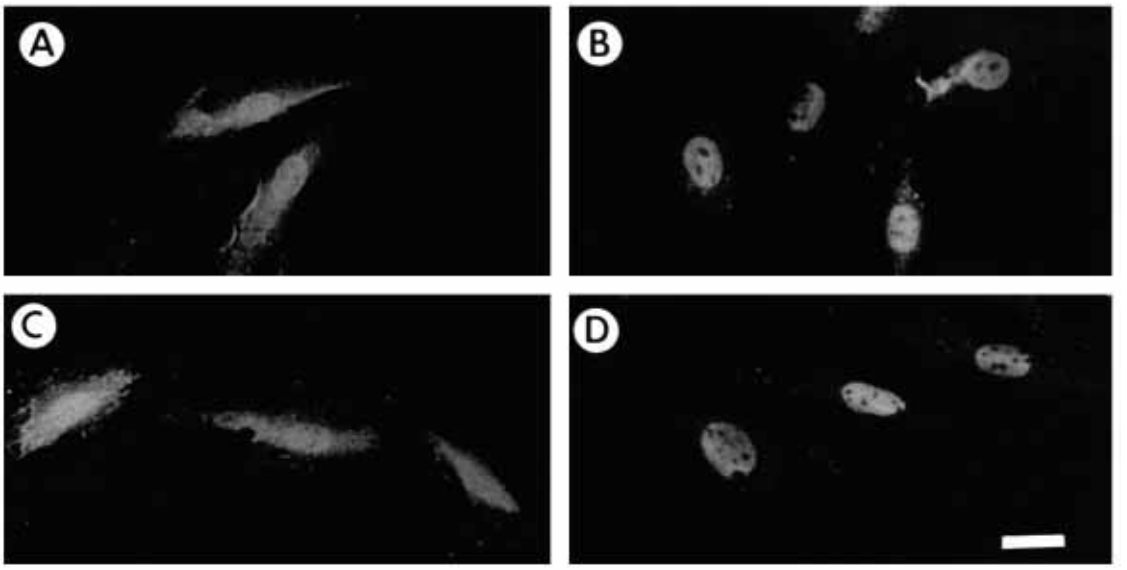

Fig. 4. Kinetics of nuclear translocation of microinjected wild-type and mutated MyoDΔ1.2.3 proteins. Purified MyoD proteins were injected into the cytoplasm of REF52. Cells were subsequently fixed at 15 and 30 minutes post-injection, before immunostaining for the distribution of injected MyoD as described in Materials and Methods. (A and B) Distribution of wild-type MyoD protein 15 and 30 minutes after injection, respectively; (C and D) distribution of mutated MyoDΔ1.2.3 protein 15 and 30 minutes after injection, respectively.

related transcription factor, r-NFIL6, to translocate to the nucleus and thus induce c-*fos* transcription (Metz and Ziff, 1991). Roux et al. (1990) have demonstrated that the nuclear accumulation of c-fos protein requires a continuous stimulation by growth factors in a cAMP-dependent manner.

The role of A-kinase in myogenesis is still both controversial and unresolved. Several early reports identified a peak of cAMP preceding myoblast fusion in primary chick myoblasts (Zalin and Montague, 1974; Zalin, 1977) and in rat myoblast (Ball and Samwal, 1980) in vitro, and agents that raise intracellular cAMP have been reported to stimulate myoblast differentiation (Betz and Changeux, 1979; Curtis et al., 1981). However, other studies have reported a negative effect of such treatment on myogenesis (Hu and Olsen, 1988; Salminen et al., 1991), and two recent reports using chronic drug treatment and overexpression of A-kinase catalytic subunit have suggested that A-kinase plays an inhibitory role in myogenesis by inhibiting the transactivation function of myogenic factors (L. Li et al., 1992a; Winter et al., 1993). Although the basis for such contradictions is unclear, both the latter studies relied upon overexpression of heterologous A-kinase catalytic subunit to reach their conclusions. In both cases, the inhibition observed by elevating A-kinase was not related to direct phosphorylation of the myogenic factors. Neither study addressed the role of the basal levels of A-kinase activity in modulating myogenesis or to what extent this level was perturbed by chronic drug treatment or overexpression of the catalytic subunit. Indeed, several studies have reported that the cAMP level was not significantly elevated following treatment of myoblasts with serum or growth factors (1.2- to 1.5-fold increase), whereas treatment with drugs that elevated intracellular cAMP result in a 4-fold increase over the basal concentration (Hu and Olson, 1988; Salminen et al., 1991). Therefore it remains plausible that physiological levels of A-kinase activity can exert a positive role on MyoD subcellular localisation and activity in vivo whereas overexpression of A-kinase may have the opposite effect. To date the consequence of inhibiting A-kinase activity on myogenesis have not been reported, probably since chronic inhibition of A-kinase for prolonged periods is not compatible with cell survival. Indeed, as we have shown previously (Lamb et al., 1991), cells must keep a basal level of A-kinase activity to maintain the interphase state of their chromatin (decondensed) and their transcriptional activity. Inhibition of this basal level with PKI results in marked changes in intracellular activity that mimic many of the events taking place upon entry into mitosis. Indeed we have estimated from microinjection experiments with PKI, that fibroblast cells contain ~0.5 μM of active A-kinase (Fernandez et al., 1991), although this level must vary during the cell cycle (Zeilig and Goldberg, 1977). If such basal activity of A-kinase is sufficient to promote nuclear import of MyoD, it implies that stimulation of A-kinase by agents that increase the intracellular cAMP level (and consequently intracellular A-kinase activity) will not result in a noticeable increase in the intensity of nuclear MyoD staining. Indeed, we observed no detectable changes in MyoD staining by treating C2 cells with forskolin or dibutyryl-cAMP (M. Vandromme and A. Fernandez, unpublished observations).

Our observations that the intracellular locale of MyoD is directly modulated by the basal levels of A-kinase strongly imply that the nuclear localisation process has been established to function within the physiological fluctuations of the cellular environment and does not require substantive changes in cellular activity. Such a process is consistent with observations showing that expression of MyoD can inhibit proliferation in non-muscle cells, an effect outside its function in the transcriptional activation of muscle-specific genes (Crescenzi et al., 1990; Sorrentino et al., 1990). If MyoD acts in a similar manner in precursor muscle cells, the level of nuclear MyoD may contribute to the withdrawal of myoblasts from the cell

cycle, which characterises the myoblast-myotube transition. In such a predictive model, the amount of MyoD translocated into the nucleus may be rate limiting in the commitment of cells to terminal differentiation. In support of such a hypothesis, the levels of cAMP (and consequently A-kinase) were found to fluctuate during the normal cell cycle in non-muscle cells (Zeilig and Goldberg, 1977), and we and others (Tapscott et al., 1988) have observed that the level of MyoD nuclear staining is highly variable in asynchronous populations of growing myoblasts.

We found that mutation of all three A-kinase putative sites did not noticably affect the nuclear localisation and nuclear import kinetics of the mutated MyoD protein, implying that the effect of A-kinase on the nuclear import of MyoD is mediated through an indirect pathway. Therefore the question remains concerning the mode of action of A-kinase in the process of MyoD nuclear transport. One possibility could be that A-kinase phosphorylation may affect the dimerisation partners of MyoD, E12 and E47, involved in the formation of heterodimers with MyoD, an event that is required for MyoD DNA-binding and transcriptional activity (Brennan and Olson, 1990; Lassar et al., 1991). However, it was showed that mutation of the region of MyoD involved in its dimerisation does not affect its nuclear localisation, implying that these two events can be uncoupled (Tapscott et al., 1988). This makes unlikely a possible effect of A-kinase through phosphorylation of E12 or E47. Another possibility relates to the general mechanism involved in nuclear transport. Most nuclear proteins contain at least one nuclear localisation sequence (NLS), which acts as a selective signal for nuclear import. We have recently determined that MyoD contains two such NLS present in the basic helix 1 region, which are necessary and sufficient to specify its nuclear locale in both muscle and non-muscle cells (Vandromme et al., unpublished data). The action of these NLSs in the nuclear transport of MyoD (as for the transport of other proteins into the nucleus) requires the specific binding to the NLS of a receptor protein (the NLS-binding protein: NBP) localised in the cytoplasm or pre-associated with the nuclear envelope, and the subsequent binding of the NBP-nuclear protein complex with the nuclear pore complex with translocation inside the nucleoplasm via the nuclear pores (reviewed by Garcia-Bustos et al., 1991). The effect of A-kinase on MyoD nuclear import revealed in this study may be mediated by phosphorylation of such intermediate cofactors, especially the NLS-binding proteins. Indeed, NLS-binding proteins have been characterised in various organisms (Silver et al., 1989; R. Li et al., 1992; Yamasaki et al., 1989; Meier and Blobel, 1990; Stochaj et al., 1991, 1993; Stochaj and Silver, 1992). One of them, NBP70, present in yeast and higher organisms, has to be phosphorylated to bind to NLS, suggesting that phosphorylation by yet unidentified kinases may constitute an important step in the regulation of nuclear import (Stochaj and Silver, 1992).

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