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MIDA1, an Id-Associating Protein, Has Two Distinct DNA Binding Activities That Are Converted by the Association with Id1: A Novel Function of Id Protein

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Id proteins not only regulate cell differentiation negatively, but they also promote growth, immortalization, and apoptosis. To know the mechanism of how Id regulates cell fate, we previously isolated an Id-associating protein, MIDA1, which positively regulates cell growth (1). Its predicted amino acid sequence consists of a Zuotin (a Z-DNA binding protein in yeast) homology region and tryptophan-mediated repeats (Tryp-med repeats). MIDA1 exhibits a sequence-specific DNA binding activity through the Tryp-med repeats (manuscript in preparation). In this study, we revealed that, like Zuotin, MIDA1 can specifically bind to Z-DNA. This suggested that MIDA is a novel DNA binding protein that has two different DNA binding activities. Furthermore, association of Id1 with MIDA1 stimulated the sequence-specific DNA binding activity, while it inhibited the Z-DNA binding activity. Therefore, we concluded that MIDA1 may act as a mediator of the growth-promoting function of Id, by switching the two DNA binding activities of MIDA1.

The Id family of helix-loop-helix (HLH) proteins has been recognized to function as negative regulators of cell differentiation in various cell types of higher eukaryotes (2). There are 4 Id family members in mammals (Id1-4) and they function by forming "non-functional" (non-DNA-binding) heterodimers with other basic HLH transcription factors, which normally drive cell lineage specific gene expression through binding to "E box" (CANNTG) transcriptional regulatory sequences in addition to the negative regulation of differentiation, it has been shown that Id proteins also function as positive regulators of cell growth, can drive apoptosis under appropriate physiological conditions, and can function as cooperating oncogenes in immortalization (3–5). In common with several other positive regulators of the cell cycle, the protein functions of Id2 and Id3, but not Id1, are regulated during the cell cycle by Cdk2-dependent phosphorylation (6, 7). Thus, Id proteins are thought to play an important role in cell fate determination in response to extracellular signaling.

We have isolated a cDNA clone encoding a protein that can associate with Id1 *in vivo* to learn how Id regulates cell fate (1). This protein was called MIDA1 (mouse Id associated 1), and its predicted amino acid sequence showed homology to Zuotin, a putative Z-DNA binding protein in yeast (Fig. 1) (8). Although MIDA1 did not have any canonical HLH motif, a conserved adjacent to a eukaryotic DnaJ motif (9), within the Zuotin homology region, can associate with the HLH region of Id1. In addition to the Zuotin homology region, we found that MIDA1 contained the Tryp-med repeats at its C-terminus, which were also found in the DNA binding domains of several transcription factors such as the c-Myb oncoprotein (10–12).

We reported previously that MIDA1 was involved in growth control, with a probable role in DNA synthesis (1), although its involvement in apoptosis and immortalization has not yet been investigated. Thus, MIDA1 is a candidate molecule to mediate the growth-promoting functions of Id. To learn the molecular mechanism of how MIDA1 acts as a growth regulator, we characterized its DNA binding activity and found that, through the Tryp-med repeats, MIDA1 can bind to DNA sequences that contain the 7-base consensus sequence (GTCAAGC) surrounded by a 1–3 bp palindromic sequence (manuscript in preparation). In this study we report that, in keeping with its homology to Zuotin, MIDA1 has a Z-DNA binding activity, in addition to its sequence specific DNA binding activity. Furthermore, we investigated how the association with Id

Abbreviations used: HLH, helix-loop-helix; GST, glutathione *S*-transferase; Tryp-med repeats, tryptophan-mediated repeats; MEL, murine erythroleukemia.

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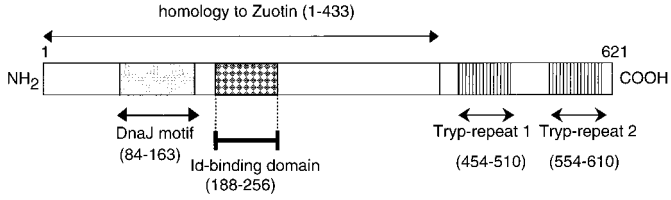

FIG. 1. Structural features of MIDA1. The predicted amino acid sequence of MIDA1 consists of two possible DNA binding domains: a Zuotin (a putative Z-DNA binding protein in yeast) homology region and Tryp-med repeats similar to the c-Myb protein. MIDA1 associates with Id1 through a conserved region adjacent to a DnaJ motif within the Zuotin homology region.

modulates the two DNA binding activities of MIDA1, to approach if or how MIDA1 mediates the functions of Id.

MATERIALS AND METHODS

Preparations of fusion proteins. To produce the GST-ΔId1 fusion protein, a plasmid was constructed that fused the C-terminal region of Id1 (corresponding to residues 50–148) to the C-terminus of glutathione S-transferase (GST) in the pET11d expression vector (Novagen). The fusion protein was produced in *E. coli* [BL21(DE3) plysS] and purified to more than 95% purity by Glutathione-Sepharose affinity chromatography (Pharmacia). To obtain MIDA1 tagged with 6 histidine residues at its N-terminus (H6-MIDA1), a plasmid was constructed that fused the entire MIDA1 cDNA to the C-terminus of 6 histidine residues in the pET11d expression vector. H6-MIDA1 was produced in *E. coli* [BL21(DE3) plysS] and purified to more than 80% purity by Heparin Sepharose chromatography (Pharmacia), followed by Ni-NTA agarose affinity chromatography (Qiagen). All purification procedures were performed as recommended by the supplier.

Gel shift analysis of Z-DNA binding. Gel shift analysis was performed by the method of Zhang *et al.* (8). The probe was linearized poly(dGme5dC) (Pharmacia), which forms stable left-handed Z-DNA in the presence of 15 mM $MgCl_2$. For competition analysis, linearized poly(dGdC) (Pharmacia) was used as B-form DNA. These DNA probes were made as follows: DNA polymer was digested with DNase I and the fragments (approximately 700 bp on average) were gel-purified and labeled with T4 DNA polymerase (Toyobo, Japan) in the presence of dGTP and [α-^{32}P] dCTP (3000 Ci/mmol; Amersham). The binding reactions contained 50 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 0.1% Triton X-100, 10 mM β-mercaptoethanol, 5% sucrose, 300 ng of sheared salmon sperm DNA, 3.2 μg of bovine serum albumin, 20 ng of H6-MIDA1, and 0.1 ng of labeled Z-DNA probe (10,000 cpm) in a 10 μl reaction volume. Complexes were allowed to form at 23°C for 20 min. For competition analysis, the indicated amount of unlabeled DNA was included in the binding reaction. For the antibody shift analysis, IgGs were purified from antisera raised against MIDA1, or the preimmune sera, by Protein A Sepharose and 2 μg of each IgG was added to the reaction. For analysis of Id interaction, the indicated amount of GST-ΔId1 was added to the binding reaction mixtures and incubated with the probes as above. The complexes were resolved on a 1.4% agarose gel in 1 × TBE containing 15 mM $MgCl_2$ for 3 h at 3.5 V/cm. The gels were dried on 3MM paper for autoradiography.

Gel shift analysis of the sequence specific DNA binding of the Tryp-med repeats of MIDA1. Gel shift analysis was performed by the method of Pollock and Treisman (13) with slight modifications. The oligonucleotides #2 and #16 (see Fig. 2a) contain synthetic single-stranded DNAs containing a core 26-base sequence flanked by two PCR primer sequences. Probe #2 but not #16 contained the 7-base consensus sequence (GTCAAGC) surrounded by a 3-bp palindromic sequence in its core region. These oligonucleotides were converted to double-stranded DNA by PCR in the presence of [α-^{32}P] dCTP (3000 Ci/mmol; Amersham), PCR product was gel purified and used as a probe. The binding reactions contained 20 mM HEPES-KOH (pH 7.6), 0.1 M KCl, 0.5 mM DTT, 0.1 mM EGTA, 1 mM $MgCl_2$, 12% glycerol, 20 μg of bovine serum albumin, 80 ng of H6-MIDA1, 22.5 ng of poly(dI-dC), and 10,000 cpm of each double-stranded oligonucleotide probe (3 ng) in a 25 μl reaction volume. Complexes were allowed to form at 23°C for 20 min. For competition analysis, the indicated amount of unlabeled oligonucleotide was included in the binding reaction. For the antibody shift analysis, IgGs were purified from antisera raised against MIDA1, or the preimmune sera, by Protein A Sepharose and 2 μg of each IgG was added to the reaction. For analysis of Id interaction, the indicated amount of GST-ΔId1 was added to the binding reaction mixtures without probes, preincubated for 20 min at 23°C and then further incubated with the probes as above. The complexes were resolved on 4% 29:1 crosslinked polyacrylamide gels in 0.5 × TBE buffer for 2 h at 8 V/cm. For supershift analysis using the anti-GST antibody, 1 μg of the antibody was included in the binding reaction containing 3 μg of GST-ΔId1, or GST, and the complexes were resolved on 3.2% gels. The gels were dried on 3MM paper (Whatman) for autoradiography.

RESULTS

Z-DNA Binding Activity of MIDA1

Almost two thirds of the MIDA1 protein, from the N-terminus, closely resembled Zuotin (1). Although the physiological meaning of Z-DNA is not clear, it has been suggested that Z-DNA is involved in transcription, DNA replication, and DNA recombination (14–16). It was reported that disruption of Zuotin in yeast resulted in a slow growth phenotype (8). This is consistent with our previous finding that a decrease in the level of MIDA1 protein, caused by the addition of antisense oligonucleotides, leads to the suppression of MEL cell proliferation (1). These results prompted us to examine the possibility that MIDA1 regulates cell growth positively through a Z-DNA binding activity. To determine whether MIDA1 has a Z-DNA binding activity like Zuotin, linearized poly(dGme5dC) was used as a stabilized Z-DNA probe for gel shift assays, according to the previous analysis of Zuotin (8). As shown in Fig. 2, nearly all of the probe was retarded in the presence of full length MIDA1 protein tagged with 6 histidine residues (H6-MIDA1), consistent with the analysis of Zuotin. This band was competed away by an excess of unlabeled poly(dGme5dC) but not poly(dGdC), a B-form DNA probe. Furthermore, the retarded band was supershifted specifically by the addition of anti-MIDA1 antibody. The band was diffuse and the retarded band was close to the free band as reported in the previous paper (8). This may be due to the properties of the Z-DNA probe. The probe itself was diffuse because it was made by partial DNase I digestion to generate an average length of 700 bp, to enable the formation of stable Z-DNA. With a probe of this length there is not a large difference in the migration of retarded and free probes. Therefore, we concluded that

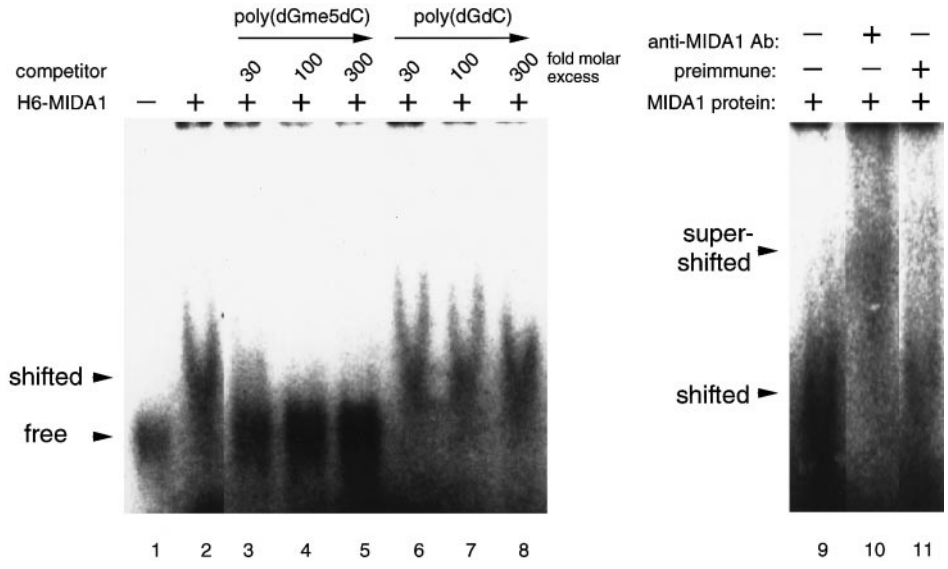

FIG. 2. Binding of full-length MIDA1 to Z-DNA. Linearized poly(dGme5dC) was ^{32}P-labeled as described under Materials and Methods and used as a Z-DNA probe. 10,000 cpm (0.3 ng) of probe was incubated with 20 ng of H6-MIDA1 and subjected to gel shift analysis. The supershifted band was observed in the presence of anti-MIDA1 antibody (lane 10), but not in the presence of control antibody (lane 11). Linearized poly(dGdC) was used as a B-DNA probe for a competition assay (lanes 6–8).

MIDA1 can specifically bind to Z-DNA, as does Zuotin, and that MIDA1 has two DNA binding activities; a Z-DNA binding activity and a sequence specific DNA binding activity.

Association of Id1 with MIDA1 Stimulates the Sequence-Specific DNA Binding Activity of the Tryp-med Repeats

Id is known to associate with several bHLH proteins such as E2A and MyoD (2), and non-HLH proteins like TCF (17), and to interfere with their DNA binding activity. Although MIDA1 was isolated as an Id-associating protein, it does not have any HLH motif and was shown to associate with Id through a short stretch within the Zuotin homology region (1). Thus, we examined whether association of MIDA1 with Id1 attenuates its two DNA binding activities. To detect the sequence specific DNA binding activity of MIDA1, we performed gel shift assays using DNA that contains its 7-base consensus sequence (manuscript in preparation) as a probe (Fig. 3a, #2). We observed a single retarded band (Fig. 3b, C1) when H6-MIDA1 was incubated with the DNA probe containing the consensus sequence (#2) but not with the DNA probe that did not contain the consensus sequence (#16) (Fig. 3b). Furthermore, this retarded band was specifically supershifted by the addition of anti-MIDA1 antibody (Fig. 3b, band C2). This indicated that the full length MIDA1 protein can bind to this specific DNA probe as well as a truncated MIDA1 protein that only contains the C-terminal Tryp-med repeats. Next, because Id1 has been shown to associate with MIDA1 (1), we examined the effect of addition of GST-ΔId1 on the formation of the DNA-H6-MIDA1 complex. Following the addition of GST-ΔId1, the band corresponding to the H6-MIDA1-DNA complex (C1) was partially shifted to a slower migrating band (Fig. 3c, C3). The band C3 was specifically supershifted by the addition of anti-GST antibody to the reaction (Fig. 3d, C4), while the addition of GST and anti-GST did not affect the formation of the DNA-H6-MIDA1 complex, indicating that Id1 is involved in the band C3. Interestingly, as shown in Fig. 3d, as the amount of GST-ΔId1, but not free GST, in the binding mixture was increased, the intensity of band C3 increased while the intensity of the free-probe band decreased. These results indicate that the MIDA1-Id1 complex shows the same DNA binding specificity as MIDA1 alone and that association with Id1 stimulates the DNA binding activity of the Tryp-med repeats of MIDA1. This is the first demonstration that Id serves also as a stimulatory factor for a DNA binding protein.

Association with Id1 Inhibits the Z-DNA Binding Activity of MIDA1

To determine whether interaction with Id affects the DNA binding activity of the Zuotin homology region of MIDA1, we again assayed the Z-DNA binding activity of MIDA1 by gel shift assays. As shown in Fig. 4, the Z-DNA binding activity of MIDA1 was reduced dramatically by the addition of an increasing amount of GST-ΔId1, but not GST. This result indicated that

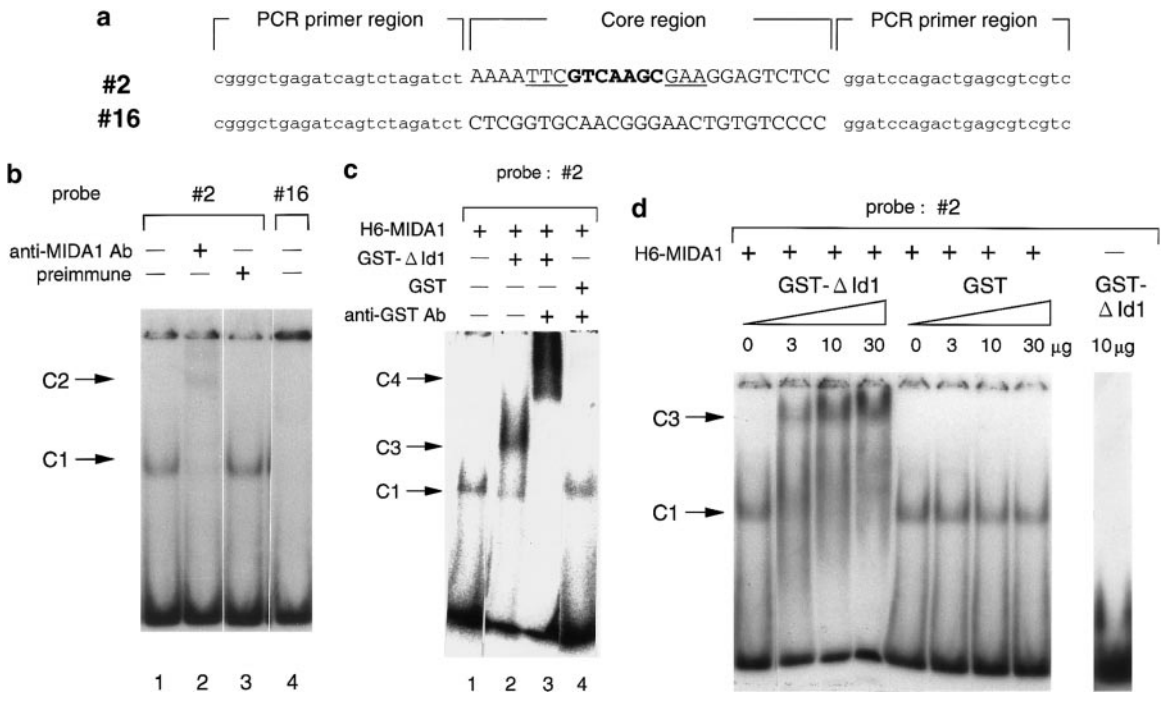

FIG. 3. Sequence-specific DNA binding of full-length MIDA1 and the effect of interaction with Id1 on its activity. (a) DNA probes used in this experiment. The sequences of the upper strands in double strands are shown. The 7-base consensus sequence that is required for the Tryp-med repeats of MIDA1 to bind DNA is indicated in bold, and the 1-3 bp palindromic sequence is underlined. The primer binding sites are indicated in lower case. (b) Gel shift assay using full-length MIDA1. H6-MIDA1 was incubated with the #2 DNA probe that contains the 7-base consensus sequence (lanes 1, 2, and 3) or the #16 DNA probe that does not contain consensus sequence (lane 4), in the presence of anti-MIDA1 antibody (lane 2) or control antibody (lane 3) and was subjected to gel shift analysis. Band C1 was formed only when H6-MIDA1 was incubated with the consensus binding sequence and band C1 was supershifted to band C2 by the addition of anti-MIDA antibody. (c) The effect of anti-GST antibody on the DNA binding reaction. The band C1, which was formed by a H6-MIDA1/#2 probe complex (lane 1), was shifted to C3 in the presence of GST-ΔId1 (lane 2). The band C3 was further supershifted to C4 by the addition of anti-GST antibody (lane 3). In lane 4, GST, H6-MIDA1, and anti-GST antibody were incubated with the #2 probe. (d) The effect of GST-ΔId1 on the sequence-specific DNA binding of MIDA1. H6-MIDA1 was preincubated with the indicated amounts of GST-ΔId1 (lanes 2–4) or GST (lanes 6–8) and a gel shift assay was performed with the #2 DNA probe. The band C1 was shifted to a slower migrating band (C3) depending on the increasing amounts of GST-ΔId1 (lanes 3 and 4). In lane 9, GST-ΔId1 alone was incubated with the #2 DNA probe.

association with Id1 inhibits the Z-DNA binding activity of MIDA1 and that this association regulates the two DNA binding activities of MIDA1 conversely.

DISCUSSION

In this study, we revealed that an Id-associating protein, MIDA1, has a Z-DNA binding activity, consistent with its homology to Zuotin. Although the physiological meaning of Z-DNA is not clear, Z-DNA has been implicated as being important in the processes of transcription, DNA replication, and DNA recombination (14, 15, 16). It has been reported that disruption of Zuotin in yeast resulted in a slow growth phenotype (8). Therefore, the present study raises the possibility that MIDA1 may act as a positive growth regulator through this Z-DNA binding activity, although the precise mechanism of this is unclear.

Since we found previously that MIDA1 exhibits a sequence specific DNA binding activity (manuscript in preparation), our present work shows that MIDA1 has two DNA binding activities; a Z-DNA binding activity and a sequence specific DNA binding activity. We believe that this is the first report of a protein that can bind to DNA both in a conformation specific and in a sequence specific manner. Since a GST fusion with a truncated MIDA1 protein that only contained the C-terminal Tryp-med repeats, had a sequence specific DNA binding activity, but did not exhibit a Z-DNA binding activity (data not shown), it is conceivable that MIDA1 exhibits the Z-DNA binding activity through the Zuotin homology region at its N-terminus and exhibits the sequence specific DNA binding activity through the Tryp-med repeats at its C-terminus.

It is not known whether the two distinct DNA binding activities of MIDA1 function synergistically or independently and how this relates to the role of MIDA1 as a regulator of cell growth. Most interestingly, we found that association of Id1 with MIDA1 stimulates the sequence specific DNA binding activity, whereas it

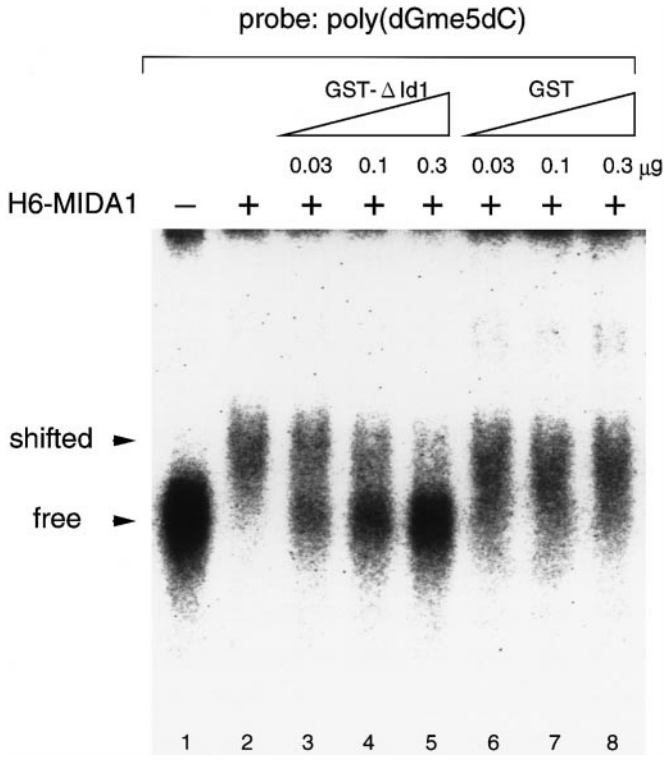

FIG. 4. Effect of interaction with Id1 on the Z-DNA binding activity of MIDA1. H6-MIDA1 was preincubated with the indicated amounts of GST-ΔId1 (lanes 3–5), or GST (lanes 6–8) and a gel shift assay was performed with the Z-DNA probe.

inhibits the Z-DNA binding activity. Our current study may present new insights into how Id regulates cell growth by switching the two DNA binding activities of MIDA1.

Whether the two DNA binding activities of MIDA1 are regulated by other Id family members, Id2-4, is currently not known. It has been reported that the growth-promoting functions of Id2 and Id3, but not Id1, are regulated during the cell cycle by CDK2-dependent phosphorylation (6, 7). It is therefore important to determine whether Id2-4 associate preferentially with MIDA1, if they regulate the two DNA binding activities of MIDA1, or how such regulation is integrated into the cell cycle control mechanisms that affect Id functions. This would establish a further important link between MIDA1 function and cell cycle control. Also, the role of MIDA1 in the apoptosis-promoting function of Id proteins, a process which appears to be tightly coupled to the growth-promoting functions of Id (4), is not known. These studies will be required to provide a more complete picture of how Id regulates cell fate.

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