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Disruption of HDAC4/N-CoR complex by histone deacetylase inhibitors leads to inhibition of IL-2 gene expression

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ABSTRACT

Previous studies have shown that HDAC inhibitors selectively inhibit IL-2 gene expression, but the mechanism of this inhibition remains to be elucidated. It was recently reported that HDAC4, a component of the nuclear hormone receptor corepressor (N-CoR) complex, associates with the IL-2 promoter via the transcription factor myocyte enhancer factor 2 (MEF2). We therefore focused on the role of HDAC4/N-CoR complex in the transcriptional regulation of IL-2. Four approaches were used to characterize this role and to investigate the relation between the regulatory function of HDAC4/N-CoR complex and HDAC4-enzymatic activity: (i) HDAC4 silencing by RNA interference, (ii) overexpression of N-CoR repression domain 3 (RD3), (iii) overexpression of HDAC4 point mutants, and (iv) treatment with HDAC inhibitors. Here, we report that HDAC4 plays an essential role in IL-2 promoter activation, and that the formation of the HDAC4/N-CoR complex, which is closely related to HDAC4-enzymatic activity, might be involved in HDAC inhibitor-mediated inhibition of IL-2 gene expression. These observations indicate that the selective inhibition of HDAC4 or the interaction of HDAC4 with N-CoR is likely a potential target for the development of novel immunosuppressants.

1. Introduction

Histone acetylation has been identified as a key regulator of gene transcription in eukaryotes [1,2]. The addition and removal of acetyl groups on specific lysine residues in histone tails are catalyzed by histone acetyltransferase (HAT) and histone deacetylase (HDAC), respectively [1,2]. In recent years, a number of non-histone proteins have been identified as substrates for these enzymes, including p53, GATA-1, RelA, signal transducer and activator of transcription 3, hypoxia-inducible factor-1α, α-tubulin, heat shock protein 90, etc. [3–5]. HATs and HDACs have been found to function in cells as large multicomponent complexes. HATs were identified as transcriptional co-activators, and include GCN5, p300/CREB-binding protein, p300/CREB-binding protein-associated factor, TAF250, and steroid receptor co-activator-1 [6–8]. By contrast, HDACs are found in complexes, including a number of transcriptional repressors and co-repressors, such as Sin3, silencing mediator for retinoid and thyroid receptors (SMRT), and nuclear hormone receptor corepressor (N-CoR) [6–8].

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Abbreviations: HDAC, histone deacetylase; N-CoR, nuclear hormone receptor corepressor; MEF2, myocyte enhancer factor 2; RD3, repression domain 3; HAT, histone acetyltransferase; SMRT, silencing mediator for retinoid and thyroid receptors; RPD3, reduced potassium dependency 3; HDA1, histone deacetylase-A 1; SIR2, silent information regulator 2; DBD, DNA-binding domain; HRP, horseradish peroxidase; SANT, SWI3/ADA2/NCoR/TFIIIB; PMA, phorbol 12-myristate 13-acetate; TBS-T, tris-buffered saline containing 0.1% Tween-20; siRNA, short interfering RNA; TSA, trichostatin A

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Protein hyperacetylation induced by HDAC inhibitors causes chromatin remodeling, transcriptional activation and repression, cell-cycle arrest, cell differentiation, and cell death [9–14].

It was recently reported that an imbalance between HATs and HDACs causes a decreased level of acetylation and leads to the development of cancer [10]. Based on these results, HDAC inhibitors are emerging as a new class of cancer chemotherapeutic agent. In addition, HDAC inhibitors are reported to possess immunosuppressive activity, such as inhibition of lymphocyte proliferation and IL-2 production [15–19], suggesting that they may also have therapeutic potential in autoimmune diseases and organ transplantation.

Multiple forms of HDAC have been cloned in humans and are classified into two classes based on primary structure and size [1,2]. Class I HDACs, homologous to yeast reduced potassium dependency 3 (RPD3), include HDAC1, HDAC2, HDAC3, HDAC8, and HDAC11 [13,20]. Class II HDACs, similar to yeast histone deacetylase-A 1 (HDA1), include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10 [20,21]. In addition, a third class of HDACs related to yeast silent information regulator 2 (SIR2) was found to require a NAD cofactor for its enzymatic activity [22]. The identification and characterization of these 11 HDACs suggest that they may play distinct roles in cellular functions. Although these roles and functional differences remain to be elucidated, a better understanding of them would help to identify isozyme-specific HDAC inhibitors that could be expected to reduce the adverse effects of non-selective HDAC inhibitors.

It was recently reported that HDAC4 associates with the IL-2 promoter via the transcription factor, myocyte enhancer factor 2 (MEF2), and represses the IL-2 promoter's activity, most likely through the recruitment of co-repressors such as N-CoR in non-stimulated T cells. It has also been reported that HDAC4 dissociates from MEF2 and the IL-2 promoter after stimulating T cells with PMA and ionomycin [23]. Although these results imply that the HDAC4/N-CoR complex may negatively regulate IL-2 transcription in T cells, to our knowledge there is no data in the literature that states that a loss of HDAC4-enzymatic activity increases IL-2 gene expression. As mentioned above, it has also been reported that HDAC inhibitors inhibit IL-2 gene expression.

Here, to characterize the inhibitory mechanism of HDAC inhibitors in IL-2 gene expression, we focused on the role of HDAC4/N-CoR complex in the transcriptional regulation of IL-2 in Jurkat cells. We used four different approaches to characterize this role and to investigate the relation between the regulatory function of HDAC4/N-CoR complex and HDAC4-enzymatic activity: (i) HDAC4 silencing by RNA interference, (ii) overexpression of N-CoR repression domain 3 (RD3), (iii) overexpression of HDAC4 point mutants, and (iv) treatment with HDAC inhibitors.

2. Materials and methods

2.1. Compounds

FR235222 [16] was biosynthesized and FR276457, [(2E)-3-[4-(1H-benzimidazol-2-ylmethyl)-phenyl]-*N*-hydroxyacrylamide hydrochloride], was chemically synthesized at Astellas Pharma Inc. (Tokyo, Japan).

2.2. Antibodies

Anti-HDAC1 antibody (sc7872), anti-HDAC4 antibody (sc5245), anti-HDAC8 antibody (sc11544), anti-N-CoR antibody (sc1609), anti-14-3-3 β antibody (sc1657), and anti-GAL4 DNA-binding domain (DBD) antibody (sc577) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-HDAC3 antibody (H3034) and anti-FLAG M2 antibody (F3165) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anti-MEF2D antibody (610774) was obtained from BD Biosciences (San Jose, CA, USA). Donkey anti-rabbit IgG (NA934V) and sheep anti-mouse IgG (NA931V) horseradish peroxidase-conjugated (HRP-conjugated) secondary antibodies were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). HRP-rabbit anti-goat IgG (611620) was obtained from Zymed Laboratories (San Francisco, CA, USA).

2.3. Plasmid construction

The plasmid pGL3-IL2pro-43 was made by inserting a PCR fragment amplified from Jurkat genomic DNA containing the IL-2 promoter from −378 to +54 into the NheI-HindIII sites of the pGL3-basic vector (Promega, Madison, WI, USA). The human IL-2 promoter sequence was obtained from GenBank (accession number X67285). pGL3-control vector carrying a SV40 promoter/luciferase expression unit was purchased from Promega. pEAK10 vector was purchased from Edge Biosystems (Gaithersburg, MD, USA). Full-length human HDAC4 (GenBank accession number NM_006037) was cloned from a human brain cDNA library using PCR and subcloned into the EcoRI/BglII site of the vector for FLAG tagging at its C-terminus. FLAG-tagged HDAC4 was obtained by EcoRI/NotI digestion and inserted into the pEAK10 vector (pEAK-HDAC4). Point mutations (H802K/H803L and H863L) of HDAC4 were introduced by the standard PCR method using pEAK-HDAC4 as a template. pBIND vector was purchased from Promega. The RD2 (amino acids 736-1004), RD3 (amino acids 1005-1498), and SWI3/ADA2/NCoR/TFIIIB (SANT) (amino acids 267-549) domains of human N-CoR (GenBank accession number NM_006311) were cloned from a human lung cDNA library using PCR and inserted in-frame into the BamHI-NotI sites of the pBIND vectors (pBIND-N-CoR (RD2), pBIND-N-CoR (RD3), and pBIND-N-CoR (SANT), respectively). Completion of all vector constructions was confirmed by sequencing.

2.4. Cell culture

Jurkat cells and HEL cells (Japanese Collection of Research Bioresources, Tokyo, Japan) were maintained in RPMI 1640 medium (Sigma–Aldrich) supplemented with 10% fetal calf serum (Moregate Biotech, Bulimba QLD, Australia), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). HEK293 cells and PEAKrapid cells (Modified HEK293 cells, Edge Biosystems) were maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were grown at 37 °C with 5% CO_2. For

activation of Jurkat cells, phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich) and ionomycin (Sigma–Aldrich) were used at final concentrations of 50 ng/mL and 1 μg/mL, respectively.

2.5. Real-time PCR analysis

Total RNA was extracted, treated with DNaseI, and purified according to the manufacturer's protocol (RNeasy Mini Kit, Qiagen, Hilden, Germany). cDNA was synthesized with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was done in an ABI Prism 7700 Sequence Detection System (Applied Biosystems) with a SYBR Green PCR kit (Applied Biosystems) according to the manufacturer's instructions. The sequences of the primer pairs were: human IL-2 sense, 5′-CAGTGCACCTACTTCAAGTTCGA-3′, and human IL-2 antisense, 5′-CCAGCAGTAAATGCTCCAGTTG-3′; human GAPDH sense, 5′-GCCATCAATGACCCCTTCATT-3′, and human GAPDH antisense, 5′-GACGGTGCCATGGAATTTG-3′. A threshold was set in the linear part of the amplification curve, and the number of cycles needed to reach it was calculated for each gene. Melting curve analysis was done to test the purity of the amplified bands. Normalization was done using amounts of human GAPDH mRNA (GenBank accession number NM_002046) as an internal control for human IL-2 mRNA (GenBank accession number NM_000586). Values are expressed as arbitrary units.

2.6. Reporter gene assay

Jurkat cells (1×10^7) were transiently transfected with 1 μg of the plasmid pGL3-IL2pro-43 by electroporation using a GenePulser II (975 microfarads, 300 V; Bio-Rad Laboratories, Hercules, CA, USA) at 4 °C. Cells were stimulated after 10 h of transfection with PMA and ionomycin. After 14 h of stimulation, cells were lysed and analyzed using a Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. In the overexpression study, FLAG-tagged HDAC4s (wild-type HDAC4, HDAC4 H802K/H803L mutant, or HDAC4 H863L mutant) or GAL4 DBD-fused N-CoRs (RD2, RD3, or SANT domain) expression vectors were co-transfected with the plasmid pGL3-IL2pro-43. In the compound treatment study, various concentrations of HDAC inhibitors were added at the time of stimulation.

2.7. Cell viability assay

Cell viability was evaluated by the modified MTT assay using a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

2.8. Purification of HDAC4 and HDAC4 mutants

PEAKrapid cells ($1–2 \times 10^6$) were transiently transfected with 10 μg of the vector pEAK-HDAC4 for human HDAC4, pEAK-HDAC4 (H802K/H803L) for human HDAC4 mutant (H802K/H803L), or pEAK-HDAC4 (H863L) for human HDAC4 mutant (H863L), using the calcium phosphate method (CalPhos™ Mammalian Transfection Kit; Takara Bio Inc., Shiga, Japan). The cells were harvested 48 h after transfection and lysed in protein lysis buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Protein lysates were cleared by centrifugation at $35{,}000 \times g$ for 20 min at 4 °C. The supernatants were allowed to bind to Anti-FLAG M2 agarose-conjugated beads (A2220, Sigma–Aldrich) overnight at 4 °C in columns. The columns were washed with Tris-buffered saline (25 mM Tris base, pH 7.4, 150 mM NaCl). The FLAG-tagged HDAC4 or HDAC4 mutants were eluted with 0.1 M glycine–HCl (pH 3.5) and neutralized with 1 M Tris base (pH 8.0). Purified HDACs were kept at −80 °C until use.

2.9. HDAC activity assay

Preparation of [^{3}H] acetyl-labeled histones and assays for histone deacetylase activity were performed essentially as described previously [14].

2.10. Western blot analysis

Equal amounts of protein lysates were resolved by size on NuPAGE™ Bis-Tris (Invitrogen) or SDS-polyacrylamide gels (Daiichi Pure Chemicals, Tokyo, Japan) and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) using a Trans-Blot SD Semi-Dry Transfer Cell System (Bio-Rad Laboratories) according to the manufacturer's instructions. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (20 mM Tris base, pH 7.5, 150 mM NaCl) containing 0.1% Tween-20 (TBS-T) and probed with primary antibodies. After washing in TBS-T, membranes were incubated with HRP-conjugated secondary antibodies and developed using a chemiluminescence detection system (ECL Plus Western Blotting Detection System, GE Healthcare Bio-Sciences) according to the manufacturer's instructions.

2.11. Immunoprecipitation with anti-GAL4 DBD antibody

Transiently transfected PEAKrapid cells were harvested 48 h after transfection. The cells were lysed in protein lysis buffer containing a protease inhibitor cocktail. Protein lysates were placed on ice for 30 min, vortexed every 10 min, and then cleared by centrifugation at $12{,}000 \times g$ for 20 min at 4 °C. The supernatants were retrieved and frozen at −80 °C until use in immunoprecipitation assays. Protein concentration was measured using a DC protein assay kit. Anti-GAL4 DBD agarose-conjugated beads (sc510AC, Santa Cruz Biotechnology) were added to the supernatant and incubated overnight at 4 °C in the presence or absence of HDAC inhibitors. The beads were washed twice with tris-buffered saline, eluted with SDS sample buffer, and analyzed by immunoblotting.

2.12. siRNA transfection

siRNA specific for HDAC4 (siGENOME SMART pool) was purchased from Dharmacon (Chicago, IL, USA). Non-specific control Duplex IX (5′-AAATTGTATGCGATCGCAGAC-3′, Dharmacon) was used as a negative control. In IL-2 reporter gene assays, Jurkat cells (1×10^7) were transiently co-transfected with 1 μg of the plasmid pGL3-IL2pro-43 and 2 μM siRNAs by electroporation using a GenePulser II (975 microfarads, 300 V)

at 4 °C. Forty hours after transfection, cells were stimulated with PMA and ionomycin. After 8 h of stimulation, cells were lysed and analyzed using a Bright-Glo Luciferase Assay System according to the manufacturer's instructions. To assess HDAC4 knockdown with siRNA in transfected cells, we used a MACSelect K^k – transfected cell selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Jurkat cells (1×10^7) were transiently co-transfected with 10 μg of the plasmid pMACS K^k.II encoding the truncated mouse H-$2K^k$ surface marker and 2 μM siRNAs by electroporation using a GenePulser II (975 microfarads, 300 V) at 4 °C. After 48 h of transfection, the transfected cells were labeled with MACSelect K^k MicroBeads and separated on MACS columns by magnetic force. Separated cells were lysed and HDAC4 knockdown was confirmed by Western blotting.

2.13. Statistical analysis

Parametric data were expressed as mean ± standard error of the mean. Comparisons of two or more means were evaluated using Student's *t*-tests with independent estimates of variance or analysis of variance followed by a Dunnett test, respectively. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of IL-2 promoter activation by the HDAC inhibitors FR235222 and FR276457

The HDAC inhibitors FR235222 [16] and FR276457, a hydroxamic derivative (see Section 2), were tested for their ability to inhibit IL-2 gene expression in Jurkat cells. We first checked the time course of IL-2 gene expression in Jurkat cells by quantitative real-time PCR. To mimic T cell antigen receptor signaling, we used PMA and ionomycin, known as a more potent stimulator of T cells than anti-CD3 antibody. When cells were stimulated with PMA and ionomycin, IL-2 mRNA expression was detected as early as 2 h and peaked at around 8 h following activation by quantitative real-time PCR (data not shown). We next analyzed the effect of FR235222 on IL-2 gene expression in Jurkat cells. Jurkat cells were stimulated with PMA and ionomycin for 8 h in the presence or absence of various concentrations of FR235222 (1–300 nM). Analysis showed that FR235222 suppressed IL-2 mRNA expression in these cells in a concentration-dependent manner, with an IC_{50} value of approximately 30 nM (Fig. 1A).

To further investigate the effect of FR235222 and FR276457 on IL-2 promoter activation in Jurkat cells, a luciferase reporter construct containing human IL-2 promoter (pGL3-IL2pro-43) was subjected to transient transfection. Transfected Jurkat cells were stimulated with PMA and ionomycin for 14 h in the presence or absence of various concentrations of FR235222 (1–300 nM) and FR276457 (10–3000 nM). Consistent with the result obtained by quantitative real-time PCR, IL-2 promoter activation was inhibited by FR235222 in a concentration-dependent manner, with an IC_{50} value of approximately 30 nM (Fig. 1B). FR276457 suppressed it with an IC_{50} value of approximately 300 nM (Fig. 1B). In this experiment, cell viability was not

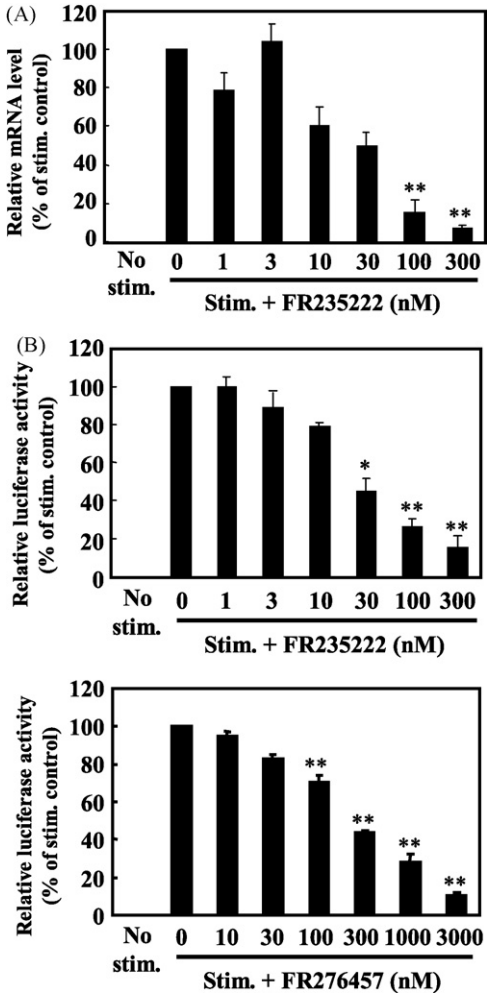

Fig. 1 – Inhibition of IL-2 gene expression by the HDAC inhibitors FR235222 and FR276457. (A) Quantitative real-time PCR analysis. Jurkat cells were unstimulated or stimulated with PMA/ionomycin in the presence or absence of various concentrations of FR235222. After 8 h stimulation, total RNA was prepared and analyzed for IL-2 mRNA expression levels by real-time PCR. GAPDH mRNA expression levels were analyzed as controls. Results are presented as a percentage of the stimulated control. Values are the mean ± S.E.M. of three independent experiments performed in duplicate. Asterisks (**) indicate statistical significance ($p < 0.01$) vs. the stimulated control. (B) IL-2 reporter gene analysis. Jurkat cells were transfected with the luciferase reporter construct pGL3-IL2pro-43, and after 10 h of transfection, the cells were unstimulated or stimulated with PMA/ionomycin in the presence or absence of various concentrations of FR235222 and FR276457. Luciferase activity was determined after 14 h stimulation. Results are presented as a percentage of the stimulated control. Values are the mean ± S.E.M. of three independent experiments performed in triplicate. Asterisks (* and **) indicate statistical significance ($p < 0.05$ and $p < 0.01$, respectively) vs. the stimulated control.

influenced by FR235222 and FR276457 (data not shown). These results indicate that FR235222 and FR276457 inhibit IL-2 gene expression at the transcriptional level without cytotoxicity.

3.2. Inhibition of IL-2 promoter activation by RNA interference-mediated HDAC4 silencing

To elucidate the inhibitory mechanism of HDAC inhibitors in IL-2 gene expression, we focused on the role of the HDAC4/N-CoR complex in the transcriptional regulation of IL-2. We first evaluated the impact of HDAC4 silencing by RNA interference on IL-2 promoter activation. Jurkat cells were cotransfected with pGL3-IL2pro-43 and HDAC4 short interfering RNA (siRNA), or non-specific control siRNA by electroporation, and after 40 h of transfection, the cells were stimulated with PMA and ionomycin. Luciferase activity and cell viability were determined after 8 h stimulation. As expected, IL-2 promoter activation was specifically inhibited by HDAC4 siRNA transfection (Fig. 2A). Cell viability was not affected by HDAC4 siRNA transfection (data not shown). In addition, to verify that HDAC4 siRNA induced the specific down-regulation of HDAC4 protein expression, siRNA-transfected cells were enriched using a MACSelect K^k—transfected cell selection kit. Jurkat cells were cotransfected with the plasmid pMACS K^k .II encoding the truncated mouse H-$2K^k$ surface marker and HDAC4 siRNA, or non-specific control siRNA, and after 48 h of transfection, transfected cells were labeled with MACSelect K^k MicroBeads and separated on MACS columns by magnetic force. Separated cells were lysed and specific knockdown of HDAC4 was confirmed by Western blotting. As shown in Fig. 2B, the amount of HDAC4 protein was markedly decreased in cells transfected with HDAC4 siRNA only. In contrast, expression levels of the other HDACs tested (HDAC1, HDAC3, and HDAC8) were not affected by HDAC4 siRNA transfection (Fig. 2B). These data show that HDAC4 plays an essential role in the transcriptional regulation of IL-2 gene expression.

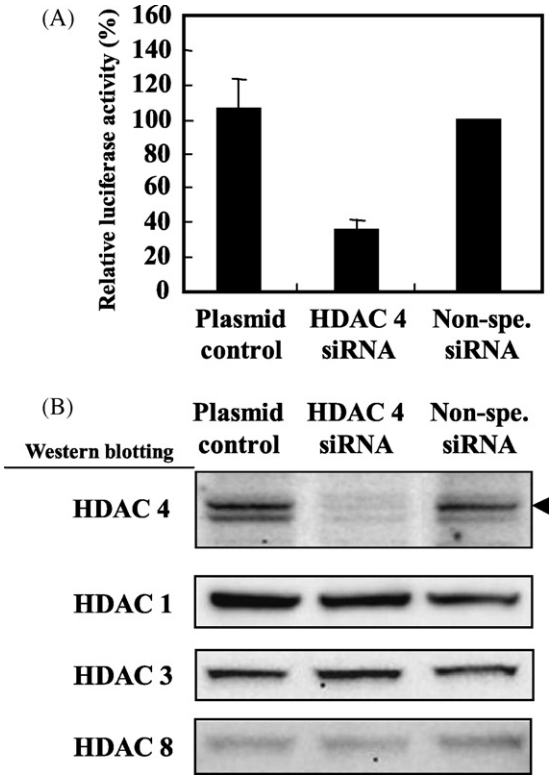

Fig. 2 – Inhibition of IL-2 promoter activation by RNA interference-mediated HDAC4 silencing. (A) Jurkat cells were cotransfected with the luciferase-IL-2 reporter construct and HDAC4 siRNA, or non-specific control siRNA, and after 40 h of transfection, the cells were stimulated with PMA/ionomycin. Luciferase activity was determined after 8 h stimulation. Values are the mean ± S.E.M. of three independent experiments performed in triplicate. (B) Jurkat cells were cotransfected with the plasmid pMACS K^k .II (MACSelect™ K^k System) and HDAC4 siRNA, or non-specific control siRNA. After 48 h of transfection, transfected cells were labeled with MACSelect K^k MicroBeads and separated using MACS columns. Separated cells were lysed and specific knockdown of HDAC4 was confirmed by Western blotting using antibodies specific for HDAC1, HDAC3, HDAC4, and HDAC8. Experiments were performed twice with similar results.

3.3. Inhibition of IL-2 promoter activation by overexpression of N-CoR RD3

Our observation that HDAC4 silencing by RNA interference inhibits IL-2 promoter activation in Jurkat cells suggested that the formation of endogenous HDAC4/N-CoR complex might be essential for IL-2 promoter activation. Previous analyses of HDAC4/N-CoR complex revealed that HDAC4 was able to bind specifically to RD3 of N-CoR [24–26]. We therefore assessed the effect of overexpression of N-CoR RD3 in IL-2 reporter gene assay. As shown in Fig. 3, IL-2 promoter activation was strongly inhibited by overexpression of N-CoR RD3. In addition, surprisingly, partial inhibition of cell viability was observed (Fig. 3). In contrast, overexpression of N-CoR RD2, known as the HDAC3-binding domain [26], increased IL-2 promoter activation (cell viability was not changed) (Fig. 3). Further, overexpression of the SANT domain of N-CoR, also known as the HDAC3-binding domain [26,27], showed almost no effect on either IL-2 promoter activation or cell viability in Jurkat cells (Fig. 3). These results indicate that the inhibition of IL-2 promoter activation and, to some extent, viability of Jurkat cells by N-CoR RD3 overexpression may be due to disruption of the interaction between endogenous HDAC4 and N-CoR.

3.4. Inhibition of IL-2 promoter activation by overexpression of HDAC4 mutants

To investigate the relation of HDAC4-enzymatic activity on the regulatory function of HDAC4/N-CoR complex in IL-2 promoter activation, we constructed expression vectors encoding FLAG-tagged HDAC4 mutants in which point mutations were introduced into the catalytic domain of HDAC4 (Fig. 4A). The characteristics of HDAC4 mutants were first confirmed by performing immunoprecipitation using PEAKrapid cells transiently expressing FLAG-tagged HDAC4 mutants. As previously reported [24,25,28], the presence of point mutations in the catalytic domain of HDAC4 markedly diminished its enzymatic activity (Fig. 4B) and disrupted the formation of the

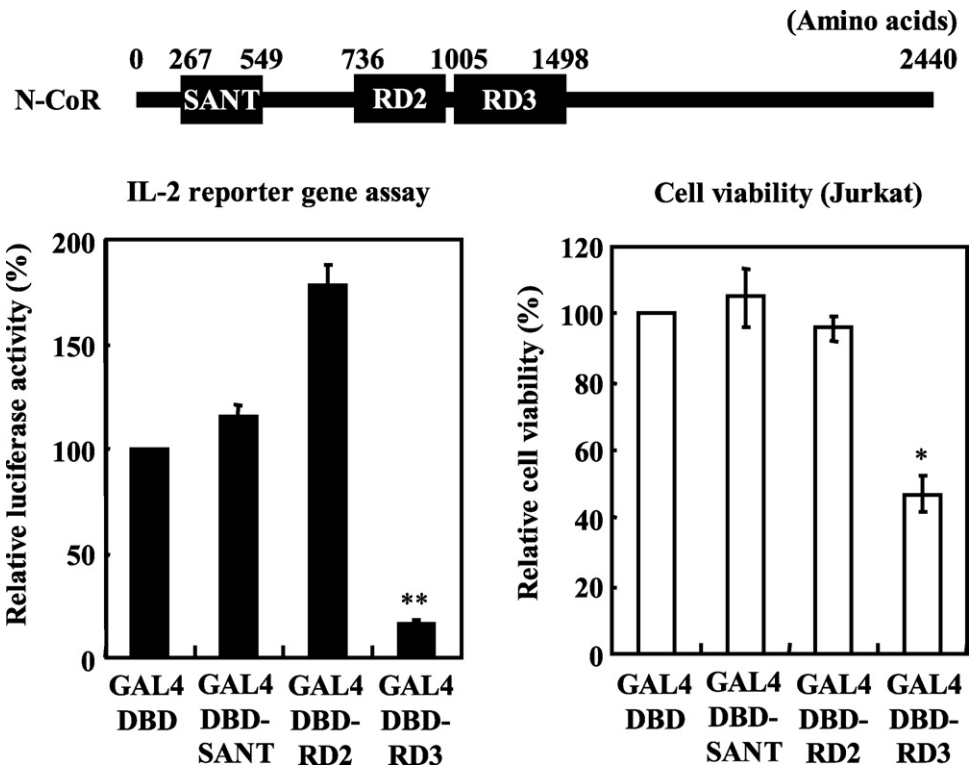

Fig. 3 – Inhibition of IL-2 promoter activation by overexpression of N-CoR RD3. Jurkat cells were cotransfected with the luciferase-IL-2 reporter construct and the expression vectors encoding GAL4 DBD, GAL4 DBD-SANT, GAL4 DBD-RD2, or GAL4 DBD-RD3 proteins, and after 10 h of transfection, the cells were stimulated with PMA/ionomycin. Luciferase activity and cell viability were determined after 14 h stimulation. Values are the mean ± S.E.M. of two independent experiments performed in triplicate. Asterisks (* and **) indicate statistical significance ($p < 0.05$ and $p < 0.01$, respectively) vs. GAL4 DBD-expressing control cells.

N-CoR complex (Fig. 4C). Given that previous studies [29,30] have shown that HDAC4 also interacts with MEF2D transcription factor via its N-terminal region (Fig. 4A), we examined whether MEF2D binding was also influenced by point mutations in the catalytic domain of HDAC4. Results showed that MEF2D binding was unchanged in the HDAC4 mutant (H863L) relative to the wild-type control (Fig. 4C). These data show that the reduced enzymatic activity of HDAC4 mutants (H802K/H803L and H863L) and their impaired ability to form a complex with N-CoR might not be due to any global change in conformation but rather to a simple alteration in catalytic site residue or local structural perturbation that disrupts the interaction site for N-CoR, and suggest that the HDAC4 mutants (H802K/H803L and H863L) may be able to act as dominant negative mutants.

We next assessed the effect of overexpression of FLAG-tagged HDAC4 and HDAC4 mutants (H802K/H803L and H863L) in the IL-2 reporter gene assay. As shown in Fig. 4D, IL-2 promoter activation was only slightly increased by overexpression of wild-type HDAC4. In contrast, both overexpressed HDAC4 mutants caused a decrease in IL-2 promoter activation (Fig. 4D). We interpret these results to suggest that it is not enough to simply induce further activation of the IL-2 promoter by overexpressing HDAC4; the association of the endogenous HDAC4/N-CoR complex with IL-2 promoter via MEF2D, which is probably affected by overexpression of HDAC4 point mutants, may also be needed to properly activate the IL-2 promoter. Further, we also evaluated the effect of HDAC4 mutant overexpression on cell viability. Jurkat cells, megakaryocytic HEL cells, and HEK293 cells were transiently transfected with the expression vectors encoding FLAG-tagged HDAC4 or HDAC4 mutant (H863L), and cell viability was quantified by the modified MTT assay using a cell counting kit-8 after 9, 22, or 48 h of transfection, respectively (Jurkat cells were stimulated with PMA and ionomycin after 5 h of transfection). Even though relative transfection efficiencies in Jurkat cells were similar to those in HEL cells or much lower than those in HEK293 cells, results showed that overexpression of the HDAC4 mutant caused in a partial, but specific, decrease in the viability of Jurkat cells, as is the case with overexpression of the N-CoR RD3 (Fig. 4E). We assume that these specific decreases in the viability of Jurkat cells may represent the specific role of the HDAC4/N-CoR complex not only in IL-2 promoter activation but in T cell growth control, because HDAC inhibitor also has an ability to induce cell-cycle arrest and apoptosis in Jurkat cells (data not shown). However, there was a possibility that overexpression of the N-CoR RD3 or the HDAC4 mutant itself is toxic for Jurkat cells. It was therefore confirmed that the decrease in IL-2 promoter activation caused by overexpression of the

HDAC4 point mutant (H863L) was not due to global defects in transcriptional control. We compared the effect of overexpression of the HDAC4 point mutant (H863L) on the activity of the IL-2 promoter with that of the SV40 promoter after normalization by the cell viability. As a result, IL-2 promoter activation was selectively down-regulated compared to SV40 promoter activity (Fig. 4F). Overall, the findings described above suggest that the formation of HDAC4/N-CoR complex is closely associated with HDAC4-enzymatic activity, and that the overexpression of HDAC4 point mutants may affect the interaction between endogenous HDAC4/N-CoR complex and MEF2D. This will eventually cause a decrease in IL-2 promoter activation and, to some extent, the viability of Jurkat cells.

3.5. *Disruption of HDAC4/N-CoR complex formation by the HDAC inhibitors FR235222 and FR276457*

Previous studies [24,25] and our finding that enzymatically inactive HDAC4 mutants lose their ability to interact with N-CoR suggested that HDAC inhibitors may have an inhibitory effect on HDAC4/N-CoR complex formation. To test this hypothesis, we evaluated the effect of FR235222 and FR276457 on the interaction between endogenous HDAC4 and overexpressed N-CoR RD3 by immunoprecipitation analysis. Various concentrations of FR235222 and FR276457 were added to lysates from GAL4 DBD-RD3-expressing PEAKrapid cells at the time of immunoprecipitation using anti-GAL4 DBD antibody, and the amount of HDAC4 in the coimmunoprecipitates was analyzed by Western blotting. As expected, both HDAC inhibitors inhibited the interaction between endogenous HDAC4 and GAL4 DBD-RD3 in a concentration-dependent manner (Fig. 5). In this coimmunoprecipitation experiment, inhibitory concentrations of FR235222 and FR276457 were much higher than the in IL-2 reporter gene assay (Fig. 1B; 80% inhibitory concentration values in the IL-2 reporter gene assay were approximately 300 and 3000 nM, respectively). We therefore further investigated the effect of FR276457 on the interaction between endogenous N-CoR and overexpressed FLAG-tagged HDAC4 in PEAKrapid cells. FLAG-tagged HDAC4 or HDAC4 mutant (H863L) were transiently expressed into PEAKrapid cells, and after 44 h of transfection, the cells were treated with or without FR276457 (3000 nM). After 4 h incubation, whole cell lysates were prepared and separated using a FLAG-affinity column. Eluted fractions were analyzed for the presence of FLAG-tagged HDAC4s (wild-type or H863L mutant) and N-CoR by CBB staining and Western blotting. As a control, the amount of 14-3-3β, known as a HDAC4 binding protein [31,32], was detected by Western blotting. Results showed the clear interaction of endogenous N-CoR with FLAG-tagged HDAC4 but not with enzymatically inactive HDAC4 mutant (H863L) (Fig. 6). Further, FR276457 inhibited the interaction between N-CoR and FLAG-tagged HDAC4 in cells at a concentration around the 80% inhibitory concentration values in IL-2 reporter gene assay (Fig. 6). These results suggest that HDAC inhibitors have an inhibitory effect on HDAC4/N-CoR complex formation which presumably involves the transcriptional regulation of IL-2 gene expression.

4. Discussion

In this study, we demonstrate the essential role of HDAC4 in the transcriptional regulation of IL-2 gene expression using three molecular biological approaches—HDAC4 silencing by RNA interference, overexpression of N-CoR RD3, and overexpression of HDAC4 point mutants. We also show that the HDAC4/N-CoR complex formation, which is closely related to HDAC4-enzymatic activity, is disrupted by treatment with HDAC inhibitors and this disruption may lead to the inhibition of IL-2 promoter activation.

We first observed that the HDAC inhibitors FR235222 and FR276457 and the specific knockdown of HDAC4 by RNA interference inhibited IL-2 promoter activation. These findings suggest that inhibition of HDACs causes a decrease in IL-2 promoter activation, and that HDAC4 plays an essential role in the transcriptional regulation of IL-2 gene expression. We next observed that overexpression of N-CoR RD3, known as the HDAC4-binding domain [24–26], also inhibited IL-2 promoter activation. This inhibition was RD3-specific, because overexpression of the SANT domain or RD2, known as the HDAC3-binding domain [26,27], had no inhibitory effect on IL-2 promoter activation. We interpret these results to suggest that overexpressed N-CoR RD3 acts as a dominant-negative inhibitor of endogenous HDAC4/N-CoR complex formation, and that this complex formation is essential for IL-2 promoter activation.

Further, to better clarify the importance of HDAC4/N-CoR complex in IL-2 promoter activation, and to investigate the relationship between the regulatory function of HDAC4/N-CoR complex and HDAC4-enzymatic activity, we examined the effects of the overexpression of wild-type HDAC4 and HDAC4 mutants on IL-2 promoter activation. Results showed that IL-2 promoter activation was increased only slightly by the overexpression of wild-type HDAC4, and inhibited by the overexpression of HDAC4 mutants. These HDAC4 mutants are enzymatically inactive and lose their ability to interact with N-CoR, but retain the ability to interact with MEF2D, a transcription factor known to be indispensable for activation of the IL-2 promoter [23]. We interpret these results to suggest that the decrease in IL-2 promoter activation caused by the overexpression of HDAC4 point mutants is probably due to a negative impact on the interaction of the endogenous HDAC4/N-CoR complex with MEF2D. It is assumed that the loss of HDAC4-enzymatic activity caused by treatment with HDAC inhibitors may affect the MEF2-dependent expression of other genes. Another observation that supports this interpretation is that, in addition to IL-2, the HDAC inhibitor FR235222 has the ability to concentration-dependently inhibit the expression of Nur77, which is known to be transcriptionally regulated by MEF2, in activated Jurkat cells (unpublished data). Previously, it was reported that the binding of HDAC4 to MEF2 is calcium-dependent [28,30]. It was also found that HDAC4, which is believed to be associated with the IL-2 promoter via MEF2 in non-stimulated T cells, dissociates from MEF2 and the IL-2 promoter after the T cells have been stimulated by PMA and ionomycin [23]. In addition, it has been reported that HDAC activity is required for RNA polymerase II to associate with the PU.1 promoter [33], and that N-CoR and a specific deacetylase are required for the transcriptional activation of one class of

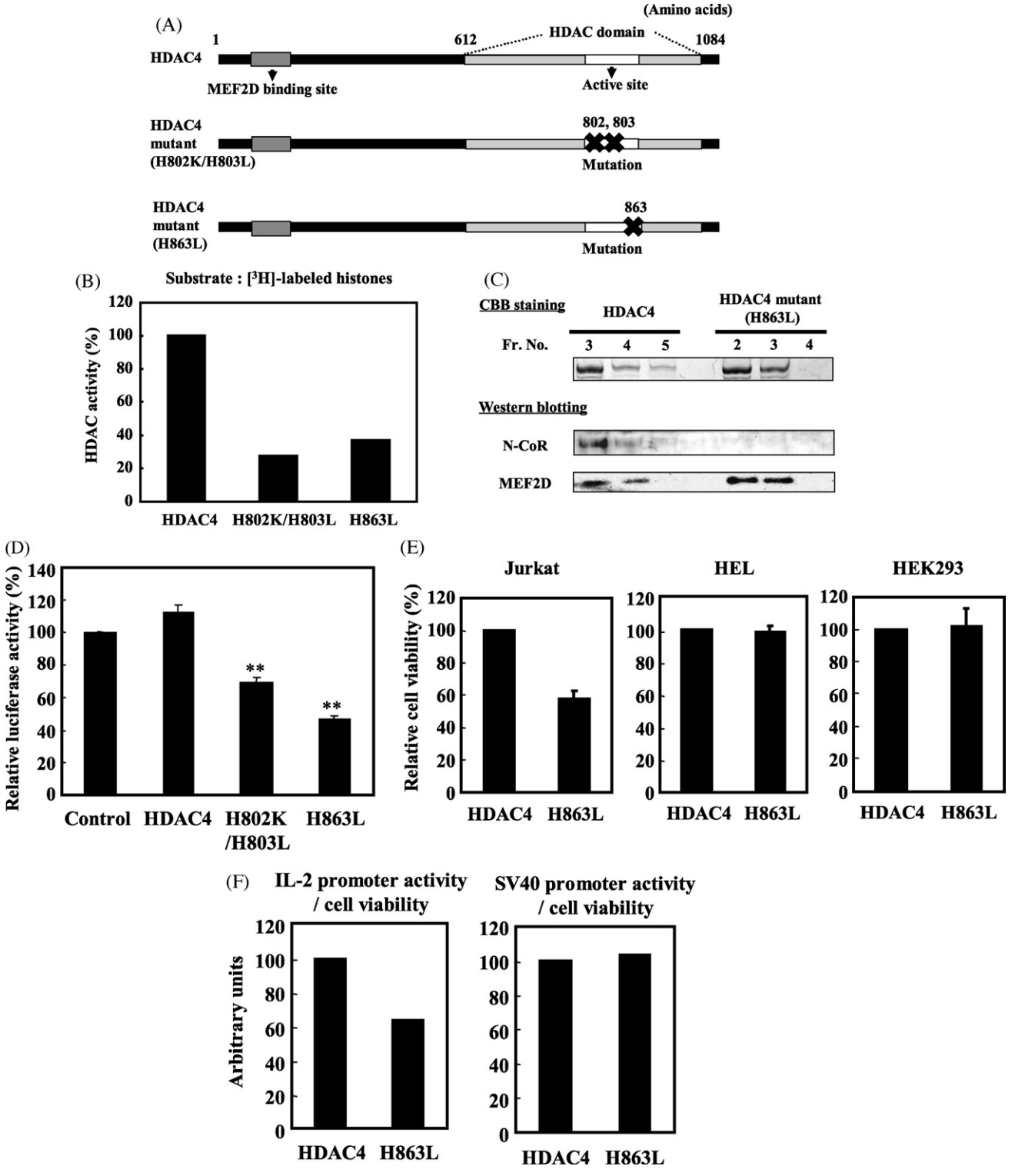

Fig. 4 – Inhibition of IL-2 promoter activation by overexpression of HDAC4 mutants. (A) Schematic representations of HDAC4 and HDAC4 mutants. Mutations were introduced into active site residues of the HDAC domain in HDAC4 by PCR methods and checked by DNA sequencing. (B) Enzymatic activities of HDAC4 and HDAC4 mutants. HDAC4 and HDAC4 mutants (H802K/H803L and H863L) were FLAG-tagged and transiently transfected into PEAKrapid cells. Immunoprecipitated proteins with anti-FLAG M2 antibody were tested for enzymatic activity as described in Section 2. Experiments were performed in duplicate. (C) HDAC4 mutant (H863L) was unable to form the N-CoR complex. FLAG-tagged HDAC4 and HDAC4 mutant (H863L) were purified from transiently transfected PEAKrapid cells by anti-FLAG M2 agarose. Fractions were analyzed by CBB staining and Western blotting using anti-N-CoR and anti-MEF2D antibodies. (D) Jurkat cells were co-transfected with the luciferase reporter construct containing human IL-2 promoter and 10 μg of either an empty vector or

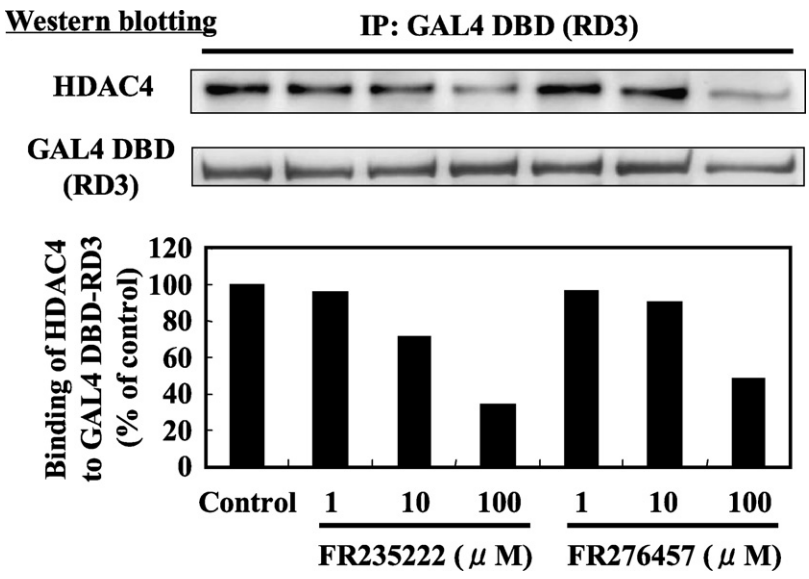

Fig. 5 – Disruption of interaction between HDAC4 and N-CoR RD3 by the HDAC inhibitors FR235222 and FR276457. Binding of HDAC4 to N-CoR RD3 is inhibited by HDAC inhibitors (FR235222 and FR276457). Various concentrations of FR235222 and FR276457 were added to lysates from GAL4 DBD-RD3 expressing PEAKrapid cells at the time of immunoprecipitation using anti-GAL4 DBD antibody, and immunoprecipitated proteins were analyzed by Western blotting using the anti-HDAC4 and anti-GAL4 DBD antibodies. The graph depicts the amount of HDAC4 proteins normalized by the amount of GAL4 DBD-RD3 proteins. Data were quantitated by densitometry. Experiments were performed twice with similar results.

retinoic acid response element [34]. These previous studies and our findings also suggest that the association of the endogenous HDAC4/N-CoR complex with the IL-2 promoter via MEF2D in non-stimulated T cells, which is probably regulated by HDAC4-enzymatic activity, may be needed to properly activate the IL-2 promoter. In some cases, this may involve the recruitment of RNA polymerase II.

Finally, we ascertained whether HDAC inhibitors had an inhibitory effect on the interaction between HDAC4 and N-CoR. FR235222 and FR276457, HDAC inhibitors which can inhibit IL-2 gene expression, showed inhibitory effects on the interaction of HDAC4 with N-CoR RD3 or endogenous N-CoR. Conversely, however, despite its ability to inhibit IL-2 gene expression, inhibition of wild-type HDAC4 enzymatic activity with TSA did not block interaction with N-CoR [24]. This apparent discrepancy may be due to differences in the HDAC inhibitor's concentration and the treatment protocols. In the previous study [24], 300 nM TSA was added to cell lysates at the N-CoR and myc-tagged HDAC4 coimmunoprecipitation step. Our results indicate that much higher concentration of HDAC inhibitor is needed to disrupt the interaction of HDAC4 with N-CoR in an *in vitro* assay than in an assay using intact cells. Thus, although a comprehensive understanding of the molecular mechanism of HDAC inhibitor-mediated inhibition of IL-2 gene expression awaits further study, we consider that transcriptional repression of IL-2 gene by HDAC inhibitors is at least partly caused by HDAC4 inhibition involving the disruption of HDAC4/N-CoR complex formation. In this study, it was also shown that overexpression of the N-CoR RD3 or HDAC4 point mutant (H863L) caused a partial, but specific, decrease in Jurkat cell viability. Our interpretation of these results is that an excessive amount of the free N-CoR RD3 itself or aberrant recruitment of HDAC4/N-CoR complex, caused by overexpression of the HDAC4 point mutant (H863L), affects

the expression vectors encoding FLAG-tagged HDAC4s (wild-type HDAC4, HDAC4 H802K/H803L mutant, or HDAC4 H863L mutant), and after 10 h of transfection, the cells were stimulated with PMA/ionomycin. Luciferase activity was determined after 14 h stimulation. Values are the mean ± S.E.M. of at least five independent experiments performed in triplicate. Asterisks (**) indicate statistical significance ($p < 0.01$) vs. control cells transfected with an empty vector. (E) Jurkat, HEL, and HEK293 cells were transiently transfected with the expression vectors encoding FLAG-tagged HDAC4 or HDAC4 mutant (H863L) and cell viability was quantified by the modified MTT assay using a cell counting kit-8 after 9, 22, or 48 h of transfection, respectively (Jurkat cells were stimulated with PMA/ionomycin after 5 h of transfection). Values are the mean ± S.E.M. of two independent experiments performed in duplicate. (F) Jurkat cells were co-transfected with the luciferase reporter construct containing either human IL-2 promoter or SV40 promoter and 10 μg of the expression vectors encoding FLAG-tagged HDAC4s (wild-type HDAC4 or HDAC4 H863L mutant). After 10 h of transfection, the cells were stimulated with PMA/ionomycin. Luciferase activity and cell viability were determined after 14 h stimulation. The graphs depict the activity of IL-2 promoter or SV40 promoter normalized by the cell viability. Values are the mean of two independent experiments performed in duplicate.

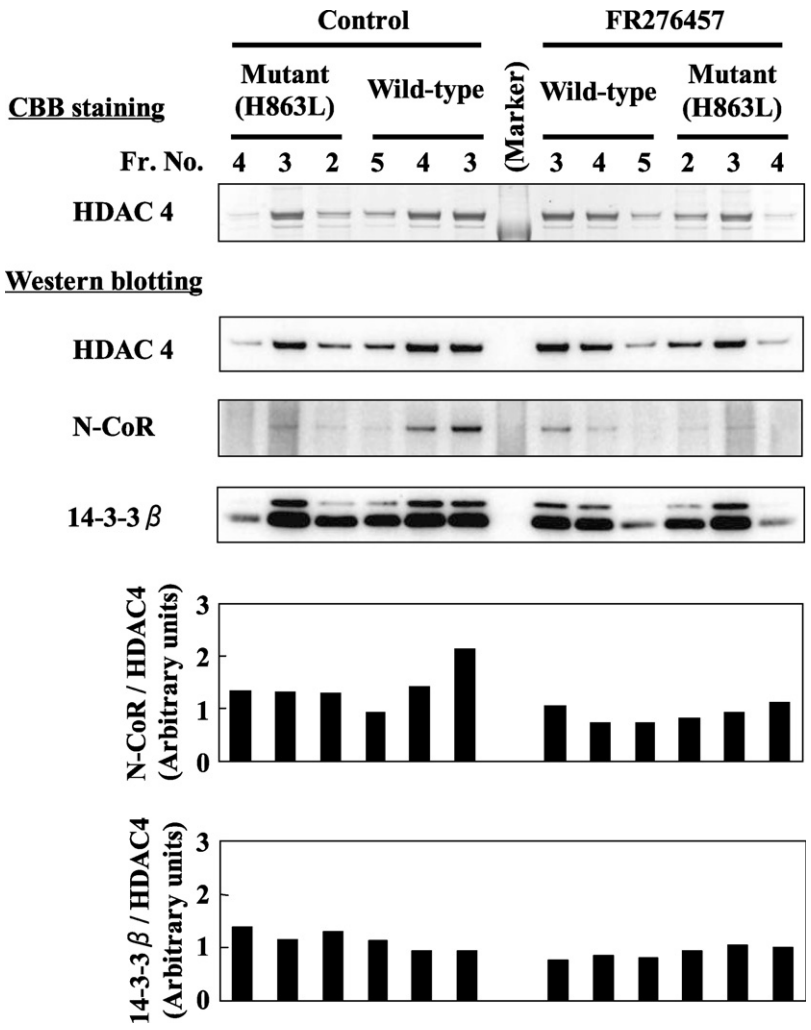

Fig. 6 – Disruption of HDAC4/N-CoR complex formation by the HDAC inhibitor FR276457 in PEAKrapid cells. FLAG-tagged HDAC4 or HDAC4 mutant (H863L) were transiently transfected into PEAKrapid cells, and after 44 h of transfection, the cells were treated with or without FR276457 (3000 nM). After 4 h incubation, whole cell lysates were prepared and immunoprecipitated with anti-FLAG M2 antibody. The immunoprecipitated proteins were analyzed by Western blotting with antibodies specific for HDAC4, N-CoR, and 14-3-3β. The graphs depict the amount of N-CoR proteins normalized by the amount of HDAC4 proteins (upper) and the amount of 14-3-3β proteins normalized by the amount of HDAC4 proteins (lower). Data were quantitated by densitometry. Experiments were performed twice with similar results.

Jurkat cell viability either directly or through the functional disruption of endogenous N-CoR. Consistent with this interpretation, it has been reported that defective T cell development occurs in N-CoR−/− mice [34] and that aberrant N-CoR recruitment plays a pathogenic role in acute myeloid leukemia [35]. However, even if this growth retardation is taken into account, our results still show that overexpression of the N-CoR RD3 or HDAC4 point mutant (H863L) exactly causes a decrease in IL-2 promoter activation. In addition, such growth retardation was not observed in Jurkat cells transfected with HDAC4 siRNA. These results convincingly demonstrate that the HDAC4/N-CoR complex, especially HDAC4, is essential for IL-2 promoter activation.

In this study, we used Jurkat cells as a model of normal T cells to study the mechanism of HDAC inhibitor-mediated transcriptional repression of the IL-2 gene. The HDAC inhibitors FR235222 and FR276457 have an ability to suppress IL-2 expression without cytotoxicity not only in Jurkat cells but in normal T cells ([16,17], and unpublished data). In addition to inhibition of IL-2 expression, FR235222 and FR276457 also have an anti-proliferation activity, and longer incubation time (over 24 h) leads both Jurkat cells and normal T cells to cell-cycle arrest and apoptosis ([16,17], and unpublished data). We therefore assume that Jurkat cells represent a useful model for T cell research, and that our findings in Jurkat cells can apply to normal T cells. In addition, transcriptional repression by HDAC inhibitors was relatively selective for the IL-2 gene as previously reported [15,19]. For example, the expression of stimuli-induced IFN-γ, a representative of Th1 cytokines, was hardly affected by FR235222 in Jurkat cells (unpublished data).

Although HDAC inhibitors hold great promise in the fields of cancer, organ transplantation, and autoimmune diseases, many have undesirable side effects caused by the non-selective inhibition of HDAC isozymes. A better understanding

of the individual roles and functional differences of HDAC isozymes would help identify isozyme-specific HDAC inhibitors with fewer or none of the adverse effects of non-selective inhibitors. In view of this, the present data may help our understanding of the molecular mechanism of inhibition of IL-2 gene expression by HDAC inhibitors and provide insight into strategies to produce more effective and safer new immunosuppressants.

In conclusion, our findings suggest that HDAC4 plays an essential role in the transcriptional regulation of IL-2 gene expression, and that HDAC inhibitors inhibit IL-2 gene expression partly by HDAC4 inhibition involving the disruption of HDAC4/N-CoR complex formation. The selective inhibition of HDAC4 or the interaction of HDAC4 with N-CoR is likely a potential target for the development of novel immunosuppressants.

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