Nuclear localization signal domain of HDAC3 is necessary and sufficient for the expression regulation of MDR1

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Histone deacetylase-3 (HDAC3) is ubiquitously expressed and conserved in a wide range of species (1). HDAC3 has been shown to form large corepressor complexes containing N-CoR/SMRT (2). HDAC3 regulates the JNK pathway (3), MAPK activation (4), and apoptosis (5). HDAC3 represses CREB3-mediated transcription, and the migration of metastatic breast cancer cells (6).

A phase I trial revealed that albumin-bound paclitaxel shows encouraging activity against advanced metastatic melanomas (7). Resistance to taxol, a microtubule-targeting drug, in hepatoma cells is related to JNK activation (8). The inhibition of MAPK enhances taxol-induced apoptosis (9).

Taxol-resistance is correlated with the expression level of MDR1 (10). Elevated levels of histone acetylation in the MDR1 promoter in drug-resistant cancer cells have been described (11).

In this study, the effect of HDAC3 on the expression of MDR1 and acetylated histones was examined. The necessity of HDAC3 activity for regulating the expression of MDR1 was also examined. The domain of HDAC3 that was necessary for exerting regulation on the expression of MDR1, and for the binding to the promoter sequences of MDR1, was identified.

RESULTS AND DISCUSSION

HDAC3 regulates the expression of MDR1 by affecting histone acetylation

Apicidin, an inhibitor of HDAC, induces anti-cancer drug-resistance via the up-regulation of MDR protein (12). The depletion of HDAC3 increases the acetylation of histone H3 at Lys9/14, and histone H4 at Lys12 (13). The expression level of acetylated H3K9/14 is elevated 100 fold in the promoter of the MDR1 gene, in drug-resistant breast cancer cell lines, compared to drug-sensitive cell lines (14). In this study, we found lower expression of HDAC3, among HDAC(s), in various cancer cell lines naturally resistant (HpeG2, Hpe3B and WM266-4), or made resistant to celastrol (SNU387R and Malme3MR), taxol (SNU387R-Taxol and Malme3M\textsuperscript{Taxol}), vinblastine (SNU387VBL), than anti-cancer drug-sensitive cancer cell lines such as SNU387 and Malme3M cells (Fig. 1A). In these anti-cancer drug-resistant cancer cell lines, the expression level of HDAC3 showed an inverse relationship with the expression level of MDR1 (Fig. 1A). This led to the hypothesis that HDAC3 would exert a negative regulation on the expression of MDR1. First, the effect of HDAC3 on histone acetylation/deacetylation to regulate the expression of MDR1 was examined. The MDR1 gene promoter contains the binding sites for various transcription factors such as p53, ETS, and AP1 (Fig. 1B). However, the binding of HDAC(s) and modified histones around the MDR1 promoter in hepatoma or melanoma cells remains unknown. The down-regulation of HDAC3 induced the expression of MDR1 (Fig. 1C). Malme3M\textsuperscript{Flag} HDAC3-Flag and SNU387/HDAC3-Flag cells stably expressing HDAC3 showed lower expression of MDR1 than their counterparts (Fig. 1C). The down-regulation of MDR1 did not affect the expression of HDAC3 (Fig. 1D). The down-regulation of HDAC3 induces the expression of Ac-H3K9/14 and Ac-H4K16 (Fig. 1D).

INTRODUCTION

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Fig. 1. HDAC3 regulates the expression of MDR1, Ac-H3\(_{K9/14}\), and Ac-H4\(_{K16}\), and the binding of modified histones to MDR1 promoter sequences. (A) Cell lysates were subjected to Western blot analysis. (B) Human MDR1 gene proximal promoter sequences. Numbers indicate their positions relative to the transcription initiation site (+1). Two sets of primers, designated as P1 and P2, were used for amplification of the proximal MDR1 promoter, respectively. (C) 48 h after transfection with the indicated siRNA, Western blot was performed. Lysates from SNU387-HDAC3-Flag or Malme3M-HDAC3-Flag cells were also subjected to Western blot. * denotes exogenous HDAC3. (D) 48 h after transfection, Western blot was performed. (E) 48 h after transfection control vector or Flag-tagged HDAC3 wild-type construct, Western blot was performed. (F) 48 h after transfection, ChIP assays were performed.

However, the down-regulation of HDAC3 did not affect expression of histone H3, or histone H4 (Fig. 1D). HDAC3 exerted a negative regulation on the expression of Ac-H3\(^{K9/14}\) and Ac-H4\(^{K16}\) (Fig. 1E). However, over-expression of HDAC3 did not affect the expression of histone H3 or histone H4 (Fig. 1E). The down-regulation of HDAC3 induced the binding of Ac-H3\(^{K9/14}\) to a site of the MDR1 promoter sequences and the binding of Ac-H3\(^{K9/14}\) to site 2 of the MDR1 promoter sequences, in SNU387 cells (Fig. 1F). The down-regulation of HDAC3 also induced the binding of Ac-H3\(^{K9/14}\) and Ac-H3\(^{K16}\) in Malme3M cells (Fig. 1F). Acetyl-H3 and Acetyl-H4 show binding to the MDR1 gene proximal promoter sequences (-116 to -11), in MCF7 cells made resistant to Adriamycin (15).

Taken together, these results suggest that HDAC3 regulates the expression of MDR1, by modulating the expression of the modified histones, and the binding of these modified histones to the MDR1 promoter sequences. It would be necessary to identify more modified histones that are regulated by HDAC3.

HDAC3 activity is necessary for the binding of modified histones to the MDR1 promoter sequences
Wild-type HDAC3, but not HDAC3\(^{S424A}\), decreased the expression of MDR1, Ac-H3\(^{K9/14}\), and Ac-H4\(^{K16}\) (Fig. 2A). This suggests that HDAC3 activity is necessary for the expression regulation of Ac-H3\(^{K9/14}\) and Ac-H4\(^{K16}\). HDAC3 did not affect the expression of histone H3 or histone H4 (Fig. 2A). HDAC3\(^{S424A}\) lacks histone deacetylase activity (16). The protein kinase CK2 phosphoacceptor site in HDAC3 is at Ser424 (16). HDAC3 activity is regulated by interaction with protein serine/threonine phosphatase 4 (PP4c) (16). The expression level of phospho-
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HDAC3<sup>S424</sup> was correlated with the expression level of HDAC3 (Fig. 2A). Wild-type HDAC3, but not HDAC3<sup>S424A</sup>, exerted negative effects on the binding of Ac-H3<sup>9/14</sup> to site 1 of the MDR1 promoter sequences in SNU387<sup>R</sup> cells, and the binding of Ac-H4<sup>16</sup> to site 2 of the MDR1 promoter sequences in SNU387<sup>R</sup> cells (Fig. 2B). Wild-type HDAC3, but not HDAC3<sup>S424A</sup>, exerted negative effects on the binding of Ac-H3<sup>9/14</sup> to site 2 of the MDR1 promoter sequences in Malme3MR cells, and the binding of Ac-H4<sup>16</sup> to site 1 of the MDR1 promoter sequences in Malme3MR cells (Fig. 2B). Wild-type HDAC3, but not HDAC3<sup>S424A</sup>, showed binding to sites 1 and 2 of the MDR1 promoter sequences in SNU387<sup>R</sup> cells and binding to site 1 of the MDR1 promoter sequences in Malme3MR cells (Fig. 2B), suggesting an essential role of HDAC3 activity in the expression regulation of MDR1. These results suggest that the expression regulation of MDR1 by HDAC3 involves the regulated binding of modified histones to the MDR1 promoter sequences. For better understanding of the mechanism of the expression regulation of MDR1, it would be necessary to identify miRNAs that are regulated by HDAC3, for better understanding of the mechanism of the expression regulation of MDR1.

The nuclear localization signal domain of HDAC3 is necessary and sufficient for conferring sensitivity to microtubule-targeting drugs
HDAC3 consists of functional domains, such as the oligomerization, nuclear export signal sequence and nuclear localization signal sequence (18). There have been no reports concerning the domain of HDAC3 that confer sensitivity to microtubule-targeting drugs. Therefore, a series of HDAC3 deletion constructs were made, to determine the functional domain of HDAC3 that confers sensitivity to microtubule-targeting drugs. The full-length HDAC3 (HDAC3-FL) decreased the expression of MDR1 in Malme3MR cells (Fig. 3A). HDAC3<sup>△C1</sup> (1-313 of HDAC3) or HDAC3<sup>△C2</sup> (1-180 of HDAC3) did not affect the expression of MDR1 (Fig. 3A). HDAC3-NES (180-313 of HDAC3), which possesses a nuclear export signal, did not affect the expression of MDR1 (Fig. 3A). HDAC3-NLS (nuclear localization signal sequence, 313-428 of HDAC3), which possesses Ser424, decreased the expression of MDR1 (Fig. 3A). HDAC3-NLS, but not other deletion constructs, decreased expression of Ac-H3<sup>9/14</sup> and Ac-H4<sup>16</sup> (Fig. 3A). The full-length HDAC3 or HDAC3 deletion constructs did not affect the expression of histone H3 or histone H4 (Fig. 3A). HDAC3-FL and HDAC3-NLS, but not other deletion constructs, conferred sensitivity to microtubule-targeting drugs (Fig. 3B). HDAC3-NLS
Fig. 3. The nuclear localization domain of HDAC3, which encompasses Ser424, is necessary and sufficient for conferring sensitivity to microtubule-targeting drugs. (A) HDAC3-Myc/His, serial deletion constructs. FL denotes full-length. NES denotes nuclear export signal domain. Numbers in parentheses represent amino acid residues of HDAC3. NLS denotes nuclear localization signal domain. 48 h after transfection, Western blot was performed. (B) Malme3MR cell line was transfected with the indicated construct. The next day, cells were treated with various concentrations of the indicated drug for 24 h, followed by MTT assays. Comparison was made between SNU387R or Malme3MR cells transfected with the control vector, and the same cells transfected with HDAC3-FL. "*P < 0.05; **P < 0.005. Comparison was also made between SNU387R or Malme3MR cells transfected with control vector, and the same cells transfected with HDAC3-NLS. "*P < 0.05; **P < 0.005. (C) Malme3MR cell line was transfected with the indicated construct. The next day, cells were treated with celastrol (1 μM) or taxol (1 μM) for 24 h, followed by Western blot. C-PARP denotes cleaved PARP. V denotes control vector. Caspase-3* denotes pro-caspase-3 and caspase-3** denotes active caspase-3. (D) The indicated cell line was transfected with the indicated HDAC3-Myc/His construct. 48 h after transfection, ChIP assays were performed, as described. SNU387R or Malme3MR cells transfected with full-length HDAC3 were also subjected to ChIP assays, employing isotype-matched anti-IgG antibody (2 μg/ml).

Fig. 4. The localization of HDAC3 deletion constructs. (A) The indicated cell line was transiently transfected with the indicated HDAC3-Myc/His construct. 48 h after transfection, immunofluorescence staining was performed, as described. SNU387R and Malme3MR cell lines transfected with full-length HDAC3 were also subjected to immunofluorescence staining employing only isotype-matched secondary antibody. (B) Cell lysates from each fraction were subjected to Western blot analysis.
conferred apoptotic effects against celestrol and taxol (Fig. 3C). HDAC3-NS, among HDAC3 deletion constructs, showed binding to the MDR1 promoter sequences (Fig. 3D). It is reasonable that nuclear localization of HDAC3, is necessary for the expression regulation of MDR1 by HDAC3. Immunofluorescence staining revealed the localization of the full-length HDAC3, in both the nucleus and cytosol (Fig. 4A). HDAC3, unlike HDAC1/2, is present in both the nucleus and cytosol of DT40 chicken B cells (19). The localization of HDAC3-ΔC1 and HDAC3-ΔC2 was mostly cytosolic (Fig. 4A). The localization of HDAC3-NES was also mostly cytosolic (Fig. 4A). HDAC3-NLS showed exclusively nuclear localization (Fig. 4A). As for the localization of these HDAC3 constructs, a cellular fractionation study showed the same results as immunofluorescence staining (Fig. 4B). Taken together, these results suggest that the NLS domain of HDAC3 may be necessary and sufficient for conferring sensitivity to microtubule-targeting drugs, and also for direct regulation of MDR1. It would be necessary to identify sequences within the NLS domain that are necessary for the expression regulation of MDR1 and modified histones.

MATERIALS AND METHODS

Cell lines and cell culture

The cancer cell lines used in this study were cultured in Dulbecco's modified essential medium (DMEM; Gibco, Gaithersburg, MD, USA), supplemented with heat-inactivated 10% fetal bovine serum (FBS, Gibco) and antibiotics, at 37°C, in a humidified incubator with a mixture of 95% air and 5% CO2.

SNU387R or Malme3MR cells stably expressing HDAC3 were generated, by co-transfection of HDAC3-Flag, along with pcDNA3.1. Stable transfectants were selected by G418 (400 μg/ml). Cancer cell lines made resistant to microtubule-targeting drugs (celastrol, taxol and vinblastine) were established by clonal expansion, with specific primers of MDR1 promoter-1 [5'-AATCCAAGGC ATCAATTTCACCCTGTCTC-3' (sense)] and [5'-AAGTGAAAT TGATGCCCTTGGACCTGTCTC-3' (antisense)]; and MDR1 promoter-2 [5'-AATTGCATACGCTAAGAGTTCCCTGTCTC-3' (sense)] and [5'-AAGAACCTTCTTGACCTATGCAACCCTGTCTC-3' (antisense) sequences were used.

Materials

Anti mouse and anti rabbit IgG-horse radish peroxidase conjugate antibodies were purchased from Pierce Company. Lipofectamin and Plus™ reagent were purchased from Invitrogen (Carlsbad, CA, USA). Bioneer (Daejeon, Korea) synthesized all primers and oligonucleotides used in this study.

Cell viability determination

The cells were assayed for their growth activity, using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma). Viable cell number counting was carried out by trypan blue exclusion assays.

HDAC3 constructs

HDAC3<sup>3224Δ,MyC/His<sub>6</sub></sup> expression plasmid (catalytically inactive HDAC3 mutant) was derived from pFlag-HDAC3, with the Quick-change site-directed mutagenesis kit (Stratagene). HDAC3 serial deletion mutant constructs were made, by cloning various PCR-amplified HDAC3 fragments into pcDNA3.1-Myc/His vector.

Immunofluorescence staining

SNU387<sup>R</sup> or Malme3M<sup>R</sup> cells were seeded onto glass cover slips in 24-well plates. Cells were fixed with 4% paraformaldehyde (v/v), for 10 min and then permeabilized with 0.4% Triton X-100 for 10 min. Nonspecific antibody binding sites were blocked by incubation with 1% BSA in TBST, for 30 min. Cells were then incubated with primary antibody specific to His (Santa Cruz, 1:100) for 24 h, followed by washing with TBST-T, three times. Anti-rabbit IgG-Alexa Fluor 546 (for detection of His) secondary antibody (Molecular Probes) was added to cells and incubated for 1 h. Cover slips were then washed, and mounted by applying Mount solution (Biomeda, Foster City, CA, USA). Fluorescence images were acquired using a confocal laser-scanning microscope and software (Fluoview ver. 2.0), with a 40X objective (Olympus FV300, Tokyo, Japan). DAPI staining was also performed, for nuclear staining.

Transfection

All transfections were performed according to the standard procedures (20). The construction of siRNA was carried out according to the instruction manual provided by the manufacturer (Ambion, Austin, TX).

ChIP assays

Assays were performed according to the standard procedures (21). PCR was done on the phenol-chloroform-extracted DNA with specific primers of MDR1 promoter-1 [5'-AATCCAAGGC ATCAATTTCACCCTGTCTC-3' (sense)] and [5'-AAGTGAAAT TGATGCCCTTGGACCTGTCTC-3' (antisense)]; and MDR1 promoter-2 [5'-AATTGCATACGCTAAGAGTTCCCTGTCTC-3' (sense)] and [5'-AAGAACCTTCTTGACCTATGCAACCCTGTCTC-3' (antisense) sequences were used.

Preparation of siRNA duplexes

The construction of siRNA was carried out according to the instruction manual provided by the manufacturer (Ambion, Austin, TX).

Statistical analysis

All data were expressed as a mean value ± standard deviation (S.D.), and differences between groups were analyzed using Student's t-test. Mean values were considered significantly different, when P < 0.05.
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