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New Yeast Cell-Based Assay System for Screening Histone Deacetylase 1 Complex Disruptor

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Abstract Histone deacetylase 1 (HDAC1) works as one of the components in a nucleosome remodeling (NuRD) complex that consists of several proteins, including metastasis-associated protein 1 (MTA1). Since the protein-protein interaction of HDAC1 and MTA1 would appear to be important for both the integrity and functionality of the HDAC1 complex, the interruption of the HDAC1 and MTA1 interaction may be an efficient way to regulate the biological function of the HDAC1 complex. Based on this idea, a yeast two-hybrid system was constructed with HDAC1 and MTA1 expressing vectors in the DNA binding and activation domains, respectively. To verify the efficiency of the assay system, 3,500 microbial metabolite libraries were tested using the paper disc method, and KB0699 was found to inhibit the HDAC1 and MTA1 interaction without any toxicity to the wild-type yeast. Furthermore, KB0699 blocked the interaction of HDAC1 and MTA1 in an *in vitro* GST pull down assay and induced morphological changes in B16/BL6 melanoma cells, indicating the interruption of the HDAC1 complex function. Accordingly, these results demonstrated that the yeast assay strain developed in this study could be a valuable tool for the isolation of a HDAC1 complex disruptor.

Key words: Histone deacetylase 1, metastasis-associated protein 1, protein-protein interaction, protein complex disruptor

Transcriptional regulation in eukaryotes requires the modification of the chromatin structure through the combination of multiple protein factors [1]. Highly conserved lysine residues of histone tails in the nucleosome can be acetylated and deacetylated by reversible enzymes, histone acetyltransferases (HATs), and histone deacetylases (HDACs) [14]. The acetylation of lysine residues in core histones neutralizes the positive charge of the lysine and potentially reduces the electrostatic interactions that tether the octamer tails to the DNA phosphate backbone, thereby causing the nucleosomes to unfold and increasing access to transcription factors [4]. As such, HATs and HDACs function as a transcription coactivator and corepressor, respectively, by interacting with specific transcriptional factors in multiple complexes to regulate gene expression [1, 5, 17, 33].

HDAC1 has been identified as one of the components in a nucleosome remodeling complex (NuRD) that consists of several other proteins, including metastasis-associated protein 1 (MTA1) [5, 26, 29]. MTA1 is overexpressed in breast cancer cells and known to interact with HDAC1 [23, 27, 28]. In addition, MTA1, which promotes tumor invasion and metastasis, is also known to mediate estrogen receptor (ER) transcriptional repression by interacting with HDAC1 [20, 34]. Similarly, MTA2, a MTA1-related protein, interacts with p53 to recruit the HDAC complex and results in the inactivation of the transcriptional activity of the protein [8, 18]. Since the protein-protein interaction of HDAC1 and MTA1 is important for the functionality of the HDAC1 complex, the interruption of the HDAC1 and MTA1 interaction may be an efficient way to regulate the biological function of the HDAC1 complex.

The protein-protein interaction has already been extensively studied using a yeast two-hybrid system [31]. The system was developed based on the idea that many eukaryotic trans-acting transcriptional regulators consist of functionally independent domains that are separated physically [3]. An interaction between two different domain-fused proteins triggers the expression of reporter genes and this makes the protein-protein interaction phenotypically detectable. Several protein complexes and components involved in cell signaling, protein synthesis, and transcription have already been identified using this method [31]. Furthermore, this system has also been applied to isolate inhibitors or stimulators for the specific protein interaction [11].

In the current study, a yeast two-hybrid system was constructed with HDAC1 and MTA1 expressing vectors to develop a screening system for the isolation of a HDAC1

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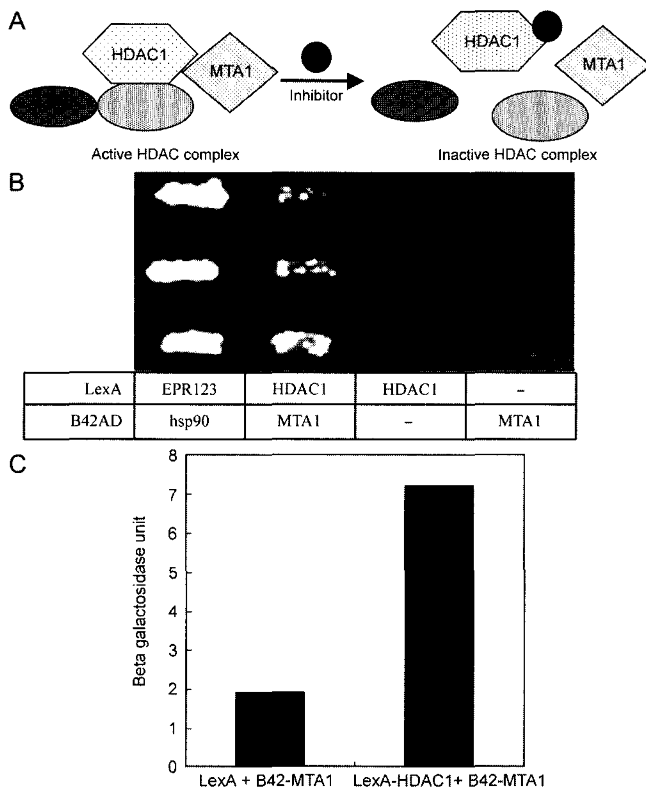

Fig. 1. HDAC1 interacts with MTA1 in the yeast two-hybrid system.

(A) Schematic model of HDAC1 and MTA1 interaction in HDAC complex and interruption of protein interaction by inhibitor. (B) The *Saccharomyces cerevisiae* reporter strain EGY48[p8op-lacZ] was transformed with a plasmid that expresses LexA DNA binding domain fused to HDAC1 or LexA DNA binding domain alone and a plasmid that expresses B42 activation domain fused to MTA1 or B42 activation domain alone. Yeast strain that expresses LexA DNA binding domain fused to EPR123 and B42 activation domain fused to hsp90 was used as positive control [9]. Three colonies from each yeast transformants were streaked on the leucine-depleted media plate and grown at 30°C until colonies expressing *LEU2* reporter gene appeared. Positive control and the yeast strains that express LexA-fused HDAC1 and B42-fused MTA1 grew in the absence of leucine. (C) Yeast reporter strains transformed LexA-fused HDAC1 and B42-fused MTA1 or LexA alone and B42-fused MTA1 were grown overnight in leu-depleted liquid media at 30°C. β-galactosidase reporter gene expression of the yeast strains was analyzed as described in Materials and Methods.

complex disruptor (Fig. 1A). The yeast assay strain transformed with HDAC1 and MTA1 expressing vectors expressed β-galactosidase as an indicator of interaction between HDAC1 and MTA1. Verification of the yeast assay strain was conducted using a known HDAC inhibitor as well as microbial metabolite libraries, and a candidate HDAC1 complex disruptor was successfully isolated from the microbial metabolites. These results demonstrate that the yeast assay strain developed in this study can provide an efficient screening method to monitor the protein-protein interaction that is crucial for the functionality and integrity of the HDAC1 complex in cells.

MATERIALS AND METHODS

Materials

The trichostatin A and cycloheximide were purchased from Sigma (St. Louis, MO, U.S.A.). The microbial metabolite libraries were prepared as previously reported [22] and kindly provided by Dr. Kim at KRIBB. All chemicals used in this study were the highest grade commercially available.

Yeast Strains and Plasmid Construction

The genotype of *Saccharomyces cerevisiae* EGY48[p8op-lacZ] is *MAT, his3, trp1, ura3, LexAop(x6)-LEU2*, and contains the p8op-lacZ reporter plasmid. The HDAC1 and MTA1 genes were amplified by a polymerase chain reaction (PCR) from Marathon ready human cDNA libraries (CLONTECH) using HDAC1 (5'-GCGCGGATCCATGG-CGCAGACGCAGGG-3' and 5'-GCGCTCGACTCAGG-CCAACTTGACCTC-3') and MTA1(5'-GCGCGGATCC-ATGGCCGCCAACATGTAC-3' and 5'-CGCGCTCGAG-CTAGTCCTCGATGACGATG-3') specific primers. The obtained PCR-amplified fragments were ligated into pLexA and pB42AD, two hybrid vectors, respectively. All the PCR-amplified cDNA products were confirmed by a sequencing analysis.

β-Galactosidase Assay

The yeast strain, EGY48[p8op-lacZ] expressing the LexA-HDAC1 and B42AD-MTA1 fusion proteins (strain EGY-HM), was grown overnight in a yeast synthetic medium (ura-, his-, trp-, 2% glucose). The cells were then transferred to a yeast induction medium (ura-, his-, trp-, 2% galactose, 1% raffinose) and incubated for 3 h at 30°C. An equal number of cells determined by absorbance at 600 nm were lysed in buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) by freezing and thawing in liquid nitrogen, then 200 μl of 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside (ONPG) was added to measure the β-galactosidase activity. When a yellow color appeared, the reaction was stopped with 500 μl of Na_2CO_3. The reaction mixture was centrifuged and absorbance measured at 420 nm. β-galactosidase unit equaled to 1,000× OD_{420}/(time×volume×OD_{600}).

Paper Disc Assay Using Yeast Assay Strain

The yeast assay strain EGY-HM transformed with both pLexA-HDAC1 and pB42AD-MTA1 was grown overnight at 30°C in a yeast induction agar medium (ura-, his-, trp-, 2% galactose, 1% raffinose), then this culture was inoculated into a medium containing 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside and 0.001% SDS at a final concentration of 0.1% v/v. Eight-mm filter paper discs (Avantec) soaked with various chemicals and microbial metabolites were placed on the agar plates after drying at room temperature. The plates were then incubated at 30°C

for 5 days and the diameters of the growth inhibitory zones around the microbial metabolite soaked papers were measured and recorded.

In vitro Binding Assay for HDAC1 and MTA1 Interaction

To generate [^{35}S]-labeled MTA1, pcDNA3-MTA1 was transcribed and translated with a TNT system (Promega) according to the manufacturer's instruction. BL21 cells transformed with GST-fused HDAC1 or GST plasmids were grown to the log phase and induced with isopropyl-thio-β-D-galactoside (IPTG) for 5 h. After sonication in PBST (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% Triton X-100, pH 7.3), the solublized proteins were recovered by centrifugation at 4°C, incubated with glutathione-agarose beads (Sigma) for 1 h at 4°C, and washed four times with PBST. For the *in vitro* binding assays, beads were mixed with the *in vitro* translated [^{35}S]-labeled MTA1 protein for 1 h at room temperature. The unbound proteins were washed with STE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 150 mM NaCl) containing 0.1% Nonidet P-40, while the bound proteins were eluted from the beads by boiling in SDS loading buffer (50 mM Tris-Cl, pH 6.8, 0.3 M β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). The final products were analyzed on 10% SDS-polyacrylamide gel and detected by autoradiography.

Animal Cell Culture

Murine melanoma B16/BL6 cells were grown in RPMI (Gibco BRL) medium supplemented with 10% fetal bovine serum (Gibco BRL), 100 units/ml penicillin, and 100 mg/ml streptomycin under 5% CO_2 atmosphere at 37°C in a humidified chamber.

RESULTS AND DISCUSSION

Construction of HDAC1- and MTA1-Expressing Yeast Two-Hybrid System

To develop a yeast assay strain expressing HDAC1 and MTA1, LexA DNA binding domain fused to HDAC1 and B42AD activation domains fused to MTA1 vectors were constructed. Since the assay strain EGY-HM contains two reporter genes, a *lacZ* reporter gene induced by 8 LexA binding sites and a *leu* gene induced by 6 LexA binding sites, the HDAC1 and MTA1 interaction could be quantified by β-galactosidase assay and cell growth tested by a leucine (leu)-depleted plate. In the plate assay, the yeast assay strain with LexA-HDAC1 and B42AD-MTA1, and the positive control strain bearing LexA-EPR123 and B42AD-hsp90, grew on the leu-depleted plate [9], however, the negative control yeast strain with LexA but without HDAC1 and B42AD-MTA1, or the LexA-HDAC1 and B42AD but without MTA1, showed no growth (Fig. 1B).

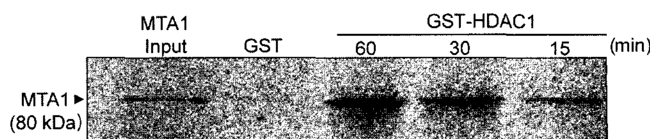

Fig. 2. Physical interaction of HDAC1 and MTA1 *in vitro*.
Overexpressed GST alone (GST) or GST fused with HDAC1 (GST-HDAC1) proteins in *E. coli* BL21 were purified and immobilized on glutathione-agarose beads. The bead-bound GST or GST-HDAC1 proteins were mixed with *in vitro* translated [^{35}S]-labeled MTA1 (MTA1 input) for different incubation times as described in Materials and Methods. After pull down of the protein with glutathione-agarose beads, bound proteins were subjected to SDS-polyacrylamide gel (10%) and autoradiography. The interaction of the HDAC1 and MTA1 proteins increased time-dependently.

In addition, the β-galactosidase activity increased 3.5-fold in the LexA-HDAC1 and B42AD-MTA1 vector expressing yeast assay strain when compared with LexA alone (Fig. 1C). These results clearly demonstrate that HDAC1 interacted with MTA1 in the yeast two-hybrid system. Moreover, this system can be used as an assay strain for screening an HDAC1-MTA1 interaction disruptor based on the inhibitory activity of the reporter phenotypes.

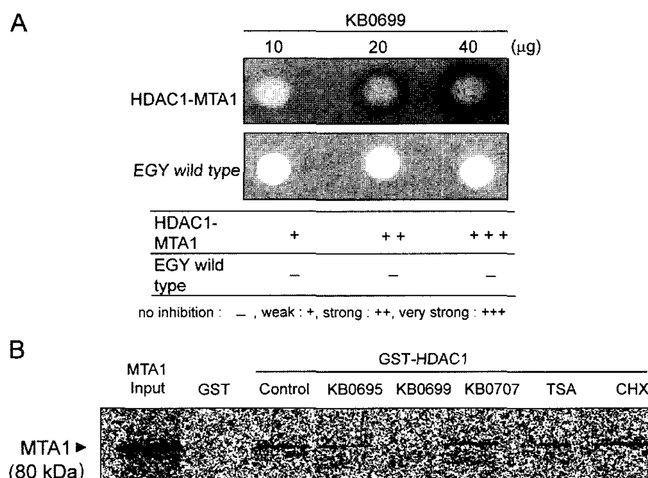

Fig. 3. Validation of yeast assay strain as screening system for HDAC1 and MTA1 interaction disruptor.
(A) Among 3,500 microbial metabolite libraries tested, KB0699 inhibited the HDAC1 and MTA1 interaction without any toxicity to the wild-type yeast. Yeast reporter strains that expresses LexA fused with HDAC1 and B42 fused with MTA1 were grown on leu-depleted and X-gal containing induction agar plate with each library soaked paper discs at 30°C. Growth inhibition around the paper disk soaked with KB0699 was compared between wild-type yeast strain and the reporter strain EGY-HM. (B) *In vitro* GST pull down assay with several microbial extracts and inhibitors. GST-HDAC1 bound glutathione-agarose beads were treated without an agent (control) and with 40 μg/ml of microbial metabolite sample, KB0695, KB0699, and KB0707, 2 μg/ml of trichostatin A (TSA), and 2 μg/ml of cycloheximide (CHX) prior to mixing *in vitro* translated [^{35}S]-labeled MTA1 (MTA1 input). Two proteins were incubated for 1 h at room temperature. The inhibitory effect of microbial metabolites on the interaction was determined by SDS-polyacrylamide gel (10%) electrophoresis and autoradiography.

HDAC1 Interacts with MTA1 *In vitro*

Although it is known that the HDAC1 complex contains MTA1 in its immunoprecipitated complex [29], there has been no report on any direct interaction between HDAC1 and MTA1 *in vitro*. Therefore, to verify direct interaction between HDAC1 and MTA1, an *in vitro* GST pull down assay was carried out. Thus, the GST or GST-HDAC1 protein induced in *E. coli* was purified and mixed with *in vitro* translated [^{35}S] methionine-labeled MTA1 in a rabbit reticulocyte lysate. As shown in Fig. 2, GST-HDAC1 and MTA1 interaction was detected 15 min after the start of the reaction and the intensity of the interaction between the two proteins increased time-dependently. This result demonstrates that HDAC1 physically interacts with MTA1 *in vitro*.

Validation of Yeast Assay Strain as Screening System for HDAC1 and MTA1 Interaction Disruptor

HDAC1 and MTA1 are highly expressed in many cancer cell lines [23, 30, 33]. HDAC1 usually coordinates with other proteins to form multiprotein complexes for its function. Therefore, the interruption of HDAC1 complex formation may have an effect on the function of HDAC *in vivo*. To date, several HDAC inhibitors, such as trichostatin A [35, 36], apicidin [2, 13], depudecin [16], trapoxin B [7, 35], MS-275 [25], and FK228 [15, 21] have been developed as competitive inhibitors of the enzyme. However, there has been no report on an inhibitor that can disrupt the HDAC1 complex without directly inhibiting the catalytic activity of HDAC1. In this sense, the yeast assay strain developed in the current study will be a useful tool to obtain a novel type HDAC function inhibitor that targets the HDAC complex. To verify this possibility, the effect of a known HDAC inhibitor, trichostatin A (TSA), and microbial metabolite libraries on the yeast assay strain was investigated.

Interestingly, TSA, an HDAC inhibitor, and cycloheximide, a protein synthesis inhibitor, showed no activity such as the inhibition of the growth and β-galacosidase expression of the yeast assay strain in a leu-depleted and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) induction plate (data not shown). These results suggested that neither the

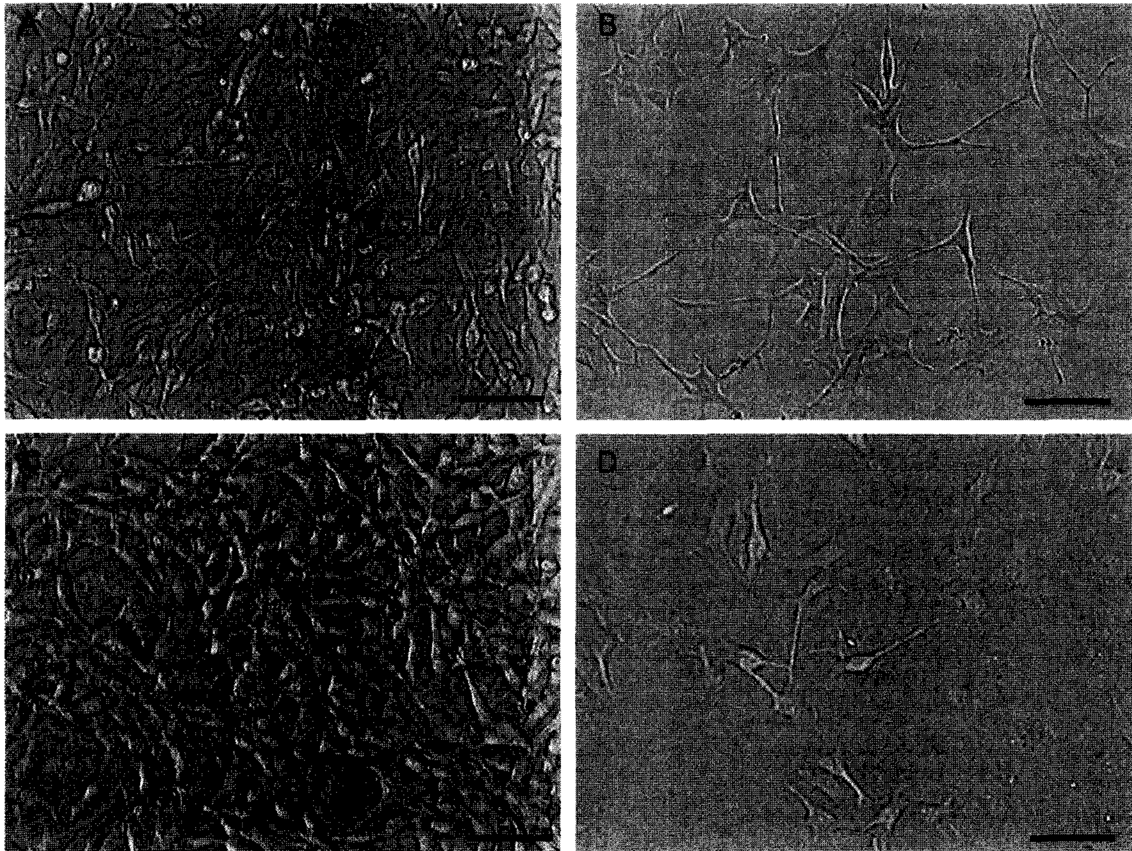

Fig. 4. Induction of morphological change in B16/BL6 melanoma cells by KB0699.

B16/BL6 melanoma cells were treated with 5 μg/ml of KB0699 and KB0707, and 0.2 μg/ml of TSA for 24 h, then the cell morphology was examined under a light microscope (Olympus, 100× magnification). Compared with the control or KB0695-treated cells, the morphology of the KB0699-treated cells was similar to that of the TSA-treated cells. (A) control, (B) TSA, (C) KB0707, (D) KB0699 (Bar=10 μm).

inhibition of HDAC activity nor protein synthesis affected the HDAC1 and MTA1 interaction. Next, the effect of microbial metabolites libraries was investigated, since these libraries have been used as diverse chemical library sources. Among 3,500 microbial metabolite libraries, three candidates, including the fungus KB0699, inhibited the growth and β-galactosidase expression of the yeast assay strain without any toxic effect on the wild EGY48[p8op-laz] strain, implying a specific inhibitory activity by the candidates on the HDAC1-MTA1 interaction (Fig. 3A). Thereafter, it was examined whether a KB0699 extract could inhibit the physical interaction of HDAC1 and MTA1 *in vitro*. As shown in Fig. 3B, the KB0699 extract efficiently disrupted the HDAC1 and MTA1 interaction *in vitro*. In contrast, negative control microbial metabolites, KB0695 and KB0707, as well as TSA and cycloheximide exhibited no activity on the HDAC1 and MTA1 interaction. These results demonstrate that the yeast assay strain expressing LexA-HDAC1 and B42AD-MTA1 can be an efficient assay system to screen cell permeable disruptors of the HDAC1 and MTA1 interaction.

Effect of KB0699 on Morphology of B16/BL6 Melanoma Cells

HDAC inhibitors are known to detransform the morphology of tumor cells into that of normal ones by inhibiting the HDAC activity in the cells [13, 16]. Since the interruption of HDAC1 and MTA1 interaction causes the inactivation of the HDAC function, an HDAC1 complex disruptor may affect the enzyme activity as well as the morphology of the cells, similar to HDAC inhibitors. Thus, the effect of KB0699, a candidate HDAC1 complex disruptor, on the morphology of B16/BL6 melanoma cells was investigated. Interestingly, B16/BL6 cells treated with KB0699 for 24 h showed dramatic changes in their morphology with elongated protrusions, similar to the changes in morphology induced by TSA. However, there were no significant changes observed with KB0707, which showed no activity both in an *in vivo* yeast assay strain and an *in vitro* binding assay. In addition, the HDAC activity in the KB0699-treated cells was significantly inhibited, whereas only a negligible inhibition was observed with KB0699 in an HDAC assay *in vitro* (data not shown). These results imply that an HDAC1 complex disruptor isolated from the yeast assay strain can affect the biological function of the HDAC1 complex in cells.

In conclusion, the current study reported on a new yeast cell-based assay strain that can monitor the interaction of HDAC1 with MTA1, one of the crucial protein-protein interactions in the HDAC1 complex. This assay strain can be utilized for the development of novel cell permeable HDAC inhibitors to refine the cellular function of the HDAC complex in gene expression.

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