The Helix-Loop-Helix Protein Id Inhibits Differentiation of Murine Erythroleukemia Cells*

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Id is considered to be a negative regulator of basic helix-loop-helix proteins, which play important roles in cell type-specific transcription and cell lineage commitment. The Id gene was first cloned in murine erythroleukemia (MEL) cells, which can be induced to differentiate toward erythrocytes with MeqSO, and its mRNA decreases after differentiation in various types of cells. In this report, we demonstrate that overexpression of Id interferes with MEL cell differentiation and that inhibition of differentiation is accompanied by reduction in expression of three erythroid-specific genes. While down-regulation of Id is an early event in the differentiation process of MEL cells, E-box binding activity of these cells increases only at a later stage of differentiation, and this late increase is reduced by the overexpression of Id in the early stage. Sequential changes in the activity of several basic helix-loop-helix proteins thus appeared to be involved in erythroid differentiation.

The basic helix-loop-helix (bHLH) proteins are a family of putative transcription factors, some of which play an important role in the differentiation to specific cell lineages (1). These proteins contain a helix-loop-helix (HLH) domain composed of two conserved amphipathic α helices separated by a variable loop region and an adjacent basic amino acid region. Acting as transcription factors, these proteins form homo- or heterodimers through HLH domains and bind to specific DNA sequences with basic regions (2). Sequences for binding and transcriptional activation for these proteins are represented by E-box motif (CANNTG), which presents pro-

The abbreviations used are: bHLH, basic helix-loop-helix; HLH, helix-loop-helix; MEL, murine erythroleukemia; MeqSO, dimethyl sulfoxide; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; HMT, human metallothionein.

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MATERIALS AND METHODS

**Plasmid Constructions**—Murine Id cDNA was obtained from H. Weintraub (Fred Hutchinson Cancer Research Center) and inserted into the pSVneoHMT-IIA vector that had been constructed in a previous work (15). Briefly, human metallothionein IIa, gene promoter and the poly(A) signal sequence from SV40 large T gene were inserted into pSVneo vector. Then, a 0.8-kilobase Smal/DraI fragment containing all coding regions of the Id gene was inserted between the HMT-IIa promoter and the poly(A) signal sequence. The resulting plasmid (pHMT-Id) contains the murine Id gene downstream of the HMT-IIa promoter and the neomycin gene as a selective marker.

**Cell Lines and Transformants**—MEL cells (DS19/3) were maintained in Eagle's minimum essential medium supplemented with 12% fetal calf serum and were induced to differentiate by the addition of 1.8% (v/v) MeqSO. The plasmid was introduced into MEL cells by a protoplast fusion method (16). Escherichia coli (HB101) cells containing pHMT-Id were grown in the presence of chloramphenicol (100 mg/ml) for 12 h to amplify the plasmid, treated with lysozyme (2 mg/ml) for 10 min at 4 °C, and then added to MEL cells fixed on Petri dishes coated with poly-L-lysine. After treatment with polyethylene glycol, the cells were washed with the medium and cultured in a CO2 incubator. After 2 days, G418 (100 μg/ml) was added to the
were analyzed by Northern blot hybridization using Id and actin cDNAs as probes. In the lower panel, the intensity of the bands was quantified by a Bio-image analyzer (Fuji BAS2000), and the relative expression of Id mRNA was scored at different distributions (lanes A, B, C, and D). The transfected clones classified as either inducible or noninducible, based on the expression of the Id gene, were scored at different distributions (lanes C and D).

The presence of Zn²⁺ to that in the absence of Zn²⁺ was scored. hne A, entafication.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Levels of Id mRNA in MEL cells after Me2SO treatment. In the upper two panels, 1 pg of poly(A⁺) RNAs from MEL cells collected at indicated times after Me2SO (DMSO) treatment were analyzed by Northern blot hybridization using Id and actin cDNAs as probes. In the lower panel, the intensity of the bands was quantified by a Bio-image analyzer (Fuji BAS2000), and the relative intensity to actin gene was plotted.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.png}
\caption{Effect of the transfected Id gene on MEL cell differentiation. In the upper two panels, 1 pg of poly(A⁺) RNAs from MEL cells collected at indicated times after Me2SO (DMSO) treatment were analyzed by Northern blot hybridization using Id and actin cDNAs as probes. In the lower panel, the intensity of the bands was quantified by a Bio-image analyzer (Fuji BAS2000), and the relative intensity to actin gene was plotted.}
\end{figure}

\textbf{DNA Binding Assays—} Nuclear extracts for DNA binding assay were prepared as described (21). After washing 1 x 10⁶ cells with phosphate-buffered saline, the cells were homogenized in hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) with a Dounce homogenizer. Homogenized cells were centrifuged at 16,000 rpm, and the nuclear pellets were resuspended in extraction buffer (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). After the centrifugation, the recovered supernatants were dialyzed against storage buffer (20 mM HEPES, 20% glycerol, 0.1 M KCl, 0.2 mM DTT, 0.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM PMSF) for 6 h. Protein was determined with a Bio-Rad protein assay kit, and the extracts were stored at -70 °C.

Electrophoretic mobility shift assay for E-box motif was performed as described (7). For this assay, a 25-base pair double-stranded oligonucleotide containing the canonical E-box motif from muscle creatine kinase enhancer (22) was used. The sequence of this nucleotide is as follows:

\begin{verbatim}
5' - GATCCCCCAACCATGCTGCCTGA - 3'  
3' - GGGGTTTGGAGCAGCCACTCTAG - 5'  
\end{verbatim}

The first strand of oligonucleotide was labeled by T4 polynucleotide kinase with [γ-³²P]ATP (8000 Ci/mmol; Amersham Japan Inc.). The oligonucleotide was then denatured and annealed to 10-fold molar excess of the opposite strand. Nuclear extracts (0.6 μg) and the oligonucleotide probe (0.1 ng) were mixed in 10 μl of binding mixture (20 mM HEPES pH 7.6, 50 mM KCl, 1 mM EDTA, 5% glycerol, 0.1 mg/ml poly(dI-dC)) and incubated at 30 °C for 15 min. The reaction mixtures were immediately electrophoresed on 5% polyacrylamide gels in 1 X TBE buffer for 3 h at 8 V/cm. Gels were dried and autoradiographed.

\textbf{Analysis of Id Binding Protein in MEL Cells—} MEL cell nuclear extracts were analyzed by SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to a nitrocellulose filter (Schleicher & Schuell). The filter was denatured with 6 M guanidinium HCl/HBB buffer (20 mM HEPES, 5 mM MgCl₂, 1 mM KCl, 5 mM DTT), renatured gradually, and blocked with 5% skim milk/HBB buffer for 1 h at 4 °C. The filter was set on Screener Blotter (Sanplatec Corp.) and incubated with bacterially produced fusion proteins (glutathione S-transferase-Myc(314-439), glutathione S-transferase-hep-tin A, and chymostatin). The extracts were mixed with anti-Id antibody and incubated for 1 h at 4 °C, and then 6 μl of protein A-Sepharose beads (Pharmacia LKB Biotechnology Inc.) was added. After incubation for an additional 1 h, the beads were washed 4 times with washing buffer (50 mM Tris-HCl, pH 7.4, 0.5 mM NaCl, 1 mM EDTA, 0.25% gelatin, 0.1% SDS) and resuspended in SDS sample buffer. The proteins were recovered by boiling for 2 min and electrophoresed in 10% SDS-polyacrylamide gel.
Fig. 3. Panel A shows induction of Id gene expression with Zn^{2+}. Seven transfected clones and parental MEL cells (DS19/3) were cultured in the presence or absence of ZnCl_{2} (160 μM) for 12 h, and levels of Id mRNA were examined by Northern blot hybridization. The four clones at left (DSld221, -261, -342, and -442) showed induction of Id mRNA in the presence of ZnCl_{2}, while in the other three transfected clones (DSld352, -361, and -422) and the parental MEL cell (DS19/3), Id mRNA was not induced in the presence of ZnCl_{2}. Panel B, shows the
Id Inhibits Erythroleukemic Differentiation

RESULTS

Decreased Expression of Id Gene Is an Early Event in MEL Cell Differentiation—When MEL cells were treated with Me₂S₀, they were committed to terminal erythroid differentiation after a latent period of 48 h (15, 23). Benezra et al. (7) reported that the level of Id mRNA was low after differentiation. In examining whether a change in Id mRNA level occurs as an early event of induction, we found that the level had dropped rapidly by 48 h after Me₂S₀ treatment to one-tenth of the initial level and remained very low thereafter (Fig. 1). These findings support the hypothesis that Id is an important factor, especially in the early event of MEL cell differentiation.

Overexpression of Id Interferes with the Differentiation of MEL Cells—If lower Id mRNA is essential for subsequent differentiation of MEL cells, forced expression of Id should alter the cell differentiation phenotype. To test this, we introduced Id gene into MEL cells under the control of HMT-2, a promoter in the pSVEneo vector. This promoter offers great advantages for analyzing the effect of the transfected gene in MEL cells because of its accurate inducibility in response to heavy metals (ZnCl₂) without any damage or undesirable effects. Furthermore, considering the previously suggested growth regulation by Id (9, 14), this promoter is useful in analyzing the effect of the transferred gene in MEL cells because of its accurate inducibility. Immunoprecipitation was done using anti-Id antibody to confirm that elevated levels of Id protein were induced in the cells with inhibitory phenotype. Fig. 4 shows that Id proteins were induced with Zn²⁺ in all such clones but not in the parent cells (DS19/3). Classification of the seven clones into either an inducible or noninducible type based on Id gene expression clearly showed that overexpression of the gene interfered with MEL cell differentiation (Fig. 2).

Inhibition of Differentiation Depends on Timing of Id Expression—The importance of the timing of a decrease in Id mRNA in the differentiation was examined by adding Zn²⁺ to cultures at different periods after Me₂S₀ treatment. Fig. 5 shows the results on clone DSld342, representing those in which the expression of Id gene was inducible. When Zn²⁺ was added either at time 0 or at 6 h after Me₂S₀ treatment, the inhibition was maximum. After 12 h, the extent of inhibition gradually decreased depending on the time of addition. Thus, the initial decrease in Id mRNA within 6 h after Me₂S₀ treatment may be critical for the induction of differentiation.

Overexpression of Id Reduces Expression of Erythroid-specific Genes—In addition to the benzidine staining, which reveals hemoglobin production, we asked whether overexpression of the Id gene interferes with expression of other erythroid-specific genes in the clone DSld342. Three erythroid-specific genes, β-globin, glycophorin, and erythroid-specific α-aminolevulinate synthase (in each of which expression is induced after 2 days of Me₂S₀ treatment (19)), were employed as probes. Northern blotting analysis of total RNAs from the cells induced with Me₂S₀ in the presence or absence of Zn²⁺ (Fig. 6) showed that in all three genes expression was reduced in the presence of Zn²⁺, which maintained the inhibitory effect of Id in differentiation of MEL cells. We then examined c-myc gene expression because its reduction is a prerequisite for the MEL cell differentiation and forced expression of the transferred c-myc gene blocks differentiation (15, 24–27). Fig. 6 shows that the expression of c-myc gene does not change in the presence or absence of Zn²⁺. Thus, inhibition of differentiation by overexpression of Id gene was not dependent on the change in c-myc gene expression. In spite of the continuous presence of Zn²⁺, Id mRNA was rapidly induced, but it dropped to a normal level by 2 days after Me₂S₀ treatment. This observation supports the idea that Id is important especially in the early event of MEL cell differentiation.

Overexpression of Id Changes E-Box Binding Activity of MEL Cells—In the muscle differentiation system, forced expression of Id inhibits the differentiation process through interaction with bHLH proteins such as MyoD and E2A (9). Assuming a similar function of Id in MEL cell differentiation, although it is not known what kind of bHLH proteins are present in MEL cells, we monitored E-box binding activity as one character of bHLH proteins. An electrophoretic mobility shift assay was performed using an oligonucleotide probe that

![Figure 4. Induction of Id proteins with Zn²⁺ in the inducible clones. Four inducible clones and parental cell cultured in the presence or absence of Zn²⁺ were metabolically labeled with [³⁵S]methionine for 2.5 h and immunoprecipitated with anti-Id antibody. Id proteins migrate as doublets between 14.4- and 21.5-kDa protein standards, which is consistent with the previous report (9).](image)
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Fig. 5. Inhibition of differentiation depends on timing of Id expression. ZnCl₂ (160 μM) was added to cultures of the inducible clone DSId342 or the parental clone SD19/3 at 0, 6, 12, and 24 h after Me₂S₀ treatment. The extent of hemoglobinized cells was monitored on day 4. Maximum inhibition was observed at 0 or 6 h after Me₂S₀ treatment in clone DSId342.

Fig. 6. Effect of the overexpression of Id on the erythroid-specific genes and c-myc gene. Twenty micrograms of total RNA was prepared from clone DSId342 in the presence or absence of Zn²⁺ at indicated times after Me₂S₀ treatment and was analyzed by Northern blot hybridization.

Fig. 7. E-box binding activity on MEL cells. Overexpression of Id reduces E-box binding activity (left panel). The labeled E-box oligonucleotides were incubated with the nuclear extracts prepared from clone DSId342 in the presence (+) or absence (−) of Zn²⁺ at indicated times after Me₂S₀ treatment. The arrowhead indicates the bands of E-box binding activity (lanes 1–6), which compete with excess (500-fold) unlabeled oligonucleotide (lane 7). In the right panel, bacterially produced glutathione S-transferase or glutathione S-transferase-ΔId fusion proteins were added to the nuclear extract. The E-box binding activity in the 4-day extract was reduced by the addition of increasing amounts of glutathione S-transferase-ΔId protein (lanes 8–12) but not glutathione S-transferase proteins (lanes 8–12).

contained an E-box from muscle creatine kinase enhancer (22). Nuclear extracts were prepared from DSId342 cells in the presence or absence of Zn²⁺ at three points of the differentiation process, 0.5, 2, and 4 days after Me₂S₀ treatment (Fig. 7, left panel). At 0.5-days, Id expression dropped. At days 2, the extent of differentiated cells began to increase, and levels of E-box binding activities were low and the same in both extracts. At 4 days, when the extent of differentiated cells reached maximum, levels of E-box binding activities increased significantly in the absence of Zn²⁺ (Fig. 7, left panel, upper band), but the increase was blocked in the presence of Zn²⁺.

To confirm that the block in the increase of E-box binding activity was due to Id protein, we added bacterially produced glutathione S-transferase or glutathione S-transferase-ΔId fusion protein to the nuclear extract in vitro. ΔId deletes 79 amino acids from the N terminus of Id to promote solubility in bacterial lysate and contains a complete HLH domain. Glutathione S-transferase-ΔId was shown to form specific heterodimers with MyoD (data not shown). Addition of glu-
s-glutathione S-transferase-Myc(314-439) proteins used as probes in this analysis. In extract in proportion to the amount of glutathione S-transferase, but not of glutathione S-transferase-Myc(314-439) or glutathione S-transferase-ΔId, dramatically reduced E-box binding activity of the 4-day differentiated MEL cells. The same bands with higher molecular weight observed in the nuclear extract (Fig. 6). The overexpressed Id in the early event did not affect the differentiation process (Figs. 2-5). Since expression of the transferred gene is transient (Fig. 6) and the commitment of MEL cell differentiation is not completely synchronous, a certain population of the cell remains to differentiate. Electrophoretic mobility shift assay analysis indicated that E-box binding activities were blocked by either Id overexpressed in MEL cells or by the excess amount of bacterially produced Id protein in the nuclear extract (Fig. 7). These suggest that Id acts as a negative regulator of bHLH proteins in MEL cells in a similar manner as reported in muscle differentiation (7, 9); in undifferentiated MEL cells, Id may form heterodimers with bHLH proteins and inhibit DNA binding activity. When the MEL cells are treated with MεSO, Id proteins rapidly decrease and the preexisting or newly synthesized bHLH proteins form functional homodimers or heterodimers, which are required for further differentiation. Our results, however, suggest a more complex mechanism of Id regulation in MEL cell differentiation. Instead of the rapid decrease in Id mRNA after induction, increase in the E-box binding activities was only detectable on day 4 after induction. This is inconsistent with myeloid differentiation, where an increase in the E-box binding activities follows a rapid decrease in Id (10). In addition in spite of the continuous presence of Zn²⁺ after Me₂SO treatment, overexpression of Id was only observed during the first day after induction (Fig. 6). The overexpressed Id in the early event did not affect the E-box binding activity but strongly reduced it on day 4 when

**Fig. 8. Analysis of Id-binding proteins in MEL cells.** MEL cell nuclear extracts were electrophoresed in SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose filter, and binding proteins to glutathione S-transferase-Myc(314-439) and glutathione S-transferase-ΔId were detected by Western blotting as described under "Materials and Methods." Panel A shows the structure of bacterially produced glutathione S-transferase (GST) glutathione S-transferase-Myc(314-439) and glutathione S-transferase-ΔId fusion proteins used as probes in this analysis. In panel B, lanes 1-3 correspond to protein probes shown in panel A. Glutathione S-transferase did not bind any proteins (lane 1), but glutathione S-transferase-Myc(314-439) bound to a 17-kDa protein, whose molecular mass is identical to Max (lane 2). Glutathione S-transferase-ΔId protein bound specifically to an approximately 30-kDa protein (lane 3). Glutathione S-transferase-Myc(314-439) and glutathione S-transferase-ΔId showed the same two high molecular weight bands (lanes 2 and 3).

**Possible Partners for Id in MEL Cells**—To identify possible partner(s) for Id in MEL cells, we searched the proteins that bind specifically to Id by Western blotting. As shown in Fig. 8, we found an approximately 30-kDa protein that binds specifically to glutathione S-transferase-ΔId protein added (Fig. 7, right panel). These results support the hypothesis that Id interferes with MEL cell differentiation through interaction with the protein(s), which possesses E-box binding activity.

**DISCUSSION**

When MEL cells are treated with inducing agents, they are committed to terminal erythroid maturation. The first changes occur in the transport of ions (28-31) and in phosphoinositide turnover (32), and changes in the expression of a group of genes including c-myc and c-myb oncogenes follow (33, 34). These changes are called early events and are required for the commitment of MEL cell differentiation. In fact, c-Myc has also been shown to inhibit differentiation in various cell lineages including MEL cells (15, 24-27, 35, 36), and it apparently acts as a negative regulator for differentiation. The immediate decrease in Id mRNA after Me₂SO treatment observed in this work suggests the involvement of Id in these early events. Our results with the transfection of the Id gene into MEL cells clearly demonstrated that Id is an important factor in MEL cell differentiation, especially in the early events, because differentiation was blocked strongly when the transferred Id gene was overexpressed earlier in the differentiation process (Figs. 2-5). Since expression of the transferred gene is transient (Fig. 6) and the commitment of MEL cell differentiation is not completely synchronous, a certain population of the cell remains to differentiate.
Id levels must drop. This time lag in MEL cells suggests the following possibilities. 1) More than two bHLH proteins are involved in MEL cell differentiation; Id proteins release the first bHLH proteins in the early event, and then these proteins either stimulate transcription of a second group of bHLH proteins or release these proteins by homo- or heterodimer formation. The first bHLH proteins are undetectable because of their limited quantity or because of different DNA binding specificity. Alternatively, Id may interact with non-bHLH protein(s) as reported in MyoD (37, 38), and these non-bHLH proteins may be involved in the commitment and differentiation. 2) The rapid drop in Id releases a small amount of bHLH proteins in the early event and leads to the autonomous transcriptional activation, which results in the accumulation of the E-box binding proteins after a time lag. Although these two possibilities must be tested by identifying the bHLH proteins interacting with Id in MEL cells, the early change in Id may lead to the commitment of differentiation, and the later accumulated E-box binding proteins may facilitate the terminal differentiation.

It has been reported that several bHLH proteins, such as SCL, LYL, and TAL2 (39–41) are expressed in hematopoietic cells. SCL restricts its expression to erythroid cells (42) and promotes spontaneous differentiation when transfected into MEL cells (43); it can, therefore, be a candidate for Id's partner in MEL cells. On the other hand, neither E-box binding activity of SCL protein nor direct interactions of SCL protein with E2A have been reported (40). We investigated the possibility of direct interaction between Id and SCL in vitro, but even if present, the binding activity of Id and SCL was far weaker than that of Id and MyoD in standard immunoprecipitation analysis. These data suggest that a distinct subset of E2A-like bHLH proteins is present and mediates regulation of SCL by Id or that unidentified bHLH proteins, which promote MEL cell differentiation, are regulated by Id. We searched for Id binding proteins in MEL cells by Westerblotting and found an approximately 30-kDa protein that binds specifically to Id (Fig. 8). Analysis of its function may clarify the regulatory mechanism of MEL cell differentiation by Id.

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