MIDA1, a Protein Associated with Id, Regulates Cell Growth*

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We have isolated cDNA clone encoding a protein that can associate with Id, a helix-loop-helix (HLH) protein. This protein is named MIDA1 (mouse Id associate 1), and its predicted amino acid sequence consists of Zuotin (a putative Z-DNA binding protein in yeast) homology region and tryptophan-mediated repeats similar to c-Myb oncprotein. MIDA1 associates with the HLH region of Id with the conserved region adjacent to eukaryotic DnaJ conserved motif within the Zuotin homology region, although it does not have any canonical HLH motif. The addition of antisense oligonucleotide of MIDA1 inhibited growth of murine erythroleukemia cells without interfering with erythroid differentiation, indicating that it regulates cell growth.

Id is a member of helix-loop-helix (HLH) proteins that play an important role in cell type-specific transcription and cell lineage commitment (1) by forming DNA-binding heterodimers such as MyoD and E2A (E12/E47). It lacks a basic region and negatively regulates other basic helix-loop-helix (bHLH) proteins by forming heterodimers that cannot bind DNA. Id gene was isolated first in murine erythroleukemia (MEL) cells (2) that could be induced to differentiate toward erythrocyte. We previously reported that Id mRNA decreased soon after induction of differentiation of MEL cells with Me2SO, and it was inhibited by the overexpression of Id gene (3). Overexpression of Id was shown to inhibit induction of differentiation in other cell systems (4–6), indicating that the inhibitory function is involved in differentiation of various cell lineages.

In addition to the regulation of differentiation, Id was shown to act as a growth regulator. HLH462 referred as Id3 shows immediate early response to serum stimulation (7), and loss of Id gene expression inhibits cell cycle induced by serum stimulation (8). Furthermore, Id2 is reported to antagonize RB protein that maintains the cells in G0/G1 phase of the cell cycle (9). Thus, Id may function as a regulator of both growth and differentiation of the cells.

In the present study, we searched the proteins that associate with Id by West-Western screening (3) to know how Id functions in the commitment of growth and differentiation of MEL cells. From an expression cDNA library constructed from mRNAs of MEL cells, we obtained a new protein that can bind Id without having HLH motif.

MATERIALS AND METHODS

West-Western Screening—The λgt11 library was plated in the Y1090 bacterial strain. After incubation at 42 °C for 3 h, 25 ml isopropyl-1-thio-β-D-galactopyranoside-impregnated nitrocellulose filters (Schleicher & Schuell) were overlaid on the plates to induce β-galactosidase fusion protein. To get good yield of protein production and transfer, the plates were incubated for 8 h at 37 °C. Filters were marked, rinsed with TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) to remove bacterial debris, and treated successively with 6, 3, and 0 m guanidine-HCl (HBB buffer 20 mM Hepes, pH 7.5, 5 mM MgCl2, 1 mM KCl, 5 mM dithiothreitol) at 4 °C for denaturation-renaturation of transferred protein. Filters were blocked with 5% skim milk/HBB buffer and incubated with 0.2 μg/ml of bacterially produced glutathione S-transferase (GST)-Id protein (3) in 1% skim milk/HBB buffer for 8 h at room temperature. Id lacks the 49 N-terminal amino acids of Id to promote solubility in bacterial lysate and contains a complete HLH domain. Then filters were washed 4 times with PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH2PO4·H2O, 1.4 mM KH2PO4, pH 7.3, 0.2% Triton X-100), and positive plaques were immunologically detected by successive treatment with anti-GST antibody (3), horseradish peroxidase-anti rabbit IgG (Zymed Laboratory), and ECL Western blotting detection reagents (Amersham Corp.).

In Vitro Association Studies—In vitro transcription and translation were performed under conditions recommended by the Promega Protocols and Application Guide. 35S-Labeled proteins were produced in the lysate from pCITE2 vectors (Novagen) that have each cDNA inserts. To check the translation products, 1 μl from each lysate was subjected directly to SDS-polyacrylamide gel electrophoresis, and 5 μl was diluted into 500 μl of HBB buffer and incubated with either GST or GST-ΔId affinity matrices in which approximately 5 μg of fusion protein adsorbed to 10 μl of glutathione-Sepharose beads (Pharmacia Biotech Inc.) for 1 h at 4 °C. The beads were washed 4 times with PBST at room temperature, and the bounded proteins were eluted with SDS-containing sample buffer, followed by SDS-PAGE and autoradiography.

In Vivo Association Studies—GST-Id (full length) cDNA was inserted under β-actin promoter and was transfected into MEL cells. One of the stable transfectants, which constitutively express GST-Id protein and parental MEL cells, were used for in vivo association studies. 1 × 108 cells were washed with phosphate-buffered saline (PBS) and incubated at room temperature for 30 min with a cross-linking agent dithiobis(succinimidyl propionate) (Pierce) at a final concentration of 2.5 mM in PBS. The cross-linking reaction was terminated by the addition of one-tenth volume of 1 mM Tris-HCl, pH 7.5, and cells were lysed by gently suspending with 5 ml of TBS-MgCl2 buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 15 mM MgCl2) containing 1% Triton X-100 and proteinase inhibitors (5 μg/ml antipine, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The cleared lysate was incubated with 100 μl of glutathione-Sepharose beads (Pharmacia) for 1 h at 4 °C, and GST-Id and its associated proteins were precipitated with beads. After 4 times washing with TBS-MgCl2 buffer, the bound proteins were eluted with SDS-containing sample buffer. In the presence of 5% β-mercaptoethanol, S-S bonds of cross-linked protein complex were cleaved, and liberated proteins were separated in SDS-PAGE. MIDA1 was detected with Western blotting using anti-MIDA1 antibody, which was obtained by immunizing bacterially produced GST-MIDA1 (residues 446–621) fusion protein. With this antiseraum, MIDA1 was detected specifically as a single band of 74 kDa (data not shown).

Northern Blotting—Total RNAs from tissues and cultured cells were prepared by guanidinium thiocyanate-phenol chloroform procedure (10). 10 μg of RNA from each sample was electrophoresed in a dena-
**Results**

Screening of Id-associated Proteins—To screen the Id-associated proteins, we employed West-Western strategy, by which several biologically interactive proteins have been identified (11, 12). We constructed the chimeric gene GST-Id (Fig. 1a) and expressed it in Schistosoma japonicum glutathione S-transferase (GST) (11, 12) (Fig. 1a) fused with the residues 78–176 of mouse Id that contains the HLH domain. b. heterodimer formation of GST-Id with MyoD. Left, in vitro translocated MyoD (lane 1) and c-Myc (lane 2) proteins. Right, in vitro translocated proteins were mixed with GST-Id and anti-GST antibody for 1 h at 4°C, and then the formed complexes were recovered by protein A-Sepharose. Myc protein (lane 4) was used as a negative control.

While MIDA1 had several interesting features in its protein structure as described above, we couldn’t find any canonical HLH motif in this protein. To search the binding domain of MIDA1 to Id, a series of deletion clones were constructed, and their RNAs were synthesized. [35S]methionine-labeled proteins from the RNAs were translated in the rabbit reticulocyte lysate system (15–17) and asked whether they could be recovered by association with GST or GST-Id affinity matrices (18). As shown in Fig. 3b, wild-type, Δ2–71, and Δ2–188 of MIDA1 could associate with Id, but Δ2–256 and Δ2–342 could not. These results indicated that the residues 189–256, a part of the Zuotin homology region, is a responsible domain for association with Id. Interestingly, Id-binding activity of Δ2–188 was stronger than that of wild-type or Δ2–71, thus the residues 2–189 may regulate Id-binding activity of MIDA1. Then we examined whether two Trp-mediated repeats affect Id binding activity of MIDA1. Clone Δ2–188 was deleted from C terminus to lack one (Δ2–188, Δ326–621) or two (Δ2–188, Δ443–621) Trp-mediated repeats and employed in the same experiment (Fig. 3c). The results showed that deletion in the Trp-mediated repeats did not affect its binding activity to Id (Fig. 3d).

Then we asked whether the HLH region of Id is essential for association with MIDA1. Binding assay with GST-Id and GST-ΔHLHId, which completely deletes its HLH domain, showed that GST-ΔHLHId could not associate with MIDA1 (Fig. 4). Thus, it is concluded that the binding domain of Id is located in its HLH region.

**MIDA1 Associates with Id in Transfected MEL Cells**—To examine whether MIDA1 associates with Id in vivo, MEL cells were transfected with the cDNA for GST-Id fusion protein, and the stable transfecants were obtained. The transfecants and the parent cells were cross-linked in vivo, using dithiothreitol (sucinimidyl propionate) (9, 19). In this procedure, we can avoid detecting reconstructed associations different from nature ones and also avoid missing unstable or transient ones by their nature. Then, the GST-Id fusion protein and its cross-linked proteins were recovered by affinity to glutathione-Sepharose. The proteins liberated from the cross-linked complex with GST-Id were successively separated by SDS-PAGE and sub-
Fig. 2. Structural features of MIDA1. a, nucleotide sequence of MIDA1 cDNA. The nucleic acid residues are numbered from the beginning of the longest cDNA insert, and amino acid residues are numbered from the beginning of the long open reading frame. Underlined residues are homologous region to Zuotin (light underline) (13), eukaryotic DnaJ conserved protein motif (dark underline) (14), Trp-mediated repeat similar to c-Myb(15–17) (box underline), b, sequence comparison of MIDA1 and Zuotin. Conserved amino acids are marked by asterisks (matched) and closed circle (related). 157/433 (36.3%) residues are identical, and 216/433 (49.9%) are closely related. c, sequence of J region from MIDA1, E. coli, DnaJ, and other eukaryotic members of DnaJ family. Conserved amino acids are reversed (14). d, sequence alignment of Trp-mediated repeats from MIDA1 and c-Myb proteins from various species. Conserved amino acids are reversed.
jected to Western blotting with the anti-MIDA1 serum made for the bacterially produced protein. As shown in Fig. 5, MIDA1 proteins, which can be detected as a 74-kDa single band, were recovered from GST-Id transfected cells but not from parent MEL cells. These results strongly suggest that MIDA1 associates with Id in vivo.

Expression of MIDA1—The levels of MIDA1 expression was measured in MEL cells, and various mouse tissues by Northern blot analysis (Fig. 6). A single 2.2-kilobase pairs signal was detected by the MIDA1 cDNA probe. The highest level was observed in undifferentiated MEL cells, but it dropped after induction of differentiation, which was correlated with that of Id mRNA (2, 3). In mouse tissues, relatively high expression was observed in spleens and testes, where population of continuously proliferating cells were abundant.

Effect of MIDA1 Antisense Oligonucleotides in MEL Cells—To know the function of MIDA1 during MEL cell differentiation, we examined the effect of the antisense oligonucleotide of MIDA1. Six-base antisense or sense (control) oligomers from translation initiation site were added to the culture. During induced differentiation of MEL cells with Me2SO, addition of the antisense oligomer (20 \( \mu \)M) as well as the sense oligomer did not show any effect on the erythroid differentiation (Fig. 7a). However, quite interestingly, the antisense oligomer, but not the sense oligomer, strongly inhibited the growth of MEL cells when the cell number was monitored for 4 days at daily intervals in the uninduced culture (Fig. 7b). The levels of MIDA1 proteins monitored by Western blotting with the anti-MIDA1 serum were dropped in the presence of the antisense oligomer (Fig. 7c). We can hardly find any dead cells with trypan blue staining in all samples, so the addition of the antisense oligomer may affect cell cycle at a certain point. Flow cytometric analysis of the DNA content showed accumulation of S phase cells (48.3–53.2%) and decrease in G0 + M phase cells (15.4–8.6%) (Fig. 8a). On the other hand, the BrdU incorporation analysis revealed strong decrease in DNA synthesizing cells (35.5–2.4%) (Fig. 8b). Thus, reduction of MIDA1 blocked the progressing DNA synthesis.

**DISCUSSION**

It had been reported that a HLH protein, Id, controls cell differentiation in several cell lineages through interacting with bHLH transcription factors such as MyoD and E2A (3–6). In addition, the role of HLH proteins for growth regulation through interacting with non-HLH proteins such as c-jun and RB has recently been reported (9, 20, 21). In this work, we have isolated a new protein, MIDA1, that associates with Id by West-Western screening of the MEL cell cDNA library. The Id-associated protein is expected to have HLH motif, but we could not find any canonical HLH motif in MIDA1, even within the Id-associated domain of MIDA1 identified by in vitro association analysis.

The predicted amino acid sequence of MIDA1, however, revealed several interesting features; almost two-thirds of the N terminus closely resembled Zuotin, isolated as a Z-DNA binding protein in yeast (13) (Fig. 2b). Z-DNA is a left-handed DNA conformation that is suggested to implicate in transcription, replication, and recombination of DNA (22–24). It was attractive to expect that MIDA1 may regulate these cellular events by affecting DNA conformation, so we tried to examine Z-DNA binding ability of the recombinant MIDA1 protein by electrophoretic mobility shift analysis. Using \(^{32}\)P-labeled poly(dG-m5dC) probe as stabilized Z-DNA, significant retardation was observed by MIDA1 protein, but Z-DNA-specific competition has not been obtained (2). Thus, the Z-DNA binding ability of MIDA1 remained to be clarified by further improvement of experimental conditions or sample preparation.

Zuotin showed overall homology with N terminus of MIDA1, but two conserved regions (residues 80–169 and 184–289 in MIDA1, residues 89–174 and 200–296 in Zuotin, respectively) were noted. The former conserved region contained “J region,” a 70-amino acid DnaJ conserved protein motif (Fig. 2c) that was found in E. coli DnaJ and several eukaryotic DnaJ members identified as essential proteins for cellular localization and folding of proteins (13, 14). Since J region is considered to mediate interaction with Hsp70s (14, 25, 26), MIDA1 may also interact with heat shock proteins and may affect conformation of proteins. Interestingly, we found that J region of MIDA1 is located adjacent to the Id binding domain. Since interaction between bHLH protein and heat shock proteins has already been reported (27), it seems possible that ternary interaction among MIDA1, Id, and heat shock proteins could regulate conformation or localization of some important proteins in the

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2 W. Shoji and M. Obinata, unpublished data.
turning point of growth and differentiation. The latter con-
served region was almost similar to the region identified by
requirement for the association with Id as demonstrated by the
in vitro analysis. Thus, the strikingly conserved
region within the Zuotin homology region of M1DA1 may con-
tain a new non-HLH protein motif that can interact with HLH
proteins such as the leucine repeat of c-Jun (20) or the pocket
domain of RB (9, 21), although a new motif for protein-protein
association motif remains to be clarified.

In the remaining C-terminal region, we found two Trp-me-
diated repeats that have similar sequence to the DNA binding
domain of c-Myb oncoprotein (Fig. 2d). This protein motif con-
tained conserved tryptophan or hydrophobic residues spaced at
intervals of approximately 20 amino acids and is proposed to
form helix-turn-helix structure (15–17). Because last two Trp-
motivated repeats from the three of c-Myb is sufficient for se-
queness specific DNA binding, two repeats of M1DA1 can be
suggested to have similar function and participate transcrip-
tional regulation. Preliminary attempts indicated that M1DA1
could not bind the 6-base pairs sequence, YAAKCG, recognized
by c-Myb, so searching for recognition sequence by a polymer-
ase chain reaction-associated DNA-binding site selection (28)
is now in progress.

We found that loss of M1DA1 by the addition of its antisense
oligonucleotide strongly interfered with growth of MEL cells,
but not with induction of differentiation. This growth suppres-
sion in antisense experiment is consistent with slow growing
phenotype of Zuotin null mutant yeast (13), suggesting that
M1DA1 can be one of counterparts of Zuotin in mammals.

3 S. Ishii, personal communication.
In vivo association between MIDA1 and Id.
Stable GST-Id-transfected MEL cells and parent cells were treated with cross-linking agent dithiobis(succinimidyl propionate), and subjected to the analysis. Each lysates were incubated with glutathione-Sepharose, and GST-Id proteins were precipitated with their associated proteins. MIDA1 protein can be detected as a single band of 74 kDa with Western blotting using anti-MIDA1 serum.

Expression of MIDA1 mRNA.
10 µg of total RNAs from MEL cells and mouse tissues were separated in denatured 1% agarose gel, and the RNAs were blotted on nylon membrane. The membrane was incubated in the hybridization buffer (50% formamide, 5 x SSC, 1 x FBP, 20 mg NaHPO4, pH 6.5, 100 µg/ml of salmon sperm DNA, 10% dextran sulfate, 0.1% SDS) containing 5 x 10^6 cpm/ml of MIDA1 cDNA probe labeled with random primer extension. A single band was observed at 2.2 kilobase pairs.

Effect of antisense or sense (control) oligomers.
MEL cells were inoculated at 4 x 10^4 cells/ml in Eagle's minimum essential medium containing 12% fetal calf serum (heat-inactivated at 65 °C for 30 min) and 1.8% Me2SO in the presence or absence of 20 µM oligomers, and were monitored for 4 days at daily intervals. □, no oligomers; ○, sense oligomers; ●, antisense oligomers.

Effect on the level of MIDA1 protein.
Cell lysates were prepared from 1 x 10^5 cells in each condition on day 2 after the addition of 20 µM oligomers, and the levels of MIDA1 protein were determined by Western blotting. Total protein amount were ascertained to be equal by Coomassie Brilliant Blue staining of SDS-PAGE electrophoresed samples. Lane 1, no oligomers; lane 2, sense oligomers; lane 3, antisense oligomers.
Furthermore we tried to establish stable transfectants that express MIDA1 gene by sense or antisense orientation. Although over 50 clones for each orientation were established, none can overexpress nor decrease MIDA1 protein expression though over 50 clones for each orientation were established, even when treated with anti-BrdU/nuclease mixed solution (Amersham Corp.), and detected by peroxidase conjugated antibody to mouse IgG, polymerizing diaminobenzidine in the presence of cobalt and nickel.

Our demonstration on the growth regulation by MIDA1 as an Id-associated protein in MEL cells supports recent reports on non-HLH proteins such as RB (9). Id may be a bifunctional protein that regulates growth and differentiation through interaction with bHLH proteins and non-HLH proteins. Such mutual interaction will be required for the commitment event between growth and differentiation during development and cellular differentiation.

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