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Reporter gene stimulation by MIDA1 through its DnaJ homology region☆

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Abstract

MIDA1 was reported as a protein that can associate with Id1. Its N-terminus has homology to Z-DNA binding protein, Zuotin, that contains DnaJ motif, considered to interact with Hsp70s, and Id binding domain. In the present study, we found that MIDA1 stimulates the transcription of the co-transfected genes. This stimulation was independent of promoter specificity because it was observed in various transfected genes. MIDA1 enhanced formation of DNA–protein complexes with E-box or TATA box without its direct binding to DNA. Analysis with deletion mutants of MIDA1 showed that the short protein fragment containing DnaJ motif within Zuotin homology region is sufficient for the stimulation of transcription and we demonstrated that MIDA1 associates with Hsp70. These data suggest involvement of MIDA1 in the stimulation of transcription in concert with Hsp70/Hsc70 molecular chaperones, thus providing a link between Hsp70/Hsc70 molecular chaperones and components of the transcriptional machinery.

Keywords: MIDA1; Zuotin; Transcription; Hsp70; DnaJ

Murine Id associated 1 (MIDA1) was first reported as a protein that can associate with Id proteins [1], which are known to lack a basic region of the DNA-binding domain and to sequester transcription factor protein targets into Id-heterodimers that are unable to bind DNA and negatively regulate differentiation [2,3]. Id proteins has been shown to function as positive regulators of cell growth, and to drive apoptosis and immortalization under appropriate physiological conditions [4–6], and MIDA1 was shown to be involved at least in growth control with a probable role in DNA synthesis [1]. In addition to its important function, MIDA1 is structurally intriguing protein. Its N-terminus showed homology to Zuotin, a Z-DNA binding protein in yeast [7]. MIDA1 can associate in vitro with the HLH region of Id1 through its conserved region adjacent to a eukaryotic DnaJ motif within the Zuotin homology region [1]. Its C-terminal domain contains c-Myb-like repeats and this region showed a sequence-specific DNA binding activity [8]. Thus, MIDA1 is a new type of DNA binding protein in exhibiting two distinct DNA binding activities; a Z-DNA binding activity and a sequence-specific DNA binding activity [9].

☆ *Abbreviations:* aa, amino acid; CMV, cytomegalovirus; DTT, dithiothreitol; EDTA, disodium dihydrogen ethylenediamine tetra-acetate; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid; HLH, helix–loop–helix; NP-40, nonidet P-40; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SANT, Swi3, Ada2, N-CoR,TFIIIB; SDS, sodium dodecyl sulfate; SV40, simian virus 40; TBE, Tris–borate EDTA buffer.

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In addition, c-Myb-like repeats of the C-terminal domain are essentially similar to recently identified SANT domain [10]. The SANT domain is a novel motif found in a number of eukaryotic transcriptional regulatory proteins that was identified based on its homology to the DNA binding domain of c-Myb. The SANT domain is essential for the in vivo functions of yeast Swi3p, Ada2p, and Rsc8p, subunits of three distinct chromatin remodeling complexes [11], and is considered to catalytically support the action of histone acetyltransferase (HAT) or histone deacetylase (HDAC) in chromatin remodeling complexes [12,13]. This multi-domain structure of MIDA1 suggests that it acts as a multi-functional protein and may be involved in the transcriptional control through interaction with several factors.

During the survey of MIDA1 function, we noticed that overexpressed MIDA1 proteins stimulated transcription of the co-transfected genes. Thus, in the present study, we investigated a new intriguing function of MIDA1 and its transcriptional stimulatory mechanisms.

Materials and methods

Cell culture and transfection. 293T and 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator. For transient transfection, 50% confluent cells were transfected using the calcium phosphate procedure or FuGENE 6 (Roche).

Luciferase reporter constructs and expression constructs. In constructing E-box-luc plasmid, oligonucleotides containing 4 tandem repeats of the E-box (CACCTG) sequence were inserted upstream of the thymidine kinase minimal promoter of the pT81 plasmid previously [14]. Firefly luciferase reporter plasmid that contains CMV promoter was prepared by that *Renilla* luciferase gene of pRL-CMV vector (Promega) exchanged with firefly luciferase, and named pCMV-luc. PicaGene Promoter Vector (Toyo Ink) contains the SV40 promoter element upstream of firefly luciferase and described as pSV40-luc. MIDA1 cDNA was subcloned into expression vector pRc/CMV (Invitrogen) at the *Not*I site to construct MIDA1 expression vector (MIDA1/pRc/CMV). Expression vector encoding E47 (E47/pcDNA3) has been described previously [15]. FLAG-tagged MIDA1, deletion mutants, and hemagglutin (HA) epitope-tagged Hsp70 cDNA were subcloned into pcDNA3 (Invitrogen) in the *Not*I and *Xba*I sites using PCR-generated fragments.

Luciferase assays. Transfection as indicated was performed in 12-well plates. After 16 h cells were lysed and assayed for luciferase activity using the luciferase assay system (Promega).

Western blot analysis. 293T cells or stable transformants were lysed in 1% NP-40 lysis buffer (1% NP-40, 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor mixture (Roche)) and mixed with SDS sample buffer. Sample was loaded into 10% SDS–PAGE gel followed by Western blot analysis using anti-MIDA1 rabbit serum, anti-α-tubulin antibody (Sigma), and horseradish peroxidase conjugated secondary antibody (Zymed).

Gel shift analysis. 293T cells were scraped from 6-well plates at 16 h post-transfection and washed with PBS. The harvested cells were lysed on ice in 0.5% NP-40, 10 mM Hepes, pH 7.6, 20 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitor mixture for 10 min. For preparation of nuclear extract, the nuclei were isolated by centrifugation at 14,000 rpm 30 s and resuspended on ice in 20 mM Hepes, pH 7.6, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitor mixture for 15 min, followed by centrifugation at 14,000 rpm for 5 min. The "E-box (CACCTG)" and the "TATA box (TATAA)" probes were end-labeled with ^{32}P using 10 U of T4 polynucleotide kinase (Takara), 10× kinase buffer (Takara), and γ-labeled ^{32}P ATP by incubating at 37 °C for 1 h. Binding reaction contained 8 mM Hepes, pH 7.6, 2.5 mM Tris–HCl, pH 7.6, 50 mM NaCl, 10% glycerol, 1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT, 500 ng poly(dI–dC), and 20,000 cpm probe in 20 μl reaction volume and incubated at room temperature for 20 min. The reactions were resolved on 4% polyacrylamide gels in 0.5× TBE buffer for 2 h at 8 V/cm. The gel was then dried onto Whatman paper, and imaging was performed by autoradiography of the dried gel.

Stable cell line. Co-transfection of 5 μg pCMV-luc with 0.5 μg pBABE/puro (puromycin resistant gene expression vector) into 293 cells was performed in 60 mm dishes by the calcium phosphate procedure. At 24 h post-transfection, cells were washed with PBS and then fresh medium was added. At 48 h post-transfection, 2 μg/ml puromycin was added in medium. After 2 weeks in selection medium, puromycin-resistant colonies were formed and evaluated by luciferase assay.

Immunoprecipitation. Five micrograms of HA-Hsp70/pcDNA3 and 5 μg of FLAG-MIDA1/pcDNA3 or mutant MIDA1 were transfected into 293T cells at 90 mm dishes. After 16 h cells were harvested and lysed at 4 °C for 30 min in 0.5% NP-40 lysis buffer (0.5% NP-40, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and protease inhibitor mixture). Insoluble cell debris were removed by centrifugation (15,000 rpm, 10 min), and the supernatants were mixed with anti-HA Agarose Conjugate (Sigma) that was swollen in 0.5% NP-40 lysis buffer supplemented with 1% BSA. The mixture was incubated at 4 °C for 2 h. Agarose was washed four times with 0.5% NP-40 lysis buffer. Bound proteins were eluted with SDS sample buffer by boiling and detected by Western blot using anti-FLAG antibody (Sigma).

Results

MIDA1 shows general stimulatory activity on the transcription of the transfected genes

During examination of MIDA1 action on the transcriptional suppression caused by Id, we found that addition of MIDA1 to E47 strongly enhanced transcriptional activity of E47, when a luciferase reporter plasmid with promoter region containing 4-tandem E-boxes was transfected in 293T cells in combination with the expression vector containing E47 and/or MIDA1 coding regions (Fig. 1). Overexpression of MIDA1 and E47 augmented luciferase activity about 6-fold more than E47 alone. Then, we examined whether this stimulation is specific for the E-box containing promoter. When the luciferase reporter plasmids containing CMV or SV40 promoter were co-transfected into 293T cells with the plasmid with or without MIDA1 coding region, dose-dependent enhancement of reporter gene expression by MIDA1was observed, regardless of kinds of promoters such as pCMV-luc and pSV40-luc. In both cases, the presence of MIDA1 enhanced the luciferase activity about 3-fold dependent on amounts of MIDA1 proteins (Figs. 2A and B). Other transfec-

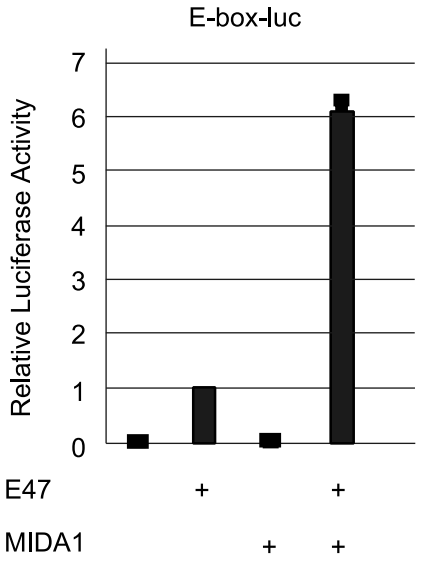

Fig. 1. Effect of MIDA1 on transcriptional stimulatory activities caused by E47. One hundred nanograms of E47/pcDNA3 (E47 expression plasmid), 1.6 μg MIDA1/pRc/CMV, and 250 ng E-box (4 tandem)-luc plasmid, as a reporter plasmid, were co-transfected into 293T cells. After 16 h cells were lysed and assayed for luciferase activity. Luciferase activity of the cells co-transfected with total 2 μg of the empty vector, E47/pcDNA3, and the reporter vector was arbitrarily set at 1, and the relative luciferase activity is shown in the figure. Error bars represent the standard deviation of the mean.

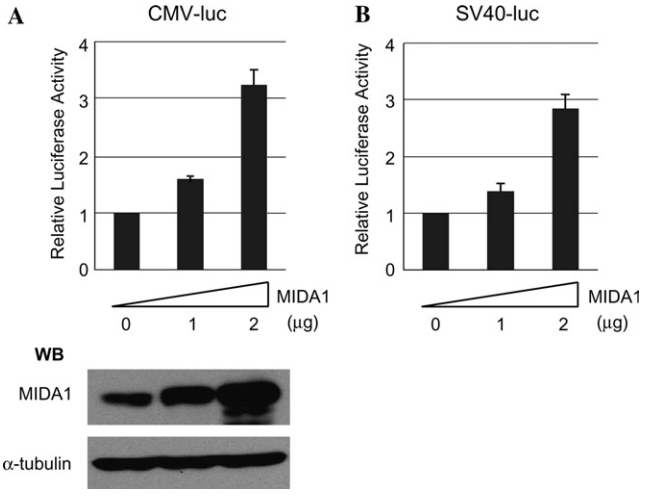

Fig. 2. MIDA1 up-regulates luciferase reporter gene expression in 293T cells. Total 2 μg of MIDA1/pRc/CMV plasmid and/or pRc/CMV plasmid (empty vector) was co-transfected into 293T cells with 30 ng pCMV-luc (A) or 50 ng pSV40-luc vector (B). After 16 h cells were lysed and assayed for luciferase activity. Shown is the relative luciferase activity. Luciferase activity of the cells co-transfected with 2 μg of the empty vector and the reporter vector was arbitrarily set at 1. Error bars represent the standard deviation of the mean. To confirm MIDA1 protein expression, Western blot analysis was performed using aliquot transiently transfected for luciferase assay (A, lower).

tion method using FuGENE6 as well as the calcium phosphate procedure indicated similar results (data not shown). Therefore, MIDA1 may have stimulatory

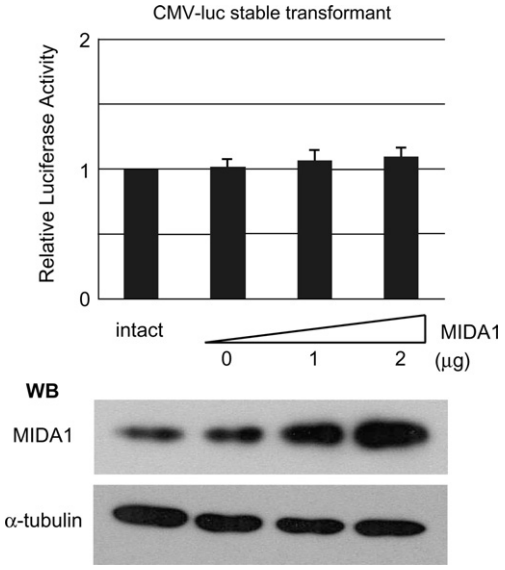

Fig. 3. MIDA1 does not enhance endogenous gene expression. Total 2 μg of MIDA1/pRc/CMV plasmid and/or pRc/CMV plasmid was transfected into 293 cells that stably express luciferase. After 16 h cells were lysed and assayed for luciferase activity. Luciferase activity of the intact stable transformant (leftmost bar) was set at 1. Error bars represent the standard deviation of the mean. In other three stable transformant clones, similar results were obtained. Western blot analysis was performed using aliquot assayed for luciferase activity (lower).

activity on the transcription of the transfected genes independent of promoter specificity.

Then, we examined whether transcriptional stimulation caused by MIDA1 is dependent on the transiently transfected genes. The CMV-luc plasmids were transfected into 293 cells and the stably transfected cells were clonally isolated by puromycin selection. The luciferase activity did not increase in the presence of MIDA1 expression in the stable transformants (Fig. 3), suggesting that MIDA1 stimulates the transcription of the transiently transfected genes, but not the endogenous genes.

MIDA1 stimulates formation of DNA–protein complexes

To elucidate how MIDA1 stimulated transcription of co-transfected genes, we examined the effect of MIDA1 on the formation of DNA–protein complexes using gel shift assay. Gel shift assay (Fig. 4) indicated that the levels of DNA–protein complexes formed on the E-box were elevated when MIDA1 was overexpressed in parallel with the transcriptional stimulation observed in Fig. 1. Then, we examined whether MIDA1 stimulated the binding of proteins to the core promoter containing TATA box (Fig. 5). Gel shift analysis showed that levels of DNA–protein complexes formed with TATA box, which were reduced by increasing amounts of the competitive DNA (lanes 5–7), were elevated in the MIDA1 overexpressed cells (lanes 2–4). These results suggest

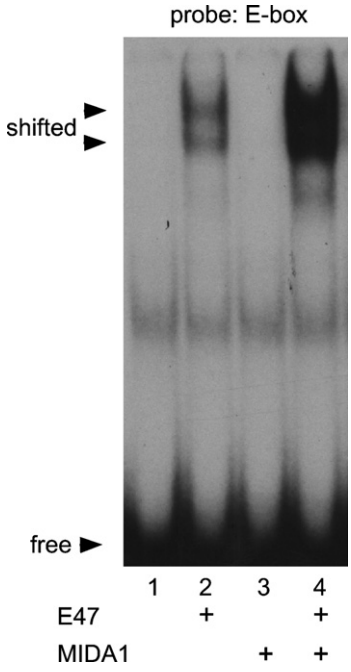

Fig. 4. MIDA1 affects binding of E47 to E-box. E47/pcDNA3 (1.6 μg) and MIDA1/pRc/CMV (6.4 μg) were co-transfected into 293T cells. After 16 h cells were collected and nuclear extracts were prepared. The ^{32}P labeled "E-box (CACCTG)" probe was reacted with these nuclear extracts and the reactants were subjected to gel shift analysis.

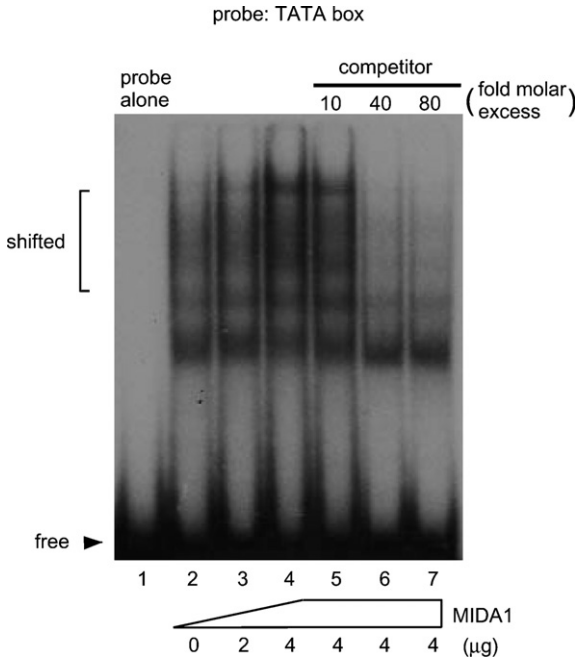

Fig. 5. MIDA1 affects TATA binding activity. Total 4 μg of MIDA1/pRc/CMV plasmid and/or the empty vector was transfected into 293T cells. After 16 h cells were collected and nuclear extracts were prepared. The ^{32}P labeled "TATA-box (TATAA)" probe was reacted with these nuclear extracts and the reactants were subjected to gel shift analysis. Competition analysis was performed by mixing the indicated amount of appropriate competitor DNA (lanes 5–7).

that MIDA1 may stimulate formation of DNA–protein complexes independent of promoter specificity.

The short protein fragment containing DnaJ motif within Zuotin homology region of MIDA1 is sufficient for the stimulation of transcription

Since MIDA1 has two distinct domains [1], we examined which domain is involved in the stimulation by using deletion mutants (Fig. 6A). The stimulation was observed with the N-terminal fragment (1–433 aa residues) containing Zuotin homology region, whereas the C-terminal fragment (434–621 aa residues) containing c-Myb-like repeat/SANT domain (Fig. 6B), which was shown to exhibit sequence specific DNA binding activity in vitro [8], did not show any stimulation. This suggests that Zuotin homology region is necessary and sufficient for its stimulation.

Expression of Id protein did not affect stimulatory activity of Zuotin homology region (data not shown), thus, Id binding region adjacent to DnaJ motif may not be necessary for this stimulatory activity. In fact, the N-terminal shortest fragment (1–187 aa residues) deleting Id binding site, but containing DnaJ motif, still retains stimulation activity (Fig. 6B). Thus, DnaJ motif may be essential and sufficient for the stimulation of transcription.

MIDA1 associates with Hsp70

Since DnaJ motif of MIDA1 is considered to be essential for stimulation of transcription and Hsp40/DnaJ protein family was reported to correlate with Hsp70/DnaK in many species, and recognized to function as chaperone [16,17], we examined whether MIDA1 can associate with Hsp70 (Fig. 7). We demonstrated that Zuotin homology region containing DnaJ motif can associate with Hsp70 (lane 3) and the association was diminished when DnaJ motif region (84–163 aa residues) was deleted (lane 4). This suggests that Hsp70 molecular chaperone may be involved in the transcriptional stimulation by MIDA1.

Discussion

MIDA1 was first reported as a protein that can associate with Id1 both in vitro and in vivo [1]. Its predicted amino acid sequence at the N-terminus shows homology to yeast Zuotin that is recognized to bind Z-DNA [7] and function as a chaperone to associate with ribosome [18,19]. The Zuotin homology region includes DnaJ motif, considered to interact with Hsp70s [16,17], and Id binding domain [1]. MIDA1 was shown to have Z-DNA binding activity, which was altered by the association with Id [9]. Additionally, its C-terminal domain

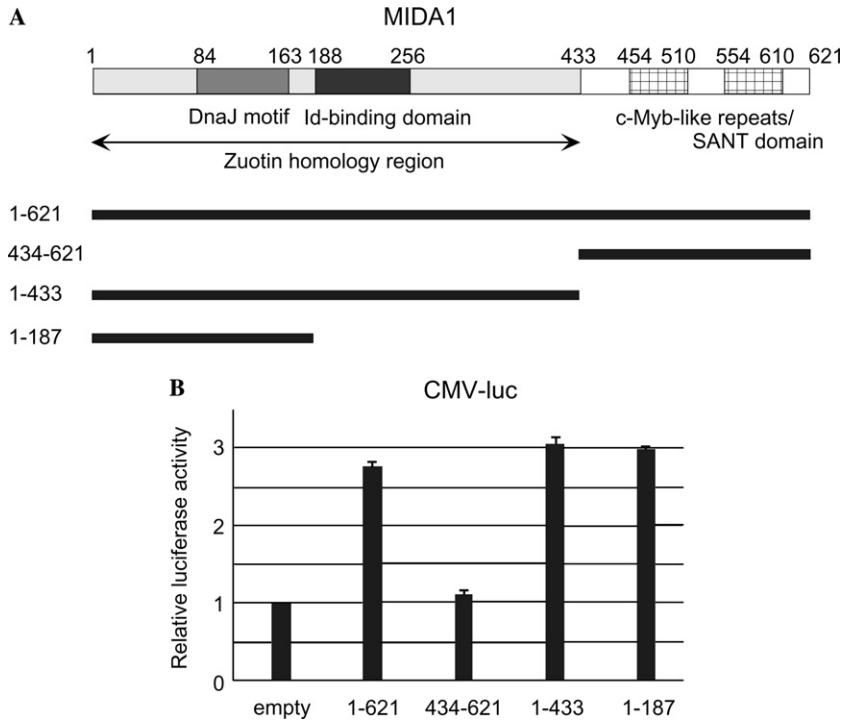

Fig. 6. Mutational analysis of transcriptional stimulatory activity of MIDA1. (A) Schematic representation of FLAG-tagged wild type (1–621) and deletion mutant MIDA1. (B) Luciferase assays. Two micrograms of FLAG-tagged wild type or mutant MIDA1 expression vector was co-transfected with 30 ng pCMV-luc vector into 293T cells followed by luciferase assays as described in the legend to Fig. 2.

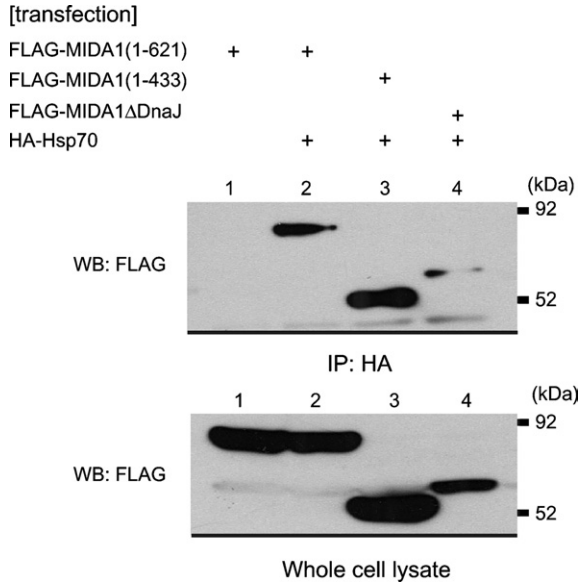

Fig. 7. MIDA1 interacts with Hsp70. Five micrograms of HA-Hsp70/pcDNA3 and 5 μg of FLAG-MIDA1/pcDNA3 or mutant MIDA1 were co-transfected into 293T cells. After 16 h cells were harvested and lysed. The cell lysates were mixed with anti-HA agarose for immunoprecipitation. Agarose binding proteins (upper) and whole cell lysate (lower) were detected by Western blot analysis using anti-FLAG antibody.

contains c-Myb-like repeats/SANT domain and we previously demonstrated that this region showed a sequence-specific DNA binding activity [8].

In the present study, we found that MIDA1 stimulated gene expression from co-transfected reporter gene. The C-terminal domain contains c-Myb-like repeats/SANT domain, which is considered to catalytically support the action of HAT or HDAC in chromatin remodeling complexes [12,13]. Thus, we expected that SANT domain may be involved in the stimulation, however, to our surprise, we found that MIDA1 mutant deleting the C-terminal domain still retained the transcriptional stimulatory activity (Fig. 6). In addition, we demonstrated by chromatin immunoprecipitation assay that localization of the acetylated histones on the transiently expressed gene chromatins was not changed by overexpression of MIDA1 (data not shown). These results indicate that C-terminal domain contains c-Myb-like repeats/SANT domain may not be involved in the stimulation observed in the present study.

We found that overexpression of MIDA1 stimulated expression of the transient transfected genes independent of promoter specificity (Fig. 2), but did not stimulate the endogenous genes (Fig. 3). Similar reporter gene stimulation by HMG box proteins was reported by Veilleux and Boissonneault [20]. Using transient transfection to overexpress two different members of the HMGB-1/2 family of DNA architectural factors, they demonstrated that these proteins provide a general enhancement in reporter gene expression irrespective of the promoter and suggested that the architectural role of these proteins can contribute to the preinitiation

complex assembly required for basal transcription. Similar to their observations in HMG box proteins, our observations suggest that MIDA1 may affect formation of the preinitiation complex assembly required for basal transcription.

Deletion mutant analysis shows that this stimulation is dependent on Zuotin homology region (Fig. 6). Zuotin homology region contains the region required for Id binding, but the stimulation was not affected by association with Id, and was dependent on the short protein fragment containing DnaJ motif. We expect that the functional domains of MIDA1 for the transcriptional stimulation and for the interaction with Hsp70 chaperone are co-localized within the short region containing DnaJ motif.

Hsp40/DnaJ protein family has been reported to correlate with Hsp70/DnaK in many species and recognized to function as a chaperone [16,17]. Because DnaJ motif of MIDA1 may be essential domain in the transcriptional stimulation, we examined whether MIDA1 interacts with Hsp70. The interaction of MIDA1 with Hsp70 was confirmed by immunoprecipitation (Fig. 7) and these data are supported by the study about *Drosophila* protein interaction map using two-hybrid system in which *Drosophila* MIDA1 homologue (CG10565) can bind with Hsp70 (CG7182) [21]. Contrary to this direct association, overexpression of Hsp70 did not enhance transcriptional stimulation by MIDA1 when we measured the reporter luciferase activity in the presence of Hsp70 and MIDA1 (data not shown). This may be simply because of abundance of Hsp70 proteins in the cells.

In this respect, recently reported function of Hap46/BAG-1M, the Hsp70/Hsc70-associating protein Hap46, also called BAG-1M (Bcl-2-associated athanogene 1), is interesting because it stimulates transcription in similar fashion [22,23]. Hap46-mediated transcriptional activation occurs through non-specific DNA binding and the C-terminal deletion variant Hap46/BAG-1M, which is unable to associate with Hsp70/Hsc70 molecular chaperones, produced greatly diminished effects on transcription, indicating a significant involvement of Hsp70/Hsc70 chaperones [22]. These data point to involvement of Hsp70/Hsc70 molecular chaperones in transcription in concert with Hap46/BAG-1M, thus providing a link between Hsp70/Hsc70 molecular chaperones and components of the transcriptional machinery. Hap46/BAG-1M contains two domains; N-terminal sequences of Hap46/BAG-1M required for binding to DNA [24,25] and the C-terminal portion involved in interactions with Hsp70s [25–27] and it can act as a molecular link between such divergent components as DNA and Hsp70/Hsc70 molecular chaperones.

In contrast, the domain for stimulation of transcription in MIDA1 is localized within the short protein fragment containing DnaJ motif (Fig. 6). Since we previously demonstrated that entire Zuotin homology region may be required for Z-DNA binding activity [1], this short fragment (1–187 aa residues) may not have DNA binding activity, thus, MIDA1 may stimulate transcription of the co-transfected genes without its direct binding to DNA. In addition, we showed that MIDA1 stimulated formation of the DNA–protein complexes with E-box (Fig. 4) or TATA box (Fig. 5), but it was not included in the DNA–protein complexes with E-box or TATA box because the size of shifted bands was the same despite MIDA1 protein overexpression (Fig. 4, lanes 2 and 4, Fig. 5, lanes 2–4). Thus, MIDA1 may stimulate transcription independent of DNA binding activity through the short N-terminal region containing DnaJ motif that associates with Hsp70/Hsc70 molecular chaperones.

Both MIDA1 and Hap46/BAG-1M show the association with Hsp70/Hsc70 molecular chaperones, although their interactive domains with Hsp70 are structurally different. MIDA1 stimulated transcription independent of DNA binding activity, whereas Hap46/BAG-1M required its DNA binding activity for stimulation [24]. Thus, both proteins may be contained with Hsp70-related transcriptional mechanisms, but their interactive molecules may be different except Hsp70.

Our present study highlights new role of DnaJ motif within MIDA1 protein for transcriptional activation. Although transcriptional activation observed in this assay required only short protein fragment containing DnaJ motif of MIDA1, MIDA1 may act as a multifunctional protein through binding to either Z-DNA or the sequence-specific DNA [8,9] and through interaction with Id [1,9] that may be required for regulation of formation of the transcriptional machinery.

Acknowledgments

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