

The nuclear localization domain of the MEF2 family of transcription factors shows member-specific features and mediates the nuclear import of histone deacetylase 4

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SUMMARY

Targeting of myocyte enhancer binding factor 2 (MEF2) proteins to the nucleus depends on a C-terminal bipartite nuclear localization signal (NLS). By expression of green fluorescent protein (GFP)/MEF2 fusion proteins in transfected myoblasts, we show that MEF2C contains an additional 13 amino acids domain, located immediately upstream of the NLS, which contributes to its nuclear retention. We also show that the NLS present in MEF2 proteins is required for efficient nuclear localization of histone deacetylase 4 (HDAC4). In muscle cells, transfected HDAC4 is largely cytoplasmic or, to a lesser extent, pan-cellular. Co-transfection of either MEF2A or MEF2C causes HDAC4 to accumulate in the nucleus in association

with MEF2. This effect strongly depends on MEF2 NLS; it also requires the specific interaction of HDAC4 with MEF2, since the isolated NLS is not sufficient for targeting HDAC4 to the nucleus and other nuclear proteins, such as NF- κ B, cannot substitute MEF2. Therefore, we demonstrate that HDAC4, different from HDAC5, is mainly a cytoplasmic resident protein, requiring a trans-acting NLS for nuclear localization. The physiological implications of MEF2 carrying its own inhibitor to the nucleus are discussed.

Key words: Transcription factors, Cell compartmentation, Muscle differentiation

INTRODUCTION

Members of the myocyte enhancer factor-2 (MEF2) family of transcription factors bind to and activate transcription through DNA response elements bearing the consensus sequence CTA(A/T)₄TAG, which were initially identified in many muscle-specific genes and are required for maximal transcriptional activity (Olson et al., 1995). Four isoforms of MEF2 are known, which are encoded by different genes and are designated MEF2A-D. They share high homology in an N-terminal MADS (MCM1, Agamous, Deficiens, Serum response factor) domain that mediates DNA-binding and dimerization and an adjacent MEF2-specific domain that influences DNA-binding affinity and interaction with transcriptional co-factors. The C-terminal regions of MEF2 factors, which are more divergent, act as transcription activation domains (TADs) and contain signals that are necessary for nuclear localization (Black and Olson, 1998). Although it is established that MEF2 factors play a critical role in muscle differentiation and development, subsequent studies have demonstrated that, with few exceptions, they are expressed ubiquitously and might have additional functions apart from their involvement in myogenesis. Indeed, the importance of MEF2 proteins has been established for diverse gene regulatory programs, including neural differentiation (Mao et al., 1999), cardiac morphogenesis (Lin et al., 1997), blood vessel formation (Lin

et al., 1998) and growth factors responsiveness (Han and Prywes, 1995; Clarke et al., 1998).

MEF2 activity is regulated by a variety of effectors in different cell types. Growth factor signalling mediated by mitogen-activated protein kinases (MAPKs), such as p38 and BMK1/ERK5, stimulates MEF2s transcriptional activity, by targeting phosphorylation to conserved sites in their TADs (Han et al., 1997; Kato et al., 1997). Calcium/calmodulin-dependent protein kinase (CaMK) and calcineurin also stimulate MEF2 activity (Woronicz et al., 1995; Liu et al., 1997; Wu et al., 2000). Five proteins have been identified so far that act as MEF2-specific transcriptional corepressors: Cabin 1 (Youn et al., 1999), MEF2 interacting transcriptional repressor (MITR) (Sparrow et al., 1999; Zhang et al., 2001) and the histone deacetylases HDAC4 (Miska et al., 1999), HDAC5 (Lemerrier et al., 2000) and HDAC7 (Dressel et al., 2001). In particular, HDAC4, HDAC5 and HDAC7 are members of the class II histone deacetylases, which specifically interact with individual members of the MEF2 family through an N-terminal dimerization domain and repress MEF2 transcriptional activator capacity at various promoters (Miska et al., 1999; Lemerrier et al., 2000; Dressel et al., 2001). Insight into the regulation and physiological significance of HDAC/MEF2 interactions has been provided by recent observations in different cell systems. In cardiomyocytes, activated CAMKIV disrupts the MEF2/HDAC complex and

causes the unmasking of MEF2 transcriptional activity (Jianrong et al., 2000). This finding indicates that in cardiomyocytes MEF2 may be the target of hypertrophic signals that act through the CAMK pathway, or the MAPK pathway, or both (as in the case of signalling triggered by phenylephrine). In fact, while CaMK activates MEF2 by dissociating HDAC/MEF2 complexes, MAPK stimulates MEF2 activity by direct phosphorylation of the TAD. Together, the CAMK and MAPK pathways synergize to activate MEF2. Export of HDAC5 to the cytoplasm, following phosphorylation of specific serine residues by activated CAMK1, was shown to be essential for efficient myogenic conversion of 10T1/2 fibroblasts by exogenous MyoD (McKinsey et al., 2000), an event that requires MEF2 (Molkentin et al., 1995) and histone acetylase (HAT) activity (Sartorelli et al., 1997). In T cells, MEF2D was shown to play a key role in T-cell receptor (TCR)-mediated apoptosis during thymic negative selection. It mediates calcium-dependent transcription of Nur77, a transcription factor involved in TCR-mediated apoptosis of thymocytes (Woronicz et al., 1994; Liu et al., 1994). MEF2D was shown to bind two calcium-responsive DNA elements in the Nur77 promoter and to mediate the calcium-dependent induction of Nur77 (Woronicz et al., 1995). It was recently demonstrated that HDAC4 (as well as MITR) contains a calmodulin binding domain that overlaps with the MEF2 binding domain. Calcium-dependent binding of calmodulin to HDAC4 leads to its dissociation from MEF2, relieving MEF2 from the transcriptional repression of the Nur77 promoter by HDAC4 (Youn et al., 2000).

In the present report we provide a thorough study of the signals that mediate nuclear localization of MEF2 and show that the NLS present in MEF2 proteins is necessary to localize the HDAC4/MEF2 complex to the nucleus.

MATERIALS AND METHODS

Plasmids

pGFP/MEF2A and pGFP/MEF2C were generated by isolation of the inserts present in pGem7zfc(+)/Mef2X/R (Yu et al., 1992) and pcDNA1/MEF2C (Martin et al., 1993) with appropriate restriction enzymes and in frame ligation to the 3' end of the sequence encoding green fluorescent protein (GFP) in pEGFP-C3 (Clontech). pGFP/MEF2A Δ -471 was obtained by insertion of the *EcoRI/BamHI* fragment of pGem7zfc(+)/Mef2X/R into the corresponding sites of pEGFP-C3. pGFP/MEF2C Δ -429, pGFP/MEF2C Δ -411, pGFP/MEF2C Δ -399 were obtained by subcloning into pGFP-C3 of the PCR products of the selected sequences present in pcDNA1/MEF2C. The same forward primer (5'-GGGAAGCTTGGGAGAAAAAGAT-3') and the following reverse primers were used: 5'-TCATCGGTC-GCTCCCATCGTA-3', to generate Δ -429; 5'-AATCTAGATTACCT-CCCCGCCTC-3', to generate Δ -411; 5'-GTATCTAGAAGGGGTG-GTGG-3', to generate Δ -399. GFP fusions to MEF2C sequences representing different extensions of the C-terminal region were obtained by subcloning into pEGFP-C3 of the PCR products of the corresponding sequences in pcDNA1/MEF2C. The following primer couples were used: 5'-AAGCTTGAGGATCACCGGAACGAA-3' and 5'-TCTAGATCATGTTGCCCATCCTTC-3', to generate MEF2C430-466; 5'-GGGAAGCTTCCACAACACACCACG-3' and 5'-TCTAGATCATGTTGCCCATCCTTC-3', to generate MEF2C401-466; 5'-GGGAAGCTTCTCCTAGAGACCGT-3' and 5'-AATC-TAGATTACCTCCCGCCTC-3', to generate MEF2C389-411; 5'-GGGAAGCTTCCACAACACACCACG-3' and 5'-TCATCG-GTCGCTCCCATCGTA-3', to generate MEF2C401-429. The same procedure was followed for constructing GFP fusions to the C-terminal region of MEF2A (pGFP/MEF2A472-507); the following

primers were used: 5'-AAGCTTGACCCACGGGGCGACTTC-3', 5'-CAGCTGTTAGGTCACCCACGCGTCCAT-3'. pFLAG/MEF2C and pFLAG/MEF2C Δ NLS were generated by in frame ligation of full length MEF2C or MEF2C Δ -429 cDNAs to the 3' end of the FLAG epitope, using the *HindIII* and *XbaI* sites in the pCMV-FLAG vector (Sigma). Plasmids encoding Myc-tagged HDAC4 (pCHDAC4-Myc) (Miska et al., 1999) HA-tagged HDAC5 (Verdel et al., 2000), NF-YB (Di Silvio et al., 1999) were kind gifts of T. Kouzarides (University of Cambridge, UK), S. Khochbin (Case Western Reserve University, Cleveland, OH) and R. Mantovani (Università di Modena e Reggio Emilia, Italy), respectively.

Cell cultures and transfections

C2.7 myoblasts (Pinset et al., 1988), kindly provided by M. Buckingham, were grown in Dulbecco's modified eagle medium (DMEM, Life Technologies) containing 10% fetal calf serum (FCS, Hyclone). Differentiation to myotubes was achieved by lowering FCS concentration to 1%. Proliferating myoblasts, grown at 60% confluence in 60 mm dishes, were transfected with a total of 2 μ g plasmid DNA/dish, using the lipid-based Lipofectamine Plus Reagent (Life Technologies) according to the manufacturer's instructions.

Detection of GFP fluorescence and immunofluorescence

36-48 hours after transfection cells were washed extensively with PBS, fixed for 20 minutes at room temperature with 3% paraformaldehyde in PBS, permeabilized with 0.05% Triton X-100 in PBS for 5 minutes, and incubated for 15 minutes with PBS containing 1% bovine serum albumin (BSA). Cells were then incubated overnight at 4°C with the primary antibody at the appropriate dilution. Antibodies used were: monoclonal anti-Myc (1:100, Sigma), polyclonal anti-NFY-B (1:50) (Mantovani et al., 1992), polyclonal anti-HA (1:50, Sigma). Cells were then washed with PBS, incubated for 15 minutes with 1% BSA in PBS and incubated for further 60 minutes with the secondary antibody. Secondary antibodies used were: goat anti-mouse IgG rhodamine conjugated (1:200, Pierce), goat anti-rabbit IgG fluorescein-conjugated (1:200, Pierce). After extensive washing with PBS, cell monolayers were mounted in 10 mM Tris-HCl, pH 9, containing 60% glycerol and examined in a Zeiss Axiophot fluorescence microscope. Images were acquired to a digital camera by the SPOT32 software package (Diagnostic Instruments Inc.) and exported into Adobe PhotoShop for processing. Prints were obtained by employing a dye-sublimation printer (Kodak). Quantitative estimates of nuclear/cytoplasmic distribution of GFP/MEF2 proteins and HDAC4 were obtained by analyzing at least 100 cells in three different experiments. Green fluorescence intensity was scored by image analysis with Adobe PhotoShop and the obtained data were statistically evaluated with SPSS 9.0 software package.

Western blot analysis and immunoprecipitation

Proliferating C2.7 myoblasts in 100 mm dishes were transfected with 5 μ g of pCHDAC4-Myc and 5 μ g of pCMV-FLAG vector, pFLAG/MEF2C or pFLAG/MEF2C Δ NLS. A fraction of the transfection cocktail was used to transfect C2.7 myoblasts grown in 30 mm dishes; these cells were subsequently analyzed by immunofluorescence to check the efficiency of transfection. 36 hours after transfection cells were collected and homogenized in 10 volumes of ice-cold buffer (10 mM Tris-HCl pH 7.9, 10 mM NaCl, 5 mM MgCl₂, supplemented with protease inhibitors). KCl was then added to 100 mM final concentration, cytoplasmic protein extracts were harvested and nuclei were collected by centrifugation at 800 *g*. Nuclei were then homogenized in 100 μ l of ice-cold 10 mM Tris-HCl pH 7.9, 5 mM MgCl₂, 0.3 M KCl; nuclear extracts were harvested by centrifugation at 13,400 *g* for 20 minutes. 5 μ g of cytoplasmic and nuclear proteins were run in an SDS/polyacrylamide gel and transferred to a poly(vinylidene difluoride) membrane (Amersham). Filters were blocked by incubation with PBST (PBS containing 0.1% Tween-20), added with 5% low fat dry milk for 1 hour and then

incubated with the primary antibodies in PBST overnight at 4°C. Antibodies used were: monoclonal anti-c-Myc antibody (1:1000, Sigma) or anti-flag M2 monoclonal antibody (1:1000, Sigma). Filters were extensively washed in PBST and then incubated with horseradish peroxidase conjugated secondary anti-mouse antibody (Amersham). After further extensive washes with PBST, antigen-antibody complexes were visualized with the enhanced chemiluminescence kit from Amersham. For the immunoprecipitation experiment, 50 µg of proteins were incubated with 50 µl of anti-flag M2 affinity gel (Sigma) in 500 µl of NET-gel buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin and 0.02% Na-azide) supplemented with protease inhibitors, for 2 hours at 4°C. The affinity gel was washed twice with NET gel buffer and once with 10 mM Tris-HCl pH 7.5 and 0.1% NP-40. The affinity gel was resuspended in 60 µl of Laemmli buffer and boiled for 5 minutes; 6 µl were subjected to western blot analysis as described above.

RESULTS

The C-terminal region in MEF2A and MEF2C contains signals that contribute differently to their nuclear localization

To study the nuclear localization of MEF2A and MEF2C we constructed a series of plasmid DNAs containing GFP fused in frame with the entire sequence or selected domains of the two proteins. Recombinant plasmids were transfected in C2.7 myoblasts and subcellular distribution of fluorescence was examined after 48 hours. As shown in Fig. 1A, GFP fluorescence is localized to both the nucleus and the cytoplasm (a), whereas GFP/MEF2A (b) and GFP/MEF2C (c) fusion proteins are strictly nuclear. Since it was previously published that MEF2A contains a bipartite nuclear localization signal (NLS) within the peptide encompassing amino acids (aa) 472-507 (Yu, 1996), we made deletions of the C-terminal region on both GFP/MEF2A and GFP/MEF2C. As expected, deletion of the 36 amino acids at the C-terminal end of MEF2A (pGFP/MEF2AΔ-471) causes its complete retention in the cytoplasm (Fig. 1Bb). On the contrary, deletion of the corresponding region (pGFP/MEF2CΔ-429; Fig. 1Bc) as well as of the upstream 18 amino acids (pGFP/MEF2CΔ-411; Fig. 1Bd), on MEF2C still allows a considerable proportion of GFP fluorescence to be localized in the nucleus. However, deletion at aa 399, causes GFP/MEF2C to localize exclusively in the cytoplasm (pGFP/MEF2CΔ-399; Fig. 1Be). Therefore the peptide encompassing aa 399-411 appears to contain a signal that is important for the complete nuclear localization or retention of MEF2C.

To determine the contribution to nuclear localization of selected domains of MEF2C C-terminal region, we examined their ability to direct GFP to the nucleus. As shown in Fig. 2A, the peptide encompassing aa 430-466 (pGFP/MEF2C430-466), containing the bipartite NLS of MEF2C, increases GFP nuclear localization (Fig. 2Ac) essentially at the same rate as the corresponding region of MEF2A (pGFP/MEF2A472-507; Fig. 2Ab). This is not surprising, since the two sequences show 60% overall homology and contain identical bipartite NLS's. MEF2C peptides encompassing aa 389-411 (pGFP/MEF2C389-411) or aa 401-429 (pGFP/MEF2C401-429), which were shown to be necessary for full nuclear localization of GFP/MEF2C, do not show any intrinsic capacity to enhance

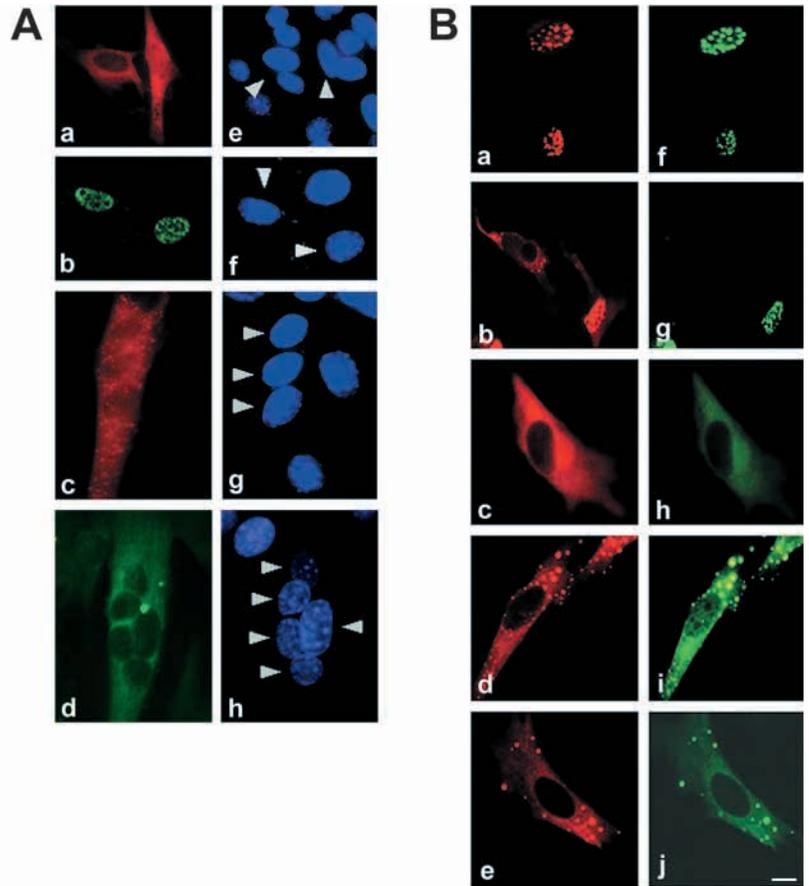
GFP nuclear localization (Fig. 2Ae and f, respectively). However, when fused to the bipartite NLS, the resulting peptide drives GFP to the nucleus, essentially at the same efficiency as the entire MEF2C protein (pGFP/MEF2C401-466; Fig. 2Ad). Fig. 2B provides a quantitative estimate of the nuclear/cytoplasmic distribution of the different GFP/MEF2-NLS constructs shown in Fig. 2A.

MEF2-NLS can act in trans and is required for efficient nuclear localization of HDAC4

HDACs deacetylate histones and transcription factors, causing transcriptional repression. In particular, specific class II HDACs, such as HDAC4 and -5, associate with MEF2 and inhibit myogenesis (Lu et al., 2000). Since it was shown that HDAC4 can shuttle between the nucleus and the cytoplasm (Wang et al., 2000) and that MEF2 and class II deacetylases can form specific complexes, we were prompted to investigate whether MEF2 could have any role in the subcellular localization of HDAC4 and/or HDAC5. Despite their vast sequence homology, HDAC4 and -5 show a very different pattern of subcellular distribution in transfected C2.7 myoblasts. In fact, in proliferating C2.7 myoblasts, HDAC4 is either cytoplasmic (64%) or pan-cellular (36%), with few cells showing nuclear staining only (Fig. 3Aa; Fig. 5); on the contrary, and in agreement with previously published observations (McKinsey et al., 2000a), HDAC5 is largely nuclear in most proliferating cells (Fig. 3Ab). Terminal differentiation to multinucleated myotubes does not alter the subcellular distribution of HDAC4 (Fig. 3Ac), while it causes HDAC5 to exit the nucleus (Fig. 3Ad) (McKinsey et al., 2000a). Co-transfection of GFP/MEF2A has a striking effect on HDAC4 subcellular localization. In fact, in cells co-expressing MEF2A or MEF2C, HDAC4 is nuclear and cytoplasmic in 80%, strictly nuclear in 20%, and cytoplasmic in less than 1% of cases (Fig. 3Ba,b; Fig. 5). In most co-transfected cells (over 95%) we confirm that HDAC4 co-localizes with MEF2 in nuclear 'speckle-like' structures (Fig. 3Ba,f,b,g) (Miska et al., 1999), thus supporting the idea that MEF2 and HDAC4 physically interact in the nuclei in vivo. Most interestingly, the increase of HDAC4 localization to the nucleus requires an intact MEF2 nuclear localization signal. In fact, co-transfection of HDAC4 with either MEF2A or MEF2C lacking the C-terminal NLS, causes the opposite effect, that is, a higher proportion of HDAC4 localizes in the cytoplasm. In fact, in 82% of the cells HDAC4 is strictly cytoplasmic, in 18% it is pan-cellular, in less than 1% it is nuclear (Fig. 3Bc,d,e; Fig. 5). Significantly, as shown in Fig. 3Bd,i,e,j, NLS-deleted MEF2s and HDAC4 often co-localize in cytoplasmic 'speckle-like' structures. This observation suggests that MEF2 factors and HDAC4 can associate in the cytoplasm but that the absence of the NLS prevents the complex to be imported into the nucleus. As would be expected, a specific dimerization domain independent of the NLS is required for complex formation, since co-transfection of HDAC4 and MEF2A (or MEF2C) GFP/NLS does not modify the subcellular distribution of HDAC4 (Fig. 4c,f; Fig. 5). Along the same line, substituting MEF2 with an unrelated transcription factor, capable of intrinsic nuclear localization, such as NFYB (Fig. 4a,d), or with GFP (Fig. 4b,e), equally does not affect HDAC4 subcellular distribution significantly.

Independent evidence that MEF2 NLS and the formation of

Fig. 3. Cellular localization of HDAC4 in C2.7 cells co-transfected with MEF2 proteins. (A) Cellular localization of Myc-tagged HDAC4 and HA-tagged HDAC5 in proliferating C2.7 myoblasts (a and b, respectively) as well as in differentiated C2.7 myotubes (c and d, respectively); corresponding Hoechst stained nuclei are indicated by arrows in e-h. (B) Cellular localization of Myc-tagged HDAC4 (red) in C2.7 myoblasts co-transfected with pGFP/MEF2A (a), pGFP/MEF2C (b), pGFP/MEF2A Δ -471 (c), pGFP/MEF2C Δ -429 (d) and pGFP/MEF2C Δ -399 (e). Green fluorescence of the same cells co-expressing the indicated GFP/MEF2 fusion proteins is shown in f-j. Bar, 4 μ m.



DISCUSSION

The identification and functional evaluation of protein domains that are responsible for targeting transcriptional factors and regulators to subcellular compartments is central to the understanding of the regulation of many genetic programs.

In this report we confirm that a C-terminal region encompassing aa 472-507 is necessary and sufficient for fully localizing GFP/MEF2A in the nucleus of C2C7 myogenic cells. Deletion of the C-terminal 36 amino acid sequence causes GFP/MEF2A to be completely retained in the cytoplasm. Indeed, as it had been pointed out previously (Yu, 1996), this region matches the bipartite NLS as originally proposed by Dingwall and Laskey (Dingwall and Laskey, 1991). Quite differently, the deletion of the corresponding region in MEF2C still allows a considerable proportion of the same protein to be retained in the nucleus. This result is surprising since the sequence homology of the C-terminal 36 amino acids in MEF2A and

MEF2C is nearly complete. Exclusion of GFP/MEF2C from the nucleus is achieved when a region encompassing aa. 399-411 is deleted, thus suggesting that this sequence might represent an additional element specifically contributing to the nuclear localization or, more likely, to the nuclear retention of MEF2C. In fact, the analysis of the isolated C-terminal region of MEF2A and MEF2C as to the ability of targeting GFP to the nucleus, has shown that the C-terminal 36 amino acids of both proteins are equally effective as NLS. However, when an extension of further 30 amino acids of MEF2C is added to the bipartite NLS, GFP localization is exclusively nuclear. In particular, we hypothesize that the amino acid sequence 399-411 might represent or contain a signal for nuclear retention, rather than localization: in fact, when it is directly tethered to GFP, in the absence of the bipartite NLS, it does not enhance nuclear targeting. Sequence analysis of the C-terminal portion of MEF2C has shown that peptide 399-411 almost completely overlaps to a region of poor sequence homology to MEF2A (the sequence underlined in Fig. 2D, corresponding to aa. 397-409). The reason for such peculiarity is not known, as well as the mechanisms underlying the functional activity of the peptide: at present we can only hypothesize that it might function as a binding domain for some not yet identified nuclear component, ultimately causing MEF2C to be retained in the nucleus.

In addition we show that both MEF2A and MEF2C provide an NLS in trans for the selective nuclear import of HDAC4. Class II histone deacetylases (such as HDAC4, HDAC5 and HDAC7) have the ability of specifically interacting with

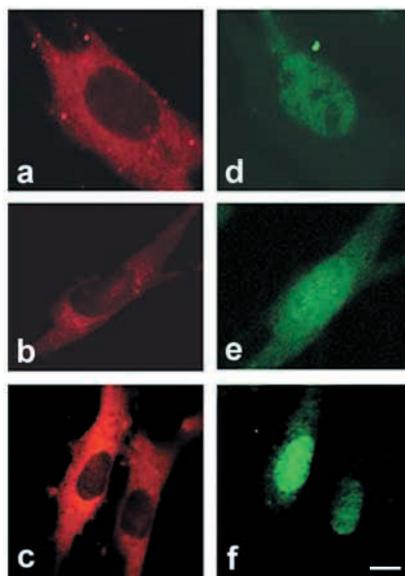


Fig. 4. Cellular localization of HDAC4 in cells co-transfected with NF-YB, GFP or MEF2NLS. Myc-tagged HDAC4 (red) localization in C2.7 myoblasts co-transfected with NF-YB (a,d), pEGFP-C3 (b,e) and pGFP/MEF2A472-507 (c,f). Bar, 4 μ m.

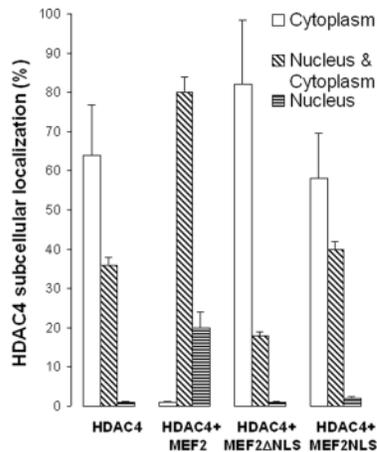


Fig. 5. Effect of MEF2 proteins on nuclear/cytoplasmic distribution of HDAC4. Myc-tagged HDAC4 (HDAC4) subcellular distribution was analyzed in C2.7 myoblasts transfected with pcHDAC4-Myc alone, or in combination with pGFP/MEF2A (MEF2), pGFP/MEF2AΔ-471 (MEF2ΔNLS), pGFP/MEF2A472-507 (MEF2NLS).

MEF2 proteins, through an N-terminal dimerization domain (Miska et al., 1999; Lemerrier et al., 2000; Dressel et al., 2001). However they show a very different subcellular distribution when transfected in C2.7 myogenic cells: in proliferating myoblasts HDAC4 is largely cytoplasmic or pan-cellular, while HDAC5 localizes almost exclusively in the nucleus. Upon differentiation to myotubes, HDAC5 moves to the cytoplasm, while HDAC4 does not significantly change its pattern of subcellular distribution. This observation is in agreement with recently published data obtained in Cos cells (McKinsey et al., 2000b) and suggests that the two deacetylases might rely on different mechanisms as far as their nuclear localization and, consequently, functional effect are concerned. As far as HDAC7 is concerned, a recent report shows that its subcellular distribution profile is essentially the same as HDAC5: nuclear in proliferating myoblasts and cytoplasmic in growth arrested cells (Dressel et al., 2001). Co-transfection of either MEF2A or MEF2C with HDAC4 causes the deacetylase to switch to a predominant nuclear localization, mostly in the form of 'nuclear speckles', where MEF2 proteins are also detected. We show that this effect heavily depends on the NLS contributed by the MEF2 proteins as well as on the formation of a MEF2/HDAC4 heterodimer: in fact co-transfection of MEF2 NLS deletion mutants causes HDAC4 to enhance its cytoplasmic localization, while co-transfection of the MEF2 C-terminal region, which contains the NLS but no dimerization domain, does not have any effect on HDAC4 subcellular distribution. Therefore, the overall picture emerging from our observations is that, in proliferating myoblasts, HDAC5 (and possibly also HDAC7) has the intrinsic property of localizing to the nucleus, while HDAC4 requires the MEF2 NLS for efficiently translocating to the nucleus. Recently reported examples of proteins requiring heterodimerization and a 'trans-acting' NLS for efficient localization to the nucleus are represented by the pituitary tumor-transforming gene product (PTTG) (Chien and Pei, 2000) and by the clock proteins mPER1 and mPER2 (Yagita

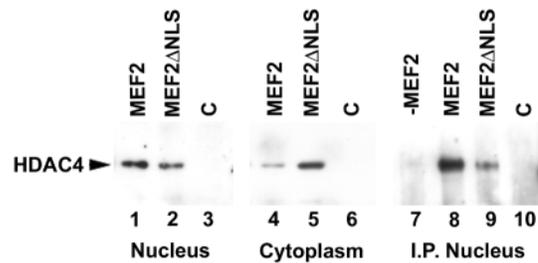


Fig. 6. Western blot analysis of HDAC4 localization. Nuclear protein extracts (lanes 1-3) and cytoplasmic protein extracts (lanes 4-6) from C2.7 myoblasts transfected with pcHDAC4-Myc and with pFLAG/MEF2C (lanes 1,4) or with pFLAG/MEF2ΔNLS (lanes 2,5) were subjected to western blot analysis with anti-Myc antibody. Nuclear protein extracts from cells transfected with pcHDAC4-Myc, alone (lane 7) or in combination with either pFLAG/MEF2C (lane 8) or pFLAG/MEF2ΔNLS (lane 9), were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were subjected to western blot analysis with anti-Myc antibody. Lanes 3, 6 and 10 (C), are control lanes showing protein extracts from mock transfected cells.

et al., 2000). Both HDAC4 and HDAC5 have been shown to efficiently inhibit MEF2 transcriptional activity (Miska et al., 1999; Lemerrier et al., 2000) and myogenesis (Lu et al., 2000). We also observed that, in C2.7 myoblasts, co-transfection of HDAC4 and MEF2A causes a tenfold reduction of the transcriptional activity at a MEF2 dependent promoter, compared with myoblasts transfected with MEF2A alone (data not shown). In this context an important role is played by 14-3-3 proteins and CaMK signalling. 14-3-3 proteins were shown to bind both HDAC4 and HDAC5 through interaction between specific domains (Wang et al., 2000; Grozinger and Schreiber, 2000) and negatively regulate their activity, apparently by sequestration in the cytoplasm or enhancement of nuclear export. However the two deacetylases are regulated differently by 14-3-3. In fact, ectopic co-expression of 14-3-3 and HDAC5 demonstrates that this deacetylase binds 14-3-3 and localizes to the cytoplasm only in the presence of CaMK; by contrast, HDAC4 appears to be constitutively bound to 14-3-3 (i.e. irrespectively of CaMK signalling) and mostly localized to the cytoplasm (McKinsey et al., 2000b). Our data are consistent with the hypothesis that HDAC4 is mostly a cytoplasmic resident protein, until its binding with 14-3-3 is substituted by MEF2, which is capable of heterodimerizing with HDAC4 in the cytoplasm and provides the signal for nuclear localization. On the contrary, HDAC5 would be a mostly nuclear resident protein, not requiring the interaction with any NLS-containing partner for localization. As it was consistently reported, HDAC4 (as well as HDAC5) is an effective repressor of MEF2 transcriptional activity; then the intriguing question arises about the functional significance of MEF2 carrying to the nucleus its own repressor. One might speculate that MEF2 factors, at least in cells expressing functionally significant levels of HDAC4, are negatively regulated by default. Repression relief might be achieved by either of the following two, non-mutually exclusive mechanisms: dissociation of HDAC4/MEF2 complexes, as was shown to be the case in HDAC5/MEF2 complexes after activation of CaMK signalling (McKinsey et al., 2000b); and substitution of HDAC4 by a transcriptional

co-activator such as p300, which was shown to compete with HDAC4 binding to MEF2D (Youn et al., 2000).

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