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14-3-3 regulates the nuclear import of class IIa histone deacetylases

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ABSTRACT

Class IIa histone deacetylases (HDACs) form complexes with a class of transcriptional repressors in the nucleus. While screening for compounds that could block the association of HDAC4 with the BTB domain-containing transcriptional repressor Bach2, we discovered that phorbol 12-myristate 13-acetate (PMA) induced the cytoplasmic retention of HDAC4 mutants lacking a nuclear export signal (NES). Although PMA treatment and PKD overexpression has been proposed to facilitate the nuclear export of class IIa HDACs by creating 14-3-3 binding sites containing phosphoserines, our experiments using HDAC mutants demonstrated that PMA greatly reduces nuclear import. PMA treatment repressed the NLS activity in a manner dependent on 14-3-3 binding. These results suggest that nuclear HDAC4 is not tethered in the nucleus, but instead shuttles between the nucleus and the cytoplasm. Phosphorylation-induced 14-3-3 binding biases the balance of nucleo-cytoplasmic shuttling toward the cytoplasm by inhibiting nuclear import.

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Histone deacetylases (HDACs) are classified into three classes based on the homology of their catalytic domains, and the class II HDACs are further divided into two subclasses, classes IIa and IIb [1,2]. Class IIa HDACs, including HDAC4, 5, 7, and 9, have an N-terminal regulatory domain and a conserved C-terminal catalytic domain [2–5]. These enzymes possess a nuclear localization signal (NLS) and a nuclear export signal (NES) at the N- and C-termini, respectively, facilitating shuttling between the cytoplasm and nucleus [6,7]; HDAC4 has two nuclear localization signals (NLS); NLS1 (residue 244–279) is the major NLS and is rich in basic

amino acids, while NLS2 (residue 1–117) is a minor NLS located at the amino terminus [7]. HDAC4 binds to myocyte enhancer factor 2 (MEF2) and represses its transcriptional activity [8]. This repression is regulated by the nucleo-cytoplasmic transport of HDAC4 [9]. Cytoplasmic localization of class IIa HDACs is promoted by phosphorylation and subsequent 14-3-3 binding [10]. Binding of 14-3-3 to HDAC4 is mediated by phosphorylation of residues Ser246, Ser467, and Ser632, which may facilitate CRM1-dependent nuclear export [11].

The silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), a corepressor protein, interacts directly with class IIa HDACs. We have previously reported that HDAC4 facilitates focus formation of Bach2, a transcriptional repressor, in a SMRT-dependent manner [12]. Bach2 forms foci around promyelocytic leukemia protein (PML) bodies under the oxidative stress conditions [13]. As the interaction of class IIa HDACs with various transcriptional repressors is associated with a variety of diseases, we screened for small molecules that inhibited the *in vivo* association of HDAC4 with Bach2 using a nuclear-localized HDAC4 mutant lacking an NES.

In this paper, we report that PMA inhibited HDAC4 nuclear import during the screening. Analysis of a series of HDAC4 mutants by nuclear import assay using leptomycin B (LMB) [14] suggested

Abbreviations: HDAC, histone deacetylase; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; MEF2, myocyte specific enhancer factor 2; NES, nuclear export signal; NLS, nuclear localization signal; CLS, cytoplasmic localization signal; PML body, promyelocytic leukemia protein body; PMA, phorbol 12-myristate 13-acetate; CHX, cycloheximide; LMB, leptomycin B; FBS, fetal bovine serum.

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that phosphorylation and subsequent 14-3-3 binding reduce nuclear import rather than enhancing nuclear export.

Materials and methods

Cells, antibodies, reagents, and plasmid construction. MCF7 and COS7 cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich). Lipofectamine 2000 (Invitrogen) was used for transfection. Stably transfected MCF7 cells were maintained in DMEM containing 10% TetSystem-approved FBS (Clontech), 450 µg/ml G-418, and 50 µg/ml Zeocin. To induce expression of Bach2 Δ CLS-HcRed and EGFP-HDAC4 Δ NES for screening, 2 µg/ml doxycycline (Sigma-Aldrich) was added to the cultures. Antibodies against FLAG (M2) and Myc (9E10) were purchased from Sigma and Santa Cruz Biotechnology, respectively. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich. Leptomycin B (LMB) was prepared as described [15]. Detail of plasmid construction in this study is described as a [Supplementary data](#).

Immunofluorescence and microscopy. Immunofluorescent staining utilized secondary antibodies conjugated to Alexa Fluor[®] 488 and Alexa Fluor[®] 594 (Molecular Probes). Cells were fixed in 3.7% formaldehyde/PBS for 15 min at room temperature, then permeabilized with 1% Triton X-100/PBS for 10 min at room temperature. Nuclear DNA was detected using VECTASHIELD[®] Mounting Medium containing DAPI (Vector Laboratories). Cells were observed by confocal fluorescence microscopy. COS7 cells expressing EGFP-HDAC4, cultured on glass-based dishes, were incubated at 37 °C in 5% CO₂ on an Olympus IX81 microscope with a UIC-QE cooled charge-coupled device camera (Molecular Devices). Time-lapse images were collected by using MetaFluor software (Universal Imaging).

Results

PMA as an inducer of cytoplasmic localization of HDAC4

Due to CRM1-dependent nuclear export, both HDAC4 and Bach2 are localized to the cytoplasm under normal, unstressed conditions [11,16]. HDAC4 and Bach2, however, form nuclear foci when localized to the nucleus in a manner dependent on the activity of a corepressor, SMRT. To identify small molecules that could inhibit the interaction, we constructed a screening system using HDAC4 and Bach2 mutants that exhibited constitutive nuclear localization due to the absence of an NES. To generate these mutants, we deleted the C-terminal region of an EGFP-labeled HDAC4 form lacking the 117 N-terminal amino acids, which is originally reported by Fischle et al. [17]. Constructs encoding the NES-deleted EGFP-HDAC4 (118–1054, EGFP-HDAC4 Δ NES) and the cytoplasmic localization signal (CLS)-deleted Bach2 bearing a C-terminal HcRed tag (1–741, Bach2 Δ CLS-HcRed) were stably introduced and expressed in MCF7 cells using the Tet-On gene expression system. We observed the colocalization of EGFP-HDAC4 Δ NES and Bach2 Δ CLS-HcRed in nuclear foci (Fig. 1A and B). We then used this cell line to screen for chemical compounds that inhibited the formation of these HDAC4/Bach2 foci. PMA induced the cytoplasmic localization of EGFP-HDAC4 Δ NES (Fig. 1B), but did not affect Bach2 localization.

As the form of HDAC4 expressed in these cells lacked its NES, it is unlikely that the intracellular localization was altered by inducing accelerated nuclear export. It was more likely that PMA induced cytoplasmic retention of newly synthesized HDAC4 and did not affect the localization of pre-existing nuclear HDAC4. To test this possibility, we analyzed the localization of HDAC4 in cells treated with PMA and a protein synthesis inhibitor, cycloheximide (CHX). Simultaneous treatment with CHX reduced the amount of protein in the cytoplasm, suggesting that only newly synthesized

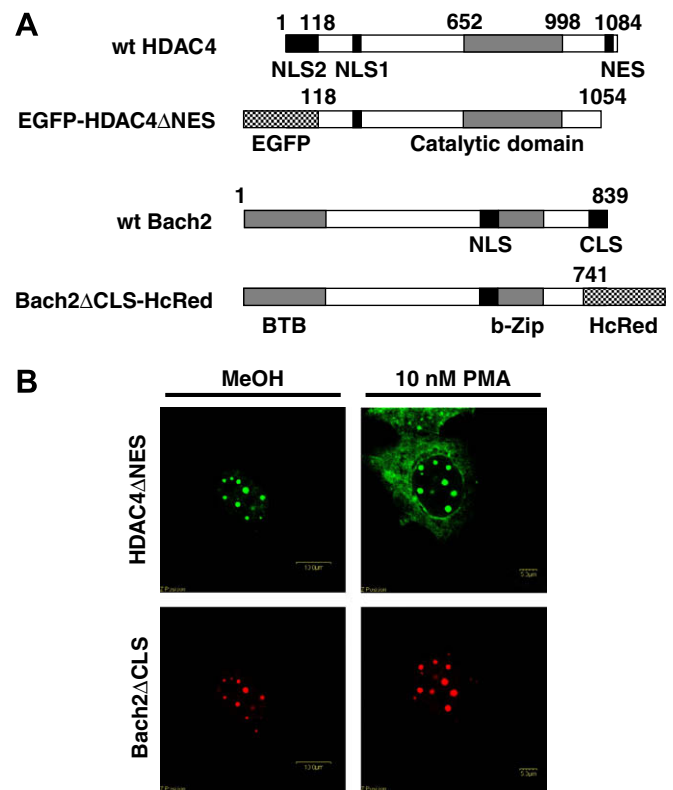


Fig. 1. Cytoplasmic localization of EGFP-HDAC4 Δ NES after PMA treatment. (A) Schematic representation of wild-type (wt) HDAC4 and EGFP-HDAC4 Δ NES as well as wt Bach2 and Bach2 Δ CLS-HcRed. (B) Fluorescent images of MCF7 cells expressing EGFP-HDAC4 Δ NES (top) or Bach2 Δ CLS-HcRed (bottom). Cells were treated with vehicle, MeOH (left), or PMA (right). (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

proteins accumulated in the cytoplasm, while pre-existing proteins were retained in the nucleus ([Supplementary Fig. 1](#)).

Regulation of HDAC4 subcellular localization by PKC δ and PKC ϵ

As PMA mimics diacylglycerol to activate cPKC and nPKC, members of these subfamilies may be involved in the cytoplasmic retention of HDAC4 by PMA. Indeed, PKC δ or PKC ϵ , members of nPKC, has been reported to upregulate a nuclear export of class IIa HDAC [18,19]. To test whether these enzymes are involved in the cytoplasmic retention of EGFP-HDAC4 Δ NES, we coexpressed in MCF7 cells wild-type or constitutively-active forms of PKC α , PKC δ , or PKC ϵ with EGFP-HDAC4 Δ NES. Coexpression of constitutively-active PKC δ or PKC ϵ but not PKC α induced the cytoplasmic localization of EGFP-HDAC4 Δ NES ([Supplementary Fig. 2](#)). *In vitro* kinase assays could not demonstrate the direct phosphorylation of HDAC4 by either PKC δ or PKC ϵ (data not shown). These results are consistent with previous observations that nPKCs are indirectly involved in the PMA-induced nuclear export of HDAC4 [18].

Inhibition of HDAC4 nuclear import by PMA treatment

Our data revealing the PMA-induced cytoplasmic retention of an NES-deficient HDAC4 were not consistent with previous studies demonstrating that PMA accelerated nuclear export by activating the NES of class IIa HDACs [18]. We therefore examined if PMA affects the rate of nuclear import, not export, using time-lapse imaging (Fig. 2). Following LMB treatment, NES-bearing proteins

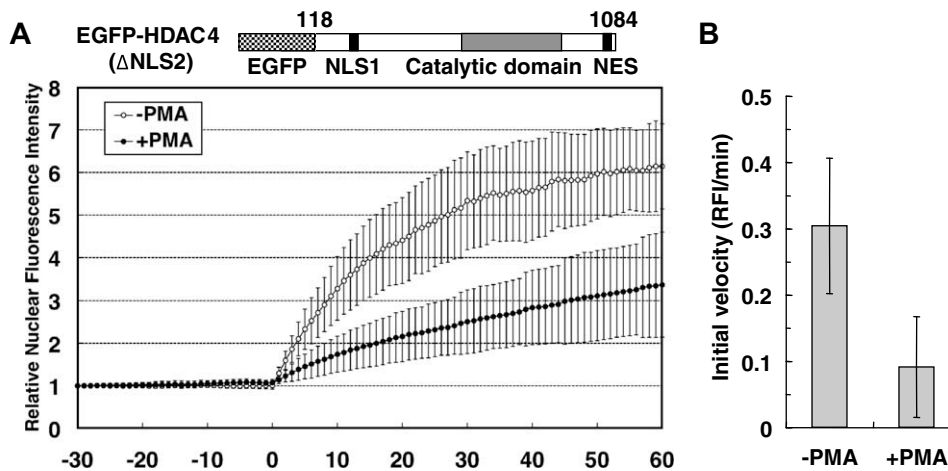


Fig. 2. The inhibition of nuclear import by PMA. (A) Quantitative analysis of nuclear fluorescence intensity. From the images (Supplementary Fig. 3), we calculated the fluorescence intensities at an arbitrary position within the nucleus using MetaFluor software (Universal Imaging). Intensities were normalized to the intensity at -30 min. Results indicated were the averages of seven cells from four independent experiments ($-$ PMA) and of 14 cells from five experiments ($+$ PMA). (B) Initial velocity of nuclear import. Average differences in the relative fluorescence intensities between 1 and 2 min were calculated as the initial velocity of nuclear import. RFI, relative fluorescence intensity. Error bars indicate standard deviations.

quickly accumulate in the nucleus [20,21], suggesting that LMB diffuses rapidly throughout the cell. Therefore, the rate at which a shuttling protein's localization changes after LMB challenge correlates with its rate of nuclear import [22]. We measured the fluorescence intensity within the nuclei of living cells expressing EGFP-HDAC4 at one minute intervals in culture. In this experiment, we used an EGFP fusion of HDAC4 (EGFP-HDAC4), which retained its C-terminal NES; this protein remains capable of shuttling between the nucleus and cytoplasm utilizing its NLS (NLS1) and NES, but is primarily localized to the cytoplasm due to the dominance of NES activity (Supplementary Fig. 3). Upon LMB treatment, nuclear fluorescence increased greater than two-fold within 5 min in the absence of PMA, reaching a plateau within 40 min (Fig. 2A). In the presence of PMA, however, the increase in fluorescence intensity was slow; the nuclear levels of EGFP-HDAC4 did not plateau within 60 min. The initial velocity of EGFP-HDAC4 nuclear import, calculated based on the results shown in Fig. 2A, was approximately three-fold slower than that seen in the absence of PMA (Fig. 2B). These results clearly indicate that the rate of HDAC4 nuclear import is reduced in PMA-treated cells.

HDAC4 possesses two NLSs, one of which is a classical NLS consisting of basic amino acids (NLS1) and the other has an unknown consensus sequence (NLS2) (Fig. 1A) [7]. The result that PMA reduced the import rate of HDAC4 was obtained using an EGFP-labeled HDAC4 lacking the N-terminal NLS2. Using immunofluorescent staining, we tested if the nuclear import of full-length HDAC4, which contains the NLS2 domain, can be inhibited by PMA treatment. Wild-type HDAC4, which was localized to both the nucleus and cytoplasm before treatment, relocalized to the nucleus within 30 min of LMB challenge (Supplementary Fig. 4, wt). The rapid nuclear accumulation induced by LMB was inhibited by PMA pretreatment, demonstrating that the import of full-length HDAC4 could be inhibited by PMA. We next tested if NLS2 activity was also affected by PMA by generating a mutant lacking NLS1 activity by introducing multiple point mutations into the protein (Fig. 3) [7]. Δ NLS1 localized almost exclusively to the cytoplasm in untreated cells. LMB treatment, however, induced the nuclear import of Δ NLS1; within 30 min of treatment, the Δ NLS1 protein exhibited diffuse localization throughout both the nucleus and cytoplasm. This nuclear import event was mediated by NLS2. Again, PMA pretreatment significantly inhibited

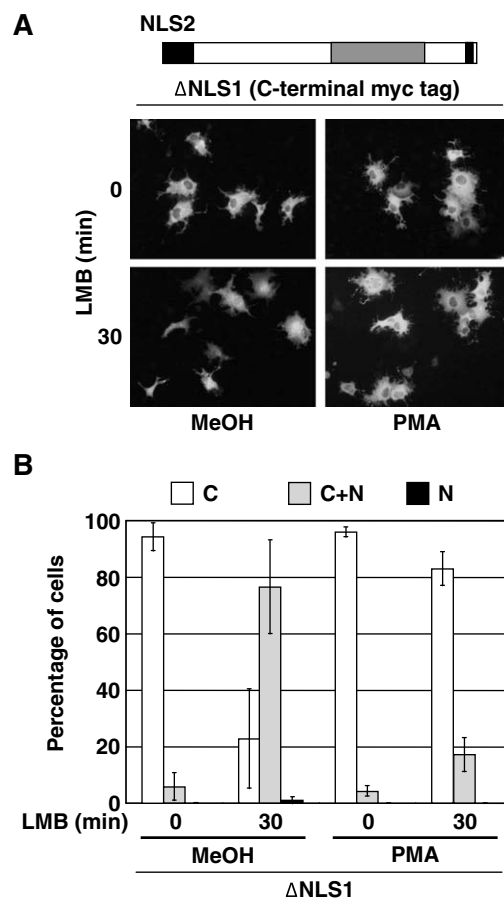


Fig. 3. Effect of NLS1 mutation on HDAC4 nuclear import. (A) Subcellular localization of Δ NLS1. COS7 cells expressing Δ NLS1 were pretreated with PMA or MeOH for 30 min. The nuclear import activity of the proteins was analyzed by staining 30 min after LMB addition. Indirect immunofluorescent microscopy was used to visualize protein localization. (B) Quantitative analysis of the subcellular localization of Δ NLS1. The nuclear import activity was determined by counting the cells with different subcellular localization patterns (C, cytoplasm; C+N, both cytoplasm and nucleus; and N, nucleus). Greater than 100 cells were counted in three independent experiments. The means of three independent experiments were indicated. Error bars indicate standard deviations.

nuclear import, indicating that PMA treatment abrogates the activity of both NLS1 and NLS2.

14-3-3 binding is required for the inhibition of HDAC4 nuclear import

PKD or nPKC, following activation by PMA, phosphorylates serine residues in class IIa HDACs to create 14-3-3 binding sites, which are necessary to maintain the cytoplasmic localization of class IIa HDACs [18]. To assess the role of 14-3-3 binding in the inhibition of HDAC4 nuclear import, we conducted a nuclear import assay using a 3SA mutant, in which the three serine residues important for 14-3-3 binding are replaced with alanine residues. In contrast to the diffused localization of HDAC4, the 3SA mutant exhibited intense localization to the nucleus [10,18]; PMA pretreatment could not induce relocalization to the cytoplasm (data not shown). Given the constitutive nuclear localization of the 3SA mutant, LMB could not be used as a tool for the import assay. To make it possible to utilize LMB to induce the nuclear import of 3SA, we mutated NLS1 of the 3SA mutant (Δ NLS1/3SA, Fig. 4A). The subcellular localization of Δ NLS1/3SA, as well as Δ NLS1, was cytoplasmic (Fig. 4B). This observation indicates that the constitutive nuclear localization of the 3SA mutant requires nuclear import mediated by NLS1, demonstrating that 3SA continues to shuttle between the nucleus and the cytoplasm. As the protein lacks NLS1 activity, Δ NLS1/3SA import into the nucleus depended entirely on NLS2 activity. LMB treatment induced the nuclear localization of a proportion of cellular Δ NLS1/3SA (Fig. 4B and C). This nuclear translocation of Δ NLS1/3SA could no longer be inhibited by PMA

treatment; the protein effectively translocated into the nucleus upon LMB treatment in the presence or absence of PMA. The PMA-resistant nuclear import of Δ NLS1/3SA contrasted the inhibition of nuclear import of Δ NLS1 mediated by PMA (Fig. 3). The marked increase in 14-3-3 binding following PMA treatment was observed for Δ NLS1; 14-3-3 binding, however, was completely lost for Δ NLS1/3SA, regardless of PMA treatment (Supplementary Fig. 5). These results indicate that PMA enhancement of 14-3-3 binding is required for the inhibition of the HDAC4 nuclear import. The nuclear transport of Δ NLS1/3SA following LMB treatment was more effective than that seen for Δ NLS1, suggesting that the 3SA mutation facilitates NLS2-mediated nuclear import, likely due to the complete loss of 14-3-3 binding. Nuclear import of HDAC5 and HDAC7 was also reduced by PMA (Supplementary Fig. 6), suggesting that cytoplasmic retention by PMA is a common mechanism in class IIa HDACs.

NES in 14-3-3 is not involved in HDAC4 cytoplasmic localization

14-3-3 possesses an NES, which may facilitate the nuclear export of a subset of proteins [23]. To see whether the binding of 14-3-3 or other associated proteins following PMA treatment accelerates the nuclear export of HDAC4, we tested the effect of LMB on the cytoplasmic retention of EGFP-HDAC4 Δ NES in the presence of PMA. If the NES activity present in HDAC4-associated proteins such as 14-3-3 is involved in the cytoplasmic retention of HDAC4, the HDAC4 protein should be relocalized to the nucleus

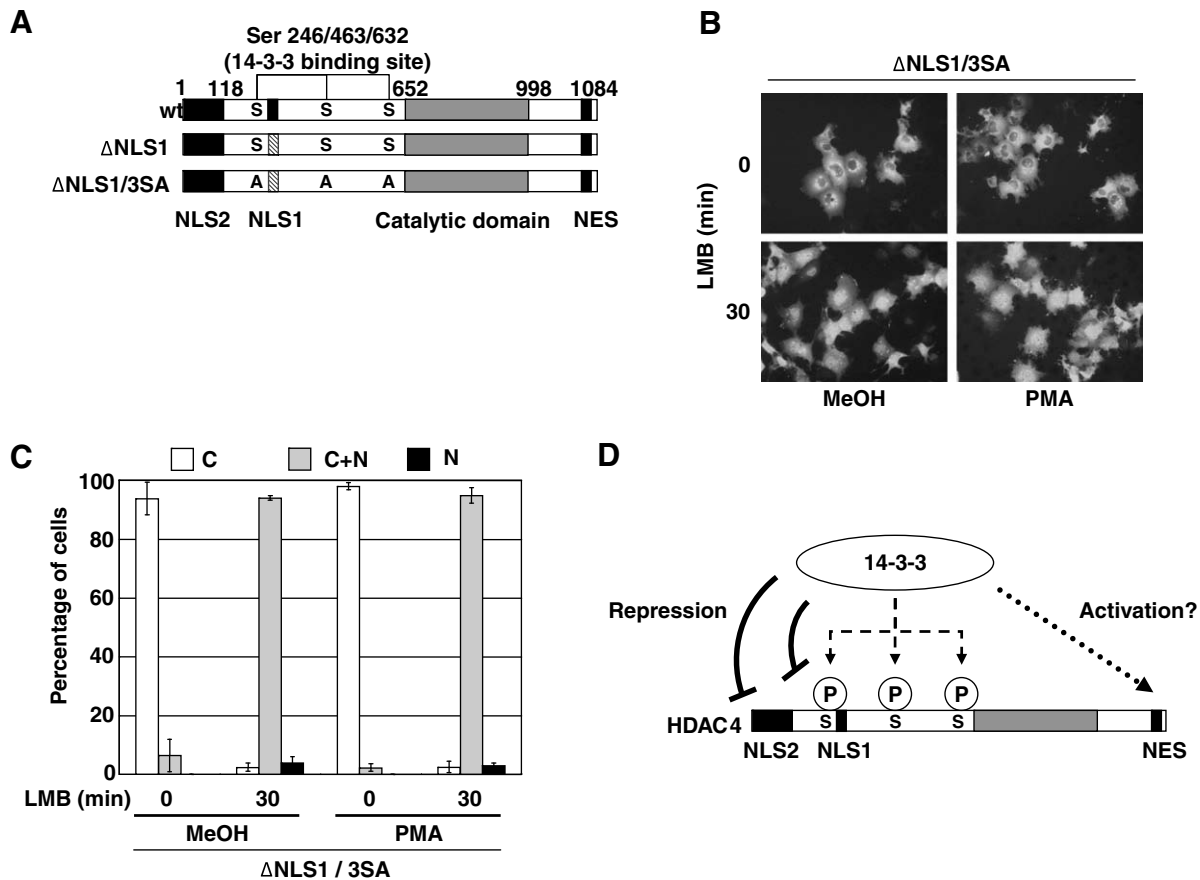


Fig. 4. A role for 14-3-3 binding in the PMA-induced cytoplasmic localization of HDAC4. (A) Schematic representation of wt, Δ NLS1, and Δ NLS1/3SA proteins. NLS mutations are indicated as diagonal boxes. (B) Immunofluorescence microscopy was used to examine the subcellular localization of the wild-type Δ NLS1/3SA. COS7 cells expressing Δ NLS1/3SA were pretreated with or without PMA for 30 min. The nuclear import activity of Δ NLS1/3SA was determined following LMB treatment. (C) Quantitative analysis of subcellular localization of Δ NLS1/3SA utilizing the same method as described in Fig. 3. (D) A model outlining the role of 14-3-3 in the subcellular localization of class IIa HDACs.

after LMB treatment. Cytoplasmic HDAC4, however, was resistant to LMB in the presence of PMA and was retained in the cytoplasm (Supplementary Fig. 7).

Discussion

14-3-3 binding has been proposed to induce cytoplasmic translocation of class IIa HDACs by masking the NLS and/or exposing the NES [24]. Although it remains controversial, it has been a more prevailing idea that the nuclear export of class IIa HDACs is facilitated by 14-3-3 binding by presentation of the NES to the export factor CRM1 via a conformation change in the C-terminus [25], as NES mutation or LMB treatment prevented the phosphorylation-induced cytoplasmic localization of class IIa HDACs [6,18,26]. In this study, however, using EGFP-HDAC4ΔNES we provided evidence that 14-3-3 binding following phosphorylation at 14-3-3 binding sites prevents the binding of nuclear import factors to both NLS1 and NLS2 (Fig. 4D) [10]. It seems likely that this discrepancy was generated from weak import activity of EGFP-HDAC4ΔNES lacking NLS2 in comparison to wild-type HDAC4, which allowed us to detect the PMA-induced cytoplasmic accumulation of the protein even in the absence of the NES. When we used an NES-deleted HDAC4 mutant containing both NLS1 and NLS2, it was difficult to observe the PMA-mediated inhibition of nuclear import, because the majority of proteins were localized to the nucleus due to the potent import activity (data not shown).

The phosphorylation of class IIa HDACs has been suggested to occur in the nucleus, leading to the dissociation of HDACs from MEF2 [9,18,27]. However, recent studies identified several cytoplasmic HDAC kinases, EMK, C-TAK1 and CaMKII, which regulate nucleo-cytoplasmic shuttling of class IIa HDACs [28,29]. Thus, HDAC4 is actively shuttling between the nucleus and cytoplasm despite a primarily nuclear localization, and once exported, phosphorylated HDAC4 upon PMA treatment may not return rapidly to the nucleus, due to decreased import activity resulting from 14-3-3 binding. This leads to the eventual accumulation of HDAC4 in the cytoplasm.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.10.079.

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