Coordinate Expression and Developmental Role of Id2 Protein and TAL1/E2A Heterodimer in Erythroid Progenitor Differentiation

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The Id proteins and basic helix-loop-helix (bHLH) proteins play major roles in specifying cell fate decisions in diverse biologic settings. A potential role for Id and TAL1/E2A bHLH genes in hematopoiesis has been suggested by studies on immortalized cell lines. However, it is uncertain whether these observations reflect normal hematopoiesis. We have investigated the expression pattern of Id2 and TAL1/E2A genes in liquid suspension culture of purified hematopoietic progenitor cell (HPCs) undergoing erythroid or granulopoietic differentiation in the first culture week and maturation to terminal cells in the second week. In quiescent, freshly purified HPCs, Id2 mRNA is detected by reverse transcriptase-polymerase chain reaction (RT-PCR), whereas TAL1 and E2A mRNAs are not. At the onset of erythroid differentiation, Id2 mRNA is downregulated, while E2A and TAL1 mRNAs are concomitantly upregulated: their expression is further increased at erythroblast level. Conversely, Id2 is not downmodulated in granulopoietic culture, except for a late decline at day 10 to 12, while TAL1 and E2A are only transiently induced in the first week of granulopoietic differentiation. The expression pattern of the TAL1/E2A heterodimer, as evaluated by mobility shift assay, is consistent with RT-PCR results (except for lower levels of the heterodimer in late erythroid maturation). TAL1 protein level, analyzed by Western blot, shows a pattern consistent with gel-shift results. Functional experiments were performed on purified HPCs treated with phosphorothioate antisense oligodeoxynucleotides to Id2 or TAL1 mRNA. The results are strictly consistent with the expression studies: anti-Id2 oligomer (α-Id2) causes a significant dose-dependent increase of erythroid colony formation, whereas α-TAL1 induces a selective dose-related inhibitory effect on erythroid colonies, as compared with untreated or scrambled oligomer-treated control HPCs. Finally, murine and human glutathione-S-transferase (GST)-Id2 polypeptides compete the TAL1/E2A-specific DNA binding activity when added to the nuclear extracts derived from erythroid culture cells, thus indicating biochemical and suggesting functional interaction of Id2 with the TAL1/E2A complex. These novel observations indicate a coordinate expression and function of an inhibitory Id protein (Id2) and a stimulatory bHLH/bHLH heterodimer (TAL1/E2A) in normal erythroid differentiation.

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erythroid differentiation; however, it is uncertain whether these studies reflect normal hematopoiesis.

At least three distinct genes encoding Id polypeptides have been identified in mammals: outside of the HLH domain, human Id1 and Id2 markedly differ, except for two homologous regions containing potential protein phosphorylation sites. The TALI/SCUT/ TCL5 gene, identified in a T-lymphocytic acute leukemia (T-ALL), encodes a bHLH protein related to erythropoiesis. Indeed, (1) TALI expression is readily detectable in K562 and HEL erythroleukemic cell lines, as well as in human fetal liver and murine yolk sac. (2) The transduced TAL1 gene enhances erythroid differentiation of MEL and K562 cell lines. However, the TAL1 expression/function in normal adult erythropoiesis is still unknown.

In vitro studies indicate that the TAL1 protein interacts with class A bHLH proteins (E12 and E47) encoded by the E2A locus to form heterodimeric bHLH complexes (TAL1/E12 and TAL1/E47) that bind E-box DNA sequences. TAL1 and E2A also associate in leukemic T cells: these studies show that TAL1 does not form homodimers but requires in vivo heterologous interaction with E12 and E47. However, corresponding in vivo studies are not yet available. Finally, recent observations indicate that Id2 protein competes with TAL1 for heterodimer formation with E2A in murine M1 leukemia cell line induced to macrophage differentiation: here again, it is uncertain whether these studies reflect normal hematopoiesis.

In an attempt to shed new light on these aspects, we have investigated the in vivo expression/function of Id2, TAL1, and E2A genes in hematopoiesis, including (1) their expression in purified HPCs induced to specific erythroid or granulocytic differentiation in liquid suspension culture; (2) their functional role by treatment of HPCs undergoing erythroid or granulocytic differentiation with antisense oligodeoxynucleotides targeting Id2 or TALI mRNA; and (3) the competing interaction between Id2 and TAL1/E2A for sequence-specific DNA-binding activity.

MATERIALS AND METHODS

Hematopoietic Growth Factors and Culture Medium

Recombinant human interleukin-3 (rHL-3) and granulocyte-macrophage colony-stimulating factor (rGM-CSF; 1.7 to 2.5 × 10⁷ U/mL) were supplied by Genetics Institute (Cambridge, MA); erythropoietin (Epo), by Angen (Thousand Oaks, CA); and e-kit ligand (KL) by Immunex (Seattle, WA). Iscove’s modified Dulbecco’s medium (IMDM; Gibco, Grand Island, NY) was freshly prepared weekly.

Adult PB

Adult PB was obtained from 20- to 40-year-old healthy male donors after informed consent. Blood (450 mL ± 10%) was collected in preservative-free CPDA-1 anticoagulant (citrate-phosphate-dextrose-adenine). Auffy coat was obtained by centrifugation (Beckman J6ME; Beckman Instruments, Palo Alto, CA; 1,400 rpm for 20 minutes at room temperature).

HPC Purification and Assay

Adult PB HPCs were purified according to a modification of the method reported by Gabbianelli et al., as described by Labbaye et al. and Giampaolo et al. (Step IA) PB samples were separated over a Ficoll-Hypaque density gradient (density [d], 1.077; Pharmac Fine Chemicals, Piscataway, NJ). (Step IB) PBMCs resuspended in IMDM containing 20% heat-inactivated fetal calf serum (FCS; Gibco) were treated with three cycles of plastic adherence. (Step II) Cells were separated by centrifugation on a discontinuous Percoll (Biochrom KG, Berlin, Germany) four-step gradient (d: 1.052, 1.056, 1.060, and 1.065). (Step III) Step III purification was performed as reported by Labbaye et al.: briefly, low density cells (1.052 and 1.056 fractions) were collected and incubated with appropriate amounts of an anti-T, anti-B, anti-natural killer (NK) lymphocyte/monocyte/granulocyte monoclonal antibody (MoAb) cocktail as described supplemented with three other MoAbs (anti-CD45, anti-CD11a, and anti-CD71; Becton-Dickinson, Mountain View, CA), as described by Labbaye et al. Cells were resuspended in IMDM containing 10 mg/mL bovine serum albumin (BSA; 97% to 99% purified, Step V; Sigma Chemical Co, St Louis, MO), incubated (1 hour at 4°C) with immunomagnetic monodisperse microspheres coated with sheep antibody to mouse IgG (different subclasses) and IgM (Dynabeads M450; Dynal, Oslo, Norway) at a ratio of four beads per cell, and then separated with a magnet. Residual cells were then counted and incubated again (1 hour at 4°C) with the same type of magnetic beads at a ratio of 10 beads per cell. Finally, the cells were counted again and incubated (30 minutes at 4°C) with magnetic beads coated with affinity-purified antimouse IgG1 (Fc-specific, Dynabeads 110.04; Dynal) at a ratio of 10 beads per cell. Throughout this procedure the concentration of magnetic beads never exceeded 50 × 10⁶/mL.

Step IIIP HPCs were routinely analyzed with a phycoerythrin (PE)-conjugated anti-CD34 MoAb (Becton-Dickinson). Cells were incubated for 30 minutes at 4°C in the presence of an appropriate amount of MoAb. After three washes with cold phosphate-buffered saline (PBS), cells were resuspended in PBS containing 2.5% formaldehyde and then analyzed by fluorescence-activated cell sorter (FACS; FACScan, Becton-Dickinson) for fluorescence intensity using a program for double immunofluorescence evaluation. At least 4,000 cells were analyzed for each determination.

The clonogenetic assay of purified HPCs in FCS culture was performed as previously reported after addition of KL (10 ng/mL), IL-3 (100 U), GM-CSF (10 ng), and Epo (3 U), unless otherwise indicated.

HPC Liquid Differentiation Culture

Step IIIP HPCs grown in FCS liquid culture (5 × 10⁶/mL in IMDM, supplemented as indicated below) were induced to specific erythroid or granulocytic differentiation by appropriate HGF combinations [saturating Epo and low IL-3/GM-CSF dosage (3 U/mL, 0.01 U, and 0.001 ng, respectively) for unilineage erythroid differentiation; saturating IL-3/GM-CSF dosage (100 U/mL and 10 ng, respectively) for selective granulocytic differentiation] as previously reported. In a fully humidified 5% CO2/5% O2/90% N2 atmosphere, FCS was substituted by BSA (fraction V, 10 mg/mL), pure human transferrin (0.7 mg/mL), human low-density lipoproteins (40 μg/mL), insulin (10 μg/mL), sodium pyruvate (10 mM), glucose (2 × 10⁻⁵ M), rare inorganic elements supplemented with iron sulfate (4 × 10⁻⁸ M), and nucleosides (10 μg/mL of each).

Morphology analysis. Cells were harvested at different days, smeared on glass slides by cytosin centrifugation, and stained with May-Grünwald-Giemsa.

Membrane phenotypes. Cells were incubated for 30 minutes at 4°C with PE- or fluorescein isothiocyanate (FITC)-labeled anti-CD34, anti-glycoporin A (Immunotech, Marseille, France), anti-CD11b (Becton-Dickinson) MoAbs and analyzed as described above.

The clonogenetic assay of Step IIIP HPCs differentiating in ery-
thyroid or granulopoietic culture was performed as described below after addition of the human growth factor (HGF) combination used in the corresponding liquid phase culture.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) mRNA Analysis**

Methodology for semiquantitative RT-PCR analysis has been previously reported.\(^{38-43}\) Briefly, total RNA, extracted from the guanidinium isothiocyanate-CsCl method from the same number of cells in the presence of 12 µg of Escherichia coli rRNA carrier, was quantitated by dot hybridization with a human rRNA probe.\(^{39}\) After densitometric analysis, the normalized amount of RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) with oligo (dT) as primer. The cDNAs were normalized by the \(\beta_2\)-microglobulin gene (5' primer, 5'-AACCACGTTGAACCTCACAGGACTGAA-3'; 3' primer, 5'-GCCCGTCTGCCCGGTCCGGTCCCGTCC-3'; internal probe from 311 bp to 360 bp).\(^{40}\) Based on amplification dose-response results, \(\beta_2\)-microglobulin normalization was obtained by amplifying an aliquot of approximately 5 ng of RT-RNA by 20 PCR cycles; ie, the cycle number allowing a linear RT-RNA dose-response (denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 45 seconds) in a Perkin-Elmer Cetus (Norwalk, CT) thermocycler.

To further insure semiquantitative RT-PCR assay for Id2, TALI, and E2A templates, several dilutions of RT-RNA from multiple HPC samples were amplified within the linear range by 40 PCR cycles; the dose-response curves consistently showed linearity for all points (see Results).

To evaluate the expression of Id2, TALI, and E2A genes, an aliquot of RT-RNA (approximately 20 ng) was amplified within the linear range by 40 PCR cycles. An aliquot of RNA (approximately 20 ng) of each sample and a mock reaction (negative controls) were amplified to exclude the presence of contaminant DNA. The amplification procedure involved denaturation at 95°C for 30 seconds, annealing at 54°C for Id2, at 58°C for TALI, and at 50°C for E2A; and extension at 72°C for 45 seconds for 40 PCR cycles.

RT-RNA from the K562 cell line or adult quiescent T lymphocytes, normalized together with RT-RNA from hematopoietic cells by the \(\beta_2\)-microglobulin gene, was used as internal positive control.

PCR products were analyzed by Southern blotting with an internal oligomer probe. Relative intensities of bands were quantified by scanning with a laser densitometer (Phosphorimager; Molecular Dynamics, Sunnyvale, CA).

The following 5' and 3' primers and probes were used: Id2: 5' primer, 5'-TCTTGGCAAGCTTTCTGCAA-3'; 3' primer, 5'-CCAATCATTGCTTCCCTC-3'; internal labeled probe, 5'-GCCAGTGTTGGCTGCTGTTAACAGGCTTCGCCATGAATTTAGC-3'\(^{14}\); \(\beta_2\)-microglobulin gene, 5' primer, 5'-ATGGTGCAAGCTTGGACCTCC-3'; 3' primer, 5'-TCTCATCTTCTGGAGGCCTTC-3'; internal labeled probe, 5'-GATGCTTCCTCCTTGACATTGCGGCTTC-3'; internal probe from 311 bp to 360 bp; 5'-GCCGGCTCCTTGACATTGCGGCTTC-3'; and 3' primer, 5'-TGGTGTGCGATGAGCCCTCGCG-3'; internal labeled probe, 5'-CTGCAAGTGTTAGATAGAAGACCCATCCGAGCA-3'.

**Bacterial Expression of Murine and Human Glutathione-S-Transferase (GST)-Id2 Fusion Polypeptides**

Plasmid pGEX-5X-3T (Pharmacia) was used for expression of wild-type GST protein. Plasmid Id2 pGEX-5X-3T, used for expression of the murine GST-Id2 fusion protein, was generated by inserting a 0.35-kb murine Id2 cDNA fragment\(^{40}\) into the BamHI/SalI sites of pGEX-5X-3T. The human GST-Id2 fusion protein (pGST-Id2) was obtained from Mark A. Israel (School of Medicine, University of California at San Francisco).\(^{48}\) Expression and purification of GST fusion proteins were performed as described previously.\(^{37}\)

**Rabbit Antisera**

TALI: Polyclonal antisera (no. 1080 and 370) were obtained against GST-fusion proteins containing the amino-terminal 121 residues and carboxy-terminal 94 residues, respectively.\(^{40}\)

E2A: The GST-E2A fusion protein (E2A residues 217 to 371) was used to produce polyclonal antisera.\(^{23}\)

**Electrophoretic Mobility Shift Assay (EMSA) and Western Blot Analysis**

The protein concentrations of all extracts were determined by the Bradford assay (Bio-Rad, Hertfordshire, UK). Each reaction contained, in 20 µL, 10 to 25 µg of nuclear extract, 10 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 2 mmol/L Mg Cl\(_2\), 4% Ficoll, 1 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), 0.5 µg of poly (dl-dC), and 1 to 3 pmol of \(^{32}P\)-labeled, double-stranded oligonucleotide probe containing the preferred sequence for DNA-binding by TALI/E2A heterodimers (top strand: ACCCTGAAGATGCTGTCGTC).\(^{23}\)

After a 15-minute incubation at room temperature, the assay mixture was loaded onto a 15-cm 4% polyacrylamide gel containing 0.25× TRIS-borate/EDTA electrophoresis buffer and electrophoresed at 180 V for 2 to 3 hours at 4°C. In some binding reactions, the nuclear extract was preincubated for 10 minutes at room temperature with 1 µL of TALI rabbit antisera no. 370, the E2A antiserum, or the same amount of normal rabbit immunoglobulin. Where indicated, an approximately 100-fold molar excess of unlabeled competitor oligonucleotides was included in the binding reaction. Western blot analysis was performed with the anti-TALI (no. 1080) rabbit serum by enhanced chemiluminescence, according to the manufacturer's protocol (Amersham, Buckinghamshire, UK).

**HPC Oligomer Treatment and Clonogenetic Assay**

**Oligomers.** The following oligomers were used: Id2 antisense (a-Id2), 5'-CTTTAACGGAGCTTACGGAGCTTACGAA-3' complementary to 24 nucleotides starting from the fourth codon Id2 mRNA; \(\delta\)Id2 scrambled, 5'-CAACTAGCGTGAAGACTGCGA-3'; TALI antisense (aTALI), 5'-GCTCGTGGGGCGCTTCCGCG-3'; TALI scrambled (\(\delta\)TALI), 5'-GGCGGCTCCGCTGCGCGC-3'.

**Oligomer treatment.** Step IIIP cells were diluted in FCS medium (see above) supplemented with HGFS (IL-3/GM-CSF/Epo; see Clonogenetic assay) in the presence or absence of antisense or randomly scrambled phosphorothioate oligomers at appropriate concentrations (25, 50, 100 µg/mL) and incubated overnight; cells were plated in clonogenetic culture in triplicate dishes (see below).\(^{20,24,37,41}\)

**Oligomer uptake.** Fluoresceinlabeled oligomers were obtained from National Biosciences (Plymouth, MN). Step IIIP HPCs (5 × 10^4) were treated with 100 µg/mL scrambled or antisense oligomers overnight in FCS culture with standard HGFS (IL-3/GM-CSF/Epo; see Clonogenetic assay); cells were washed and analyzed by FACS.\(^{28}\)

**Clonogenetic assay.** Step IIIP HPCs were seeded (2 × 10^5/mL per dish, three plates per point) and cultured in 0.9% methycellulose in IMDM supplemented with \(\alpha\)-thioglycerol (10^\(-4\) mol/L; Sigma) and different HGFS [IL-3 (100 U), GM-CSF (10 ng) ± Epo (3 U), unless otherwise indicated] at 37°C in a 5% CO_2/5% O_2/90% N_2 humidified atmosphere.\(^{20,24}\) PCS was substituted as indicated above for liquid suspension culture.

CFU-GEMM, BFU-E, and CFU-GM colonies were scored on day 16 to 18. In selected experiments, colonies were picked up, and the cell composition was analyzed as described.\(^{20,24}\)'
RESULTS

HPC Purification and Unilineage Differentiation in Liquid Suspension Culture

In this experimental series, purified step IIIP cells comprised 85% to 95% CD34+ cells and 75% to 95% HPCs (BFU-E, CFU-GM, and CFU-GEMM), as assayed in clonogenetic culture after optimal HGF stimulus (saturating KL/IL-3/GM-CSF/Epo dosage; Fig 1A). It is noteworthy that the HPC recovery was markedly higher (45% mean value; Fig 1A) than reported by a previous methodology.26

Step IIIP HPCs were induced to gradual unilineage erythroid or granulocytic differentiation up to terminal maturation by differential HGF stimulus: ie, saturating Epo and low IL-3/GM-CSF dosage, or saturating IL-3/GM-CSF level, respectively. These HGF combinations have been previously used25,26 in unilineage suspension culture of HPCs purified as reported by Gabbianelli et al26 and Labbaye et al27. Fig 1B and results not shown; see also Giampaolo et al28 and Sposi et al29. During the first week of culture, the HPCs undergo a gradual increase in number, paralleled by a decrease of both proliferative potential, as indicated by the decline of colony size, and CD34+ expression. In the second week, the gradual maturation of morphologically recognizable precursors to terminal cells is indicated by the progressive shift of membrane phenotype (eg, gradual rise of glycophorin A+ and CD11b+ cells in the erythroid and granulocytic pathways, respectively) and morphology. The erythroid culture comprises ≥97% to 99% erythroblasts in the second week, while the granulopoietic system includes a minority (≤5% to 10%) of monocytic cells in the terminal phase.

Id2, TAL1, and E2A mRNA Expression in Erythroid and Granulopoietic Liquid Cultures

RNA samples were generated at sequential times from step IIIP HPC liquid suspension culture induced to unilineage erythroid or granulopoietic differentiation: ie, at days 0, 1, 3, 5, 7, 10, 12, 14, and 17 (≈10⁵ cells per time point).

A series of controls insured a semiquantitative evaluation of mRNA levels (see Materials and Methods), including dose-response curves for the assayed templates (Fig 2A). The expression of Id2, TAL1, and E2A mRNA was evaluated in three separate RNA samples from three independent culture experiments. We observed a remarkably reproducible mRNA expression pattern: representative results are shown in Fig 2B.

Id2 gene was present on day 0 quiescent HPCs. The expression level gradually decreased during initial erythroid differentiation to an undetectable level from day 5 through day 17, and was progressively reinduced in terminal maturation through day 10 to 17. Conversely, the Id2 mRNA level did not fluctuate during granulocytic differentiation/maturation, except for a transient drop at day 10 to 12.

TAL1 mRNA was not detected in quiescent HPCs. In the erythroid culture, TAL1 expression was induced through differentiation and initial maturation (from day 1 through 10) and then sharply increased in advanced maturation at day 12 to 14. In the granulopoietic system, expression was induced during the first week and drastically downmodulated during the second week.

The E2A mRNA pattern mimicks that of TAL1. The E2A gene was not expressed in quiescent HPCs. In the erythroid culture, moderate expression was observed from day 7 through day 12 to 14. In the granulopoietic system E2A mRNA was detected at decreasing levels from day 1 through 7, followed by no expression through day 17.

EMSA of TAL1/E2A Heterodimer in HPC Differentiation

To evaluate the DNA-binding activity of TAL1 in HPC erythroid and granulopoietic cultures, EMSA was performed with a radiolabeled oligonucleotide probe containing the TAL1 consensus E-box sequence.23

As shown in Fig 3A, incubation of this probe with the nuclear extract from Jurkat cells (lanes 1 through 6) generated several protein-DNA complexes (lane 2). Two of these complexes, indicated by dashes, deserve detailed analysis. We observed that (1) the high mobility complex was supershifted by preincubation with antiserum raised against the carboxy terminal 94 residues of TAL1 (lane 5), but not with the corresponding preimmune serum (lane 4); and (2) both the high and low mobility complexes were supershifted by preincubation with an anti-E2A rabbit serum (lane 3), but not with the corresponding preimmune serum (lane 2). In line with the results reported by Hsu et al,23 our observations confirm that the high mobility complex represents the E2A/TAL1 heterodimer, while the low mobility one can be ascribed to the E2A/E2A homodimer,23 as further indicated by in vitro studies.22

We then assayed the nuclear extracts from day 7 erythroid culture (Fig 3A). The erythroid cells exhibit a band (lane 7) comigrating with the E2A/TAL1 complex in Jurkat cells (lane 6). This band is subtotally abrogated by incubation with the TAL1 consensus E-box oligonucleotide (lane 8). Furthermore, it is sharply supershifted by incubation with anti-TAL1 or anti-E2A sera (lanes 10 and 12, respectively), while it is not affected by the corresponding preimmune serum (lanes 9 and 11). We conclude that this band represents the E2A/TAL1 heterodimer in erythroid cells.

We then incubated the TAL1 consensus E-box sequence with equivalent amounts (10 µg) of nuclear extracts from erythroid and granulopoietic cultures (Fig 3B): the E2A/TAL1 heterodimer, not detected in quiescent HPCs at day 0 (lane 6), was present at days 7, 11, and 14 in the erythroid lineage (lanes 7, 8, and 9), but not in the granulopoietic series (lanes 10, 11, and 12). In other experiments, we also determined the presence of E2A/TAL1 heterodimers at days 7 and 12 (data not shown): we observed no difference when compared with days 7 and 11 (Fig 3B). Thus, it is apparent that, in both erythroid and granulopoietic cultures, the kinetic pattern of E2A/TAL1 heterodimer roughly correlates with that of TAL1 and E2A mRNA, as evaluated by RT-PCR (at late erythroid culture times; however, the binding activity decreases, whereas the RNA expression is sustained).

The low mobility complex has a higher molecular weight in the erythroid lineage as compared with Jurkat cells (Fig 3A and B). However, the low mobility band in erythroid cells is also diminished with the anti-TAL1 antibody, sug-
Fig 1. (A) Normal step IIIp cell purification: total cell number, percentage of CD34+ cells, HPC frequency, and recovery (mean ± SEM values from 12 separate experiments are indicated by horizontal bars). Total cell number (left panel) was evaluated after different steps of purification, including the Ficoll cut, the Percoll gradient, and the three sequential passages on magnetic beads. The percentage of CD34+ cells in step IIIp cells (right panel) was evaluated as indicated in Materials and Methods. Step IIIp HPC (BFU-E + CFU-GM + CFU-GEMM) frequency and recovery were evaluated by clonogenic assays of FCS+ cultures. HPC recovery is expressed as percentage of progenitor cells present at the end of purification (Step IIIp) as compared with their number in PBMCs (Step 1). (B) Morphology analysis of step III HPCs differentiating in the erythroid (E) or granulomonocytic (G) culture system (mean ± SEM values from three separate experiments).
suggesting that TAL1 is part of the complex (Fig 3B). The possibility exists that, in the erythroid culture, TAL1/E2A can bind to other protein(s). Further studies are required to elucidate this aspect.

**TAL1 Western Blot Assay**

Western blot analysis was performed with equivalent amounts (13 μg) of nuclear extracts from day-0 HPCs and erythroid or granulopoietic culture at days 7 and 12 (Fig 3C). Immunoblotting was performed with rabbit antiserum no. 1080. TAL1 protein, not detected in day-0 HPCs, is selectively expressed in erythroid culture on day 7 and, at a lower level, on day 12.

**Antisense Oligonucleotide Experiments**

Three separate dose-response experiments were performed with phosphorothioate antisense oligomers to Id2 or TAL1 mRNA (α-Id2, α-TAL1). Step III P HPCs were incubated with 25, 50, and 100 μg α-Id2 or α-TAL1 oligomers, while control HPCs were either untreated or incubated with...
Fig 3. (A) EMSA of DNA binding by TAL1/E2A complexes of Jurkat leukemic T cells and day 7 erythroid culture cells (representative results from several independent experiments). A double-stranded 32P-labeled oligonucleotide probe containing the TAL1-binding consensus sequence (upper strand: ACCTGAACAGATGGTCGGCT) was incubated with nuclear extracts from Jurkat cells (lanes 1 through 6) and from erythroid culture cells (lanes 7 through 12). incubation of this probe with the nuclear extract from Jurkat cells (lanes 1 through 5) generated several protein-DNA complexes. Two of these complexes, labeled E2A/E2A and TAL1/E2A, were partially supershifted (arrow) by preincubation with an anti-E2A rabbit serum (lane 3) but not with the corresponding preimmune serum (lane 2). The high mobility complex was supershifted (left arrow) by preincubation with antiserum raised against the carboxy-terminal 94 residues of TAL1 (lane 5), but not with the corresponding preimmune serum (lane 4). The erythroid cells exhibit a band (lane 7) comigrating with the E2A/TAL1 complex in Jurkat cells (lane 6). This band is subtotally abrogated by incubation with an approximately 100-fold excess of the unla- beled TAL1 consensus E-box oligonucleotides (lane 8). Furthermore, it is supershifted (right arrow) by incubation with anti-TAL1 and anti-E2A serum (lanes 10 and 12, respectively), while it is not affected by the corresponding preimmune serum (lanes 9 and 11). (B) EMSA of TAL1/E2A heterodimer in HPC erythroid and granulopoietic cultures (representative results from three independent experiments). 32P-labeled TAL1 consensus E-box oligonucleotide was incubated with nuclear extracts from erythroid and granulopoietic culture (lanes 6 through 12) or Jurkat cells (lanes 1 through 5; see supershift band in lane 5, as indicated by arrow in panel A). The E2A/TAL1 heterodimer, as compared with Jurkat cells (lanes 1 through 5), was not detected in the quiescent HPCs at day 0 (lane 6) and was present at days 7, 11, and 14 in the erythroid (E) lineage (lanes 7, 8, and 9, respectively), but not in the granulopoietic (G) series (lanes 10, 11, and 12). The Jurkat cell extracts were preincubated with anti-TAL1 serum (no. 370) (lane 5), corresponding preimmune serum (lane 4), or an approximately 100-fold excess of wild-type TAL1 binding sequence (lane 3). Protein-oligonucleotide complexes representing E2A homodimers (E2A/E2A) or TAL1 heterodimers (E2A/TAL1) are indicated.
corresponding amounts of scrambled oligomers (Fig 4A and B). The clonogenic assay of control, α-sense, or scrambled oligomer-treated HPCs was then performed in the presence of intermediate or saturating IL-3/GM-CSF dosage combined or not with intermediate or plateau Epo levels in experiments with α-lid2 or αTAL1, respectively (Fig 4A and B).

Treatment with α-lid2 caused a significant dose-dependent rise of BFU-E colonies, as compared with scrambled-treated or untreated controls. A modest, barely significant increase in CFU-GM colony number was observed only in the absence of Epo at the 100-μg α-lid2 dosage (Fig 4A). Control RT-PCR experiments were performed to verify the specific suppression of lid2 mRNA by α-lid2 treatment. Thus, step IIIP cells were grown in liquid phase culture mimicking the methylcellulose culture conditions [ie, cells were treated with saturating IL-3/GM-CSF/Epo dosage and α-TAL1 at day 0 (100 μg) and day 3 (50 μg)]. At day 5 of culture, TAL1 mRNA was significantly reduced in α-TAL1−treated cells, as compared with corresponding scrambled oligomer-treated or untreated cells; β2-microglobulin mRNA levels were similar in all three groups (data not shown). Further control studies were performed to evaluate the oligomer uptake: greater than 90% of cells were labeled after overnight treatment with fluoresceinated α-lid2 or scrambled oligomer.

Human and Murine GST-lid2 Polypeptides Compete With the TAL1/E2A-Specific DNA-Binding Activity

To test directly whether lid2 protein is capable of inhibiting specific DNA-binding by the TAL1/E2A heterodimers, murine and human GST-lid2 polypeptides were expressed in E. coli and purified by affinity chromatography on glutathione-agarose beads. Murine and human GST-lid2 polypeptides, both containing the HLH region with 98% homology at the amino acid level, competed with the DNA-binding activity of the TAL1/E2A heterodimer when added to the nuclear extracts derived from day-7 erythroid culture cells (Fig S).

These results suggest that abrogation of the DNA binding by TAL1/E2A heterodimers in late erythroid maturation, as seen in Fig 3B, is likely caused by the reinduced expression of dominant negative lid2 protein.

DISCUSSION

The discrete molecular events underlying early hematopoiesis are still poorly understood, primarily due to the extreme rarity of HPCs and HSCs, which represent less than 1/0.01% and less than 0.1/0.001% of human bone marrowα and PB11.26.44 cells, respectively. Thus, it is necessary that early HPCs/HSCs be stringently purified and then cultured under conditions allowing a gradual, homogenous wave of differentiation specifically along one or more lineage(s). We have recently developed methodology for HPC purification and unilineage differentiation in liquid phase culture. The original purification methodology20 has been recently improved to allow both stringent purification and abundant recovery of HPCs (CFU-GEMM, BFU-E, CFU-GM) from adult PB.27.28 Several lines of evidence indicate that the puri-
Fig 4. (A) Dose-response effect of Id2 antisense phosphorothioate oligomers (α-Id2) on step IIIP BFU-E and CFU-GM colony formation. Cells incubated overnight with oligomers in FCS- liquid medium were plated the next day in FCS- semisolid cultures and scored at day 17. Values are means ± SEM values from triplicate dishes. BFU-E colony number: untreated control, 63 ± 0.6; scrambled α-Id2, 62 ± 2.0, 60 ± 2.3, and 54 ± 1.7; and α-Id2, 70 ± 1.2, 73 ± 0.9, and 75 ± 2.3; at 25, 50, and 100 μg levels, respectively. A representative experiment from three independent experiments is shown. *P < .05; **P < .01 by Student’s t-test. Top inset: Control RT-PCR studies on Id2 and β2-microglobulin (β2m) mRNA levels. Representative results on control-, scrambled α-Id2- (Scrambl., 100 μg), and α-Id2- (100 μg) treated cells are shown. For other details, see Results. (B) Dose-response effect of TAL1 antisense phosphorothioate oligomers (α-TAL1) on step IIIP BFU-E and CFU-GM colony formation. Cells were treated with oligomers in FCS- liquid medium at day 0 (full dose) and day 3 (half dose) for six additional hours before plating in semisolid cultures. The day-0 dosage is indicated. For other details, see legend panel A. Values are means ± SEM from triplicate dishes; a representative experiment from three independent experiments is shown. *P < .05; **P < .01 by Student’s t-test. Top inset: Control RT-PCR studies on TAL1 and β2-microglobulin (β2m) mRNA levels. Representative results on control-, scrambled α-TAL1- (Scrambl., 100 μg), and α-TAL1- (100 μg) treated cells are shown. For other details, see Results.
expression pattern. Both genes, while silent in quiescent HPCs, are expressed in the erythropoietic pathway, particularly at advanced maturation stages. In granulopoietic differentiation, their expression is initially induced but then abrogated at the early maturation stage.

The expression pattern of the heterodimeric TAL1/E2A protein complex is basically in line with that of TAL1 and E2A mRNA. The TAL1/E2A heterodimer is absent in quiescent HPCs, present throughout erythroid maturation, and not detected in granulopoietic maturation. Similarly, TAL1 protein is detected by Western blot assay in erythroid but not granulopoietic culture.

The accumulation of TAL1 mRNA at the late erythroid maturation stage (ie, day 10 to 12) does not reflect a comparable accumulation of TAL1 protein, as evaluated by Western blot. This difference may be hypothetically ascribed to a regulatory mechanism hindering TAL1 mRNA translation in terminal erythroid maturation: further studies are required to elucidate this aspect.

It is noteworthy that TAL1 expression data are consistent with the functional results. Thus, α-TAL1 oligomer selectively causes a marked decrease of TAL1 mRNA level and a dose-related inhibitory effect on erythroid colony formation: the inhibition is dependent on repeated addition of the oligomer (at days 0 and 3), thus indicating that TAL1 is functionally required after initiation of the erythroid differentiation program. The blockade of erythroid differentiation induced by α-TAL1 is only partial: this indicates that the suppression of TAL1 expression induced by α-TAL1 was not complete, and/or other erythroid TFs cooperate with TAL1 to induce erythroid differentiation in relatively advanced stages (see below).

Studies on erythroleukemic cell lines have and erythroid ontogenetic development have suggested but not proven a possible role for TAL1 in erythropoiesis. Our results provide novel in vivo evidence on the expression and role of TAL1 and TAL1/E2A complex in normal adult erythroid progenitor differentiation.

The mechanism of TAL1 function in erythropoiesis deserves discussion. In differentiating purified HPCs, the expression pattern of TAL1 gene is remarkably similar to that of GATA-1: both patterns are characterized by cell cycle-dependent initiation and lineage-dependent abrogation in the granulopoietic series. In this regard, several lines of evidence suggest a close functional relationship between TAL1 and GATA-1 genes. TAL1 is expressed in erythroid, mast, and megakaryocytic cell lines—a profile remarkably similar to that of GATA-1. Furthermore, TAL1 may be a target for GATA-1 in that the TAL1 proximal promoter displays a GATA motif that binds GATA-1 with high affinity, GATA-1 is required for full TAL1 promoter activity in transfection experiments, and the proximal TAL1 promoter mediates reporter transactivation by overexpressed GATA-1 protein.

A key mechanism in erythroid differentiation may be represented by GATA-1 activation of the TAL1 gene via the GATA motif in the TAL1 proximal promoter: this would in turn lead to TAL1/E2A heterodimer formation and activation of erythroid-specific genes via interaction with E-box motifs in their promoters. In this regard, a survey of the nucleotide data base showed possible TAL1/E2A binding sites associated with a number of candidate erythroid target genes: eg, in the hypersensitivity site 2 of the β-globin locus control region (CCCAGATGT), near the 3'-enhancer of the human γ-globin gene (AACAGATGTT), and in the erythroid promoters of the carbonic anhydrase II (TTTCATATTT), band 3 (TCCAGATTG), and GATA-1 (ATCATATGTA) genes.

The relationship between the Id2 and TAL1/E2A expression/function in differentiating HPCs is of interest. As previously mentioned, in the murine 32D and MEL cell lines, induction of granulopoietic and erythroid differentiation is coupled with downmodulation of Id and upmodulation of an E-box–binding activity. Furthermore, Id2 protein competes with TAL1 for heterodimer formation with E2A in murine M1 leukemic cell line induced to macrophage differentiation. However, these studies on immortalized murine
cell lines do not elucidate the expression/function of Id protein in normal human hematopoiesis.

We observed that in HPC erythroid differentiation the Id2 expression/function pattern mirrors that of TAL1 and E2A. Indeed, Id2 mRNA is present in quiescent HPCs, but is gradually abrogated in early erythropoiesis up to the advanced maturation stage. Furthermore, α-Id2 treatment selectively induced a marked decrease of Id2 mRNA level and an enhanced erythroid colony formation. [Id1 and Id3 mRNAs are barely or not expressed in HPC erythroid and granulopoietic differentiation (data not shown).] More importantly, murine and human GST-Id2 polypeptides competed with the specific DNA-binding activity of the TAL1/E2A heterodimer when added to nuclear extracts from day-7 erythroid culture cells. Thus, these experiments suggest that abrogation of the TAL1/E2A-specific DNA-binding activity in late erythroid maturation is likely caused by the reinduced expression of dominant negative Id2 protein.

Collectively, these novel results suggest that in the normal adult erythropoietic system downmodulation of Id2 allows the TAL1 and E2A protein interaction to generate heterodimers activating erythroid-specific genes. In the final erythroid maturation stage, Id2 upmodulation may hamper TAL1/E2A heterodimer formation, thus leading to terminal suppression of erythroid specific genes.

Our observations indicate a coordinate expression and function of an inhibitory Id protein and a stimulatory bHLH heterodimer (TAL1/E2A) in normal adult erythroid differentiation. Similar inhibitory/stimulatory Id/bHLH protein mechanisms may operate in other, particularly granulopoietic, hematopoietic lineages.

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