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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.

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-myristate13-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 Bach2ΔCLS-HcRed and EGFP-HDAC4ΔNES 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% CO_2 on an Olympus IX81 microscope with a UIC-QE cooled charge-coupled device camera (Moleqular 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-HDAC4ΔNES) and the cytoplasmic localization signal (CLS)-deleted Bach2 bearing a C-terminal HcRed tag (1-741, Bach2ΔCLS-HcRed) were stably introduced and expressed in MCF7 cells using the Tet-On gene expression system. We observed the colocalization of EGFP-HDAC4ΔNES and Bach2ΔCLS-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-HDAC4ΔNES (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

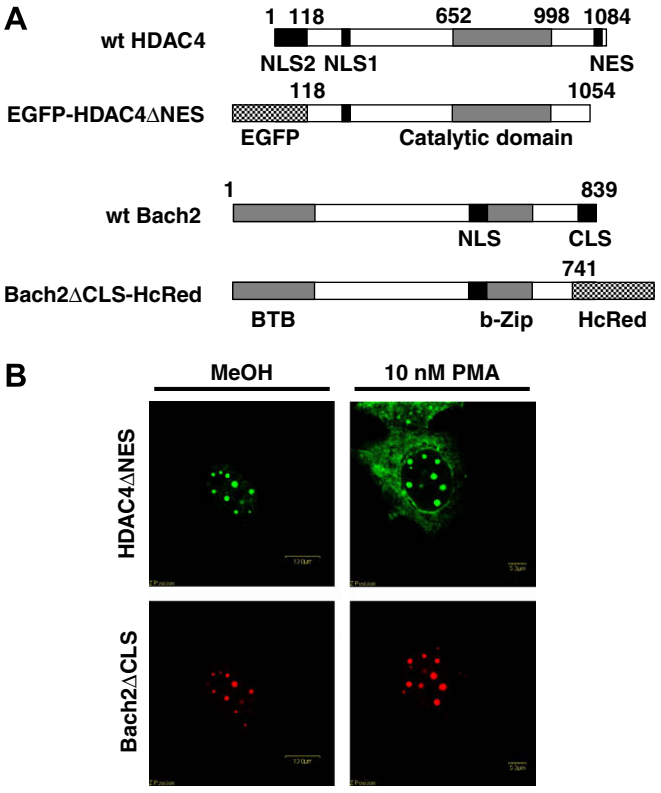

Fig. 1. Cytoplasmic localization of EGFP-HDAC4ΔNES after PMA treatment. (A) Schematic representation of wild-type (wt) HDAC4 and EGFP-HDAC4ΔNES as well as wt Bach2 and Bach2ΔCLS-HcRed. (B) Fluorescent images of MCF7 cells expressing EGFP-HDAC4ΔNES (top) or Bach2ΔCLS-HcRed (bottom). Cells were treated 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 PKCδ and PKCε

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, PKCδ or PKCε, 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-HDAC4ΔNES, we coexpressed in MCF7 cells wild-type or constitutively-active forms of PKCα, PKCδ, or PKCε with EGFP-HDAC4ΔNES. Coexpression of constitutively-active PKCδ or PKCε but not PKCα induced the cytoplasmic localization of EGFP-HDAC4ΔNES (Supplementary Fig. 2). *In vitro* kinase assays could not demonstrate the direct phosphorylation of HDAC4 by either PKCδ or PKCε (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