Suppression of Erythroid but not Megakaryocytic Differentiation of Human K562 Erythroleukemic Cells by Notch-1

(Running Title: Notch-1 and erythroleukemia cell differentiation)

Lloyd T. Lam, Chiara Ronchini, Jason Norton, Anthony J. Capobianco, and Emery H. Bresnick

1University of Wisconsin Medical School, Department of Pharmacology, Molecular and Cellular Pharmacology Program, 387 Medical Science, 1300 University Avenue, Madison, WI 53706;
2University of Cincinnati College of Medicine, Department of Molecular Genetics, Biochemistry and Microbiology, Cincinnati, OH 45267-0524

*To whom correspondence should be addressed

TEL: 608-265-6446; FAX: 608-262-1257; E-Mail: ehbresni@facstaff.wisc.edu

Copyright 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
ABSTRACT

The Notch signal transduction pathway is a highly conserved regulatory system that controls multiple developmental processes. We have established an erythroleukemia cell model to study how Notch regulates cell fate and erythroleukemic cell differentiation. K562 and HEL cells expressed the Notch-1 receptor and the Notch ligand Jagged-1. The stable expression of the constitutively active intracellular domain of Notch-1 (NIC-1) in K562 cells inhibited erythroid without affecting megakaryocytic maturation. Expression of antisense Notch-1 induced spontaneous erythroid maturation. Suppression of erythroid maturation by NIC-1 did not result from downregulation of GATA-1 and TAL-1, transcription factors necessary for erythroid differentiation. Microarray gene expression analysis identified genes activated during erythroid maturation, and NIC-1 disrupted the maturation-dependent changes in the expression of these genes. These results show that NIC-1 alters the pattern of gene expression in K562 cells leading to a block in erythroid maturation and therefore suggest that Notch signaling may control the developmental potential of normal and malignant erythroid progenitor cells.
INTRODUCTION

The Notch signal transduction pathway is a highly conserved regulatory system that controls multiple developmental processes (1,2). Notch signaling is mediated by a single-pass transmembrane Notch receptor (1). The extracellular domain of Notch contains a ligand binding site that interacts with transmembrane ligands such as Jagged and Delta (3). Ligand binding induces the site-specific proteolytic cleavage of the intracellular domain of Notch (NIC), liberating NIC from the residual membrane-bound polypeptide (4,5). NIC then enters the nucleus and associates with the DNA-bound transcription factor suppressor of hairless or CBF1. NIC binding converts CBF1 from a transcriptional repressor to an activator and alters target gene expression (6,7). The presence of multiple Notch homologs (Notch 1-4) (8), multiple ligands [e.g., Jagged-1 (9), Jagged-2 (10), and Delta (11)], and additional components that modulate Notch signaling [e.g., deltex (12), suppressor of deltex (13), and lunatic fringe (14)] endow considerable complexity to the Notch signaling system. Moreover, certain biological actions of Notch are CBF1-independent (15,16), adding further diversity to Notch signaling.

In addition to the diverse biochemical aspects of Notch signaling, Notch serves multiple developmental functions (2,8). Developmental processes regulated by Notch include somitogenesis, myogenesis, neurogenesis, and hematopoiesis. Seydoux and Greenwald (17) have described the lateral inhibition hypothesis of Notch function, in which one cell conveys inhibitory signals to its neighbor through Notch ligand-receptor interactions. These signals suppress differentiation of one lineage and permit differentiation into a distinct lineage. This activity to control cell fate is exemplified by analysis of neurogenesis in Drosophila (18). In the ventral blastoderm of Drosophila, precursor cells develop into either neuroblasts or epidermal cells. When Notch signaling is impaired by Notch mutations, the precursor cells develop exclusively into neuroblasts. The Notch ligand Delta expressed in neuroblasts generates signals to surrounding cells, suppressing further neurogenesis. Embryos with gain-of-function Notch mutations show increased numbers of epidermal cells and reduced numbers of neuroblasts. Thus, Notch signaling
regulates cell fate by controlling asymmetric cell division during stem/progenitor cell differentiation. Since Notch has multiple developmental functions, it is not surprising that components of the Notch pathway are critical for survival. Disruption of murine genes encoding Notch-1 (19), the ankyrin repeats of Notch-2 (20), Jagged-1 (21), or CBF1 (22) have embryonic lethal phenotypes. Although components of the Notch pathway are expressed in multiple hematopoietic cell types (7, 22, 23, 23-26), the role of Notch in hematopoiesis is ill-defined (27). Considering that hematopoietic stem (HSC)/progenitor cells interact functionally with stromal and endothelial cells in the hematopoietic microenvironment (28), it seems logical that Notch may be important for this intercellular communication. Several lines of evidence implicate Notch as a regulator of hematopoiesis. Infection of murine bone marrow with a retrovirus expressing constitutively active Notch-1 induced T-cell leukemia in a bone marrow reconstitution assay (29). Second, constitutively active Notch-1 and Notch-2 inhibited the myeloid differentiation of the murine 32D cell line (30). Third, expression of constitutively active Notch in T-cells of transgenic mice induced thymocytes to form CD8+ T-cells, rather than CD4+ T-cells (31). Lastly, a conditional knock-out of mouse Notch-1 (32) and disruption of Jagged-2 (33) caused defective T-cell differentiation.

While studying protein components of the β-globin locus control region (LCR), we identified CBF1 as a protein in K562 nuclear extracts that binds a conserved, functionally important region of the LCR (23). As no previous studies had investigated the role of Notch signaling in erythroid cell differentiation and function, we have now asked whether other components of the Notch pathway are expressed in erythroleukemic cells and if a constitutively active Notch receptor influences erythroleukemia cell maturation.
EXPERIMENTAL PROCEDURES

**Plasmids.** The constructs encoding sense and antisense human Notch-1 were described previously (34) (gifts from Jorge Laborda, Food and Drug Administration). The sense and antisense constructs were subcloned into the *Pvu*II site of the plasmid pEBVHisA (Invitrogen), which contains a hygromycin resistance gene. Transcripts encoded by the constructs correspond to amino acids 1176 to 2232 of human Notch-1. The pBabe-NIC-1 expression vector encoding constitutively active Notch-1 (NIC-1) was described previously (35). This vector was derived from the pBabe-puro retroviral vector (36) and includes a cDNA sequence of human *Notch-1* encoding amino acids 1758 to 2556. The expression vector encoding amino acids 1758 to 2556 of human Notch-1 with a myc epitope tag fused to its carboxy terminus was constructed from pcDNA3.1 by standard techniques. The Notch-dependent reporter plasmid containing four CBF1 binding sites and a simian virus 40 promoter fused to luciferase (4×wtCBF1Luc) was described previously (37) (gift of Diane Hayward, Johns Hopkins Medical School).

**Cell Culture.** The human erythroleukemia cell lines K562 and HEL were propagated in Iscove’s Modified Eagle’s Medium (Biofluids) containing 25 µg/ml gentamycin and 10% fetal calf serum (Gibco-BRL) (complete IMEM). The cell lines were grown in a humidified incubator at 37°C, in the presence of 5% carbon dioxide. K562 and HEL cells were treated with 40 µM hemin for 48 h or 50 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) for 6 days prior to RNA preparation. In certain experiments, TPA treatments were for shorter times as specified in the figure legends. Erythroid differentiation of K562 cells was also induced with sodium butyrate (0.5 mM) by treatment of cells for 3 days.

**Benzidine Staining.** The benzidine stock solution contained 0.2% w/v benzidine hydrochloride in 0.5 M acetic acid. Cells (1 x 10^5) were washed twice with ice-cold PBS. The cell pellets were resuspended in ice-cold PBS (27 µl). The benzidine solution (3 µl) containing hydrogen peroxide (0.0012% final concentration) was added and incubated for 10 min at room temperature. Benzidine
positive cells were quantitated by light microscopy. At least 100 cells were counted in triplicate for each condition.

**Stable Transfection and Retroviral Infection Assays.** K562 cells were stably transfected by electroporation with a Bio-Rad Gene pulser electroporator. Cells (5 x 10^6) were washed, resuspended in 0.5 ml of ice-cold PBS, mixed with linearized plasmid DNA (5 µg), and subjected to electroporation (960 µF; 220 V) in a 0.4 cm wide electroporation cuvette (BTX). pBabe and pBabe-NIC-1 were linearized with NotI. Cells were then added to 10 ml of complete IMEM, grown for two days, and diluted in complete IMEM containing 1.5 µg/ml puromycin. Cells were propagated in complete IMEM containing 1.5 µg/ml puromycin (pools of K562-Babe and K562-NIC-1 cells). Importantly, stably transfected cells were analyzed for erythroid differentiation as soon as the pools were generated (approximately 2 to 3 weeks) to reduce the probability of phenotypic changes that may result from prolonged growth.

For retroviral infection of K562 cells, pBabe-NIC-1 (5 µg) and pMD.G (2 µg) were cotransfected into modified 293 human embryonic kidney cells (2 ml, 10^5/ml) by the calcium phosphate transfection method as described previously (38). The media was changed once after 8 h of transfection to remove the calcium phosphate. The pMD.G expression vector encodes the viral envelope protein VSV-G. The modified 293 cells were previously stably transfected with pol and gag genes (gift of Shigeki Miyamoto, University of Wisconsin Medical School). K562 cells (3 ml, 2 x 10^5/ml) were added with polybrene (4 µg/ml) in complete IMEM and incubated for 48 h. The infected cells were separated from adherent 293 cells and then selected with puromycin (1.5 µg/ml).

**Transient Transfections.** K562 cells (5 x 10^6) stably transfected with pBabe-NIC-1 (Fig. 3C) or control cells (Fig. 2) were collected by centrifugation at 240 x g for 6 min at 4°C and resuspended in complete IMEM containing 1.5 µg/ml puromycin. Plasmid DNA (1 µg of 4×wtCBFILuc or 5xGAL4Luc) was suspended in 150 µl of complete IMEM, incubated with 4 µl of Superfect (Qiagen) for 15 min at room temperature and then added to cells. For the experiment of Fig. 2, 1 µg of pBabe or pBabe-NIC-1 was cotransfected with the reporter vectors. After incubating for 40 h, cells were harvested and assayed for luciferase activity. The luciferase activity
was normalized by the protein content of the lysates, determined by Bradford assay using γ-globulin as a standard.

**RNA Analysis.** Total RNA from K562 or HEL cells was extracted with Triazol (Gibco-BRL). RT-PCR was carried out with a Promega RT-PCR kit. Total RNA (0.2 ng, 2 ng, 20 ng or 200 ng) was reverse transcribed at 48°C for 45 min with 0.25 U of AMV reverse transcriptase in a 25 µl mixture containing 0.2 mM nucleotide triphosphates, 0.25 U of Tfl DNA polymerase, 1 mM MgSO$_4$, and 25 pmoles of sense-antisense primers specific for the PCR products. The resulting cDNA pool was amplified by 35 cycles of PCR. The PCR products were resolved on 1.8% agarose gels and visualized by ethidium bromide staining. The RT-PCR primers used in this study were human *Notch-1* (5’ sense, GCCGAGCGACAGGTGTGACGT, 3’ antisense, CAACGGTAGAGGGCTCTCGGAT); human *Jagged-1* (5’ sense, ATACTTCAAAAGTGCCCTCAAG, 3’ antisense, TTCCGTGAGGACCACAGACGT); human *Integrin αIib* (5’ sense, AGCTACTGTTGCAAGCTTCAC, 3’ antisense, GCGCCCCGGGCGAGGTGCACG); human *Integrin β3* (5’ sense, GCCTCTTGGCCTCACCTCAGCT, 3’ antisense, CTGGGATAGCTTCTCATGACAGCCC); human *HPRT* (5’ sense, CAGACTGAAAGCTATTGTAATG, 3’ antisense, CTAGATGCTGTCTTTGATGTG).

**Western Blotting.** Nuclear extracts were prepared from K562 cells (2.5 x 10$^5$) as described previously (39). Proteins (15 µg) were resolved by SDS-PAGE on a 9% acrylamide gel. The proteins were transferred to an Immobilon P membrane (Millipore), and TAL1 was detected by Western blotting with an anti-TAL1 polyclonal antibody (40). The TAL1 antibody was incubated with the membrane for 12 h at 4°C, and immunoreactive proteins were visualized by incubation with a mouse anti-rabbit immunoglobulin conjugated to horseradish peroxidase, followed by chemoluminescence detection.

To detect stably expressed myc-tagged NIC-1 (NIC-1-myc), whole cell lysates were prepared in NP-40 lysis buffer [50 mM Hepes (pH 7.4), 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1% NP-40]. Lysates were cleared by centrifugation at 13,000 x g for 20 min at 4°C. Supernatants
were split into two aliquots and immunoprecipitated with either preimmune sera or anti-NIC 925 polyclonal antibody. Anti-Nic 925 is a rabbit polyclonal antisera directed against amino acids 1759-2095 of human Notch-1. Immune complexes were collected by adsorption to protein A-Sepharose. Proteins were resolved by SDS-PAGE, and NIC-1-myc was detected by immunoblotting with the anti-myc tag monoclonal antibody 9E10.

Electrophoretic Mobility Shift Assay. The preparation of K562 cell nuclear extracts and DNA binding analysis were performed as described previously (39). GATA-1 DNA binding activity was measured by EMSA with a double-stranded end-labeled oligonucleotide containing a high-affinity GATA-1 binding site (TTCGGTTGCAGATAAACATTGAAT). The specificity of DNA binding was assessed by competition with a 200-fold excess of homologous or unrelated oligonucleotide (EboxGTWT) (39) containing a repetitive GT sequence and a high-affinity E-box (GCTTAGGGTGTGGCCAGATGTTCTCAGC). DNA binding activity was quantitated by PhosphorImager analysis with ImageQuant software (Molecular Dynamics).

Microarray Gene Expression Analysis. Polyadenylated RNA was isolated from untreated and hemin-treated (20 µM, 48 h) K562 cells with the OligoTex (Qiagen) RNA kit. Microarray analysis was done by Incyte Pharmaceuticals Inc. (Palo Alto, CA). Briefly, polyadenylated RNA from untreated and hemin-treated K562 cells was reverse transcribed to generate Cy3 and Cy5 labeled cDNA probes, respectively. cDNA probes were competitively hybridized to a UniGEM1 cDNA microarray (Incyte Pharmaceuticals Inc.) containing 9844 immobilized cDNA fragments (average cDNA length, 500 - 5000 bp). Cy3 and Cy5 fluorescence were imaged individually, and the normalized ratios of Cy3/Cy5 fluorescence at a given spot on the microarray were used to calculate differential gene expression. Northern blotting was used to assess the influence of NIC-1 on gene expression. Total RNA (10 µg) was resolved on 1% agarose/formaldehyde gels and analyzed by standard procedures. Blots were hybridized under stringent conditions with random-primed probes, exposed to a PhosphorImager overnight, and quantitated with ImageQuant software. The HSP70 and IL-8 cDNA probes were gifts from Rick Morimoto (Northwestern University), and David
Denhardt (Rutgers University), respectively. The DD and α-globin cDNA probes were obtained from Genome Systems (IMAGE numbers 298560 and 74275, respectively).
RESULTS

Expression of Notch-1 and Jagged-1 in K562 and HEL Erythroleukemia Cells - Treatment of K562 cells with hemin induces an erythroid phenotype (41), whereas treatment with the phorbol ester TPA induces megakaryocytic differentiation (42). This system has been used to define factors that regulate leukemic cell differentiation, which may also control normal hematopoiesis [e.g., (43-48)]. To begin to assess whether Notch signaling regulates erythroid or megakaryocytic differentiation of K562 cells, we asked whether components of the Notch pathway are expressed in these cells. We previously detected CBF1 in K562 and mouse erythroleukemia (MEL) cells (23).

RNA isolated from untreated, hemin-treated, or TPA-treated K562 cells and another human erythroleukemia cell line (HEL) was analyzed by RT-PCR with Notch-1, Jagged-1, and HPRT primers. Notch-1 transcripts were detected at similar levels in all conditions (Figs. 1A and B). Jagged-1 transcripts were detected in undifferentiated K562 and HEL cells, and hemin treatment did not influence Jagged-1 expression. In contrast, treatment of K562 cells with TPA for 6 days enhanced Jagged-1 expression (2.6 +/- 0.1 fold; mean +/- SEM, n = 3) (Fig. 1A). We compared the time course for TPA induction of Jagged-1 with the induction of Integrin β3, a megakaryocyte-specific cell surface marker (Fig. 1C) (49). The induction of Integrin β3 was apparent after one day of TPA treatment and was maximal after three days (Fig. 1C). In contrast to K562 cells, TPA treatment of HEL cells did not increase Jagged-1 expression (Fig. 1B). However, as HEL cells constitutively express Integrin β3 (data not shown), the lack of Jagged-1 induction in this system may be related to the expression of megakaryocytic markers in the uninduced state (50).

CBF1-dependent Notch Signaling is Functional in K562 cells - To assess whether the Notch signaling pathway is functional in K562 cells, we used a reporter gene assay that measures CBF1-dependent Notch signaling. We tested whether a previously described vector encoding the constitutively-active cytosolic domain of Notch-1 (NIC-1) could activate a Notch-responsive reporter gene in transient transfection assays in K562 cells. The Notch-responsive reporter contained four binding sites for the Notch-regulated transcription factor CBF1 upstream of the
simian virus 40 promoter fused to luciferase (p4xCBF1luc). Cells were also transfected with a control reporter containing five GAL4 binding sites and lacking CBF1 binding sites (p5xGAL4luc). Strong luciferase activity was apparent when pBabe-NIC-1 was cotransfected with p4xCBF1luc but not with p5xGAL4luc (Fig. 2). In contrast, very low luciferase activity was measured with either of the reporters with or without the empty expression vector pBabe. Thus, the activation of p4xCBF1luc by transiently transfected pBabe-NIC-1 confirms that pBabe-NIC-1 encodes functional NIC-1 in K562 cells, and the CBF1-dependent Notch pathway is functional in K562 cells.

Expression of NIC-1 Inhibits Erythroid Maturation of K562 cells - To determine if Notch signaling influences erythroid maturation of K562 cells, we generated stably transfected and retrovirally infected pools of K562 cells containing pBabe-NIC-1 or the control vector pBabe. The cells were treated with hemin to determine whether NIC-1 altered their responsiveness to hemin-induced erythroid maturation. After treatment of cells for 48 h with hemin, cells were assayed for hemoglobin accumulation, a marker for erythroid differentiation, by benzidine staining. A representative staining pattern of cells stably transfected with the pBabe vector (K562-Babe) or pBabe-NIC-1 (K562-NIC-1) is shown in Fig. 3A. The percentage of benzidine positive cells was considerably lower for all eleven pools of K562-NIC-1 cells versus K562-Babe cells (Fig. 3B) (p < 0.001). Similar results were seen with six pools of K562 cells in which pBabe and pBabe-NIC-1 were stably introduced by retroviral infection (Fig. 3B).

Erythroid maturation of K562 cells can be induced by certain chemicals other than hemin, including butyrate, cytosine arabinofuranoside, and hydroxyurea (42). To determine whether NIC-1 inhibits erythroid differentiation by multiple inducers, we tested whether butyrate-induced differentiation was sensitive to NIC-1. The percent of benzidine positive K562-Babe cells was 1.9 +/- 0.9% (n=6) and 10.6 +/- 1.8% (n=12) for control and butyrate treated, respectively. Thus, butyrate was less efficient in inducing erythroid maturation than hemin. Similar to the results with hemin, expression of NIC-1 strongly inhibited butyrate-induced erythroid maturation [control and butyrate treated - 1.5 +/- 0.8% (n=6) and 3.5 +/- 0.8% (n=12) benzidine positive cells,
respectively]. Even though butyrate is a weaker inducer than hemin, the inhibition was highly significant ($p < 0.001$).

The pBabe-NIC-1 expression vector used in Figs. 2 and 3 was shown previously to overexpress NIC-1 protein after stable transfection into baby rat kidney cells (35). However, we have been unable to detect NIC-1 protein expression in K562-NIC-1 cells by Western blot analysis of whole cell extracts using the same antibody used in the previous study, an anti-human Notch-1 rat monoclonal antibody that recognizes the cytosolic domain of Notch-1 (35). Despite not being able to detect NIC-1, the results of Fig. 2 provide strong evidence that functional NIC-1 protein is expressed in the cells. The inability to detect NIC-1 by Western analysis may be due to the rapid turn-over and/or low-level expression of NIC-1. It is well established that NIC is unstable and can elicit biological effects at a very low protein concentration. An epitope-tagged derivative of NIC-1 containing six copies of a myc tag could not be detected with the anti-Myc 9E10 antibody under conditions in which it elicited a strong transcriptional response (4). As an alternative assay to assess whether functional NIC-1 is expressed in the stably transfected cells, we transiently transfected K562-Babe and K562-NIC-1 cells with the Notch-responsive reporter gene p4xCBF1luc. K562-NIC-1 cells had higher luciferase activities than untransfected K562 cells, whereas K562-Babe cells had a low background activity equivalent to untransfected K562 cells (Fig. 3C). These results provide evidence that K562-NIC-1 cells, which are strongly impaired in erythroid differentiation, express functional NIC-1.

K562 cells were also stably transfected with an expression vector encoding a myc-epitope tagged derivative of NIC-1 (NIC-1-myc) or the control empty vector. NIC-1-myc was detected by Western blotting with an anti-myc antibody in lysates from cells containing the NIC-1-myc expression vector but not the blank vector (Fig. 3D). To assess whether cells stably expressing NIC-1-myc were compromised in hemin-induced erythroid maturation, differentiation assays were performed with five independently derived pools of cells. Expression of NIC-1-myc suppressed erythroid maturation by 57% (Fig. 3E), consistent with the results with the pBabe-NIC-1 vector.
Expression of Antisense Notch-1 Induces Erythroid Maturation of K562 cells - As an additional approach to assess the importance of Notch-1 in the erythroid maturation of K562 cells, we generated clonal cell lines containing sense or antisense Notch-1 expression constructs or a control vector. The antisense construct was shown previously to reduce the levels of Notch-1 mRNA in 3T3-L1 fibroblasts (34). The benzidine reactivity of the clones was assayed as a measure of erythroid maturation (Fig. 4A). Five of ten antisense Notch-1 lines had a higher percentage of benzidine positive cells than lines stably transfected with the empty vector or sense Notch-1. The average percentage of benzidine positive cells for the empty vector, sense, and antisense clones was 4.5+/0.9, 2.0+/0.3, and 11.9+/2.9 (mean +/- SE), respectively (p < 0.05). Endogenous Notch-1 was only weakly detected by Western blotting and therefore it would be very difficult to assess whether the levels of endogenous Notch-1 protein are reduced by antisense Notch-1 (data not shown). However, the specificity of the antisense effect is supported by the fact that the sense Notch-1 and empty vector controls did not induce erythroid maturation and the antisense Notch-1 vector was shown previously to decrease Notch-1 protein levels in another system (34).

The spontaneous benzidine reactivity of the five antisense lines suggested that antisense Notch-1 can overcome a block in erythroid differentiation. We reasoned that clones lacking this differentiation block might be hypersensitive to hemin-induced erythroid differentiation. To test whether the antisense clones were altered in their sensitivity to hemin treatment, two clones containing each construct were incubated with increasing concentrations of hemin, and benzidine positive cells were scored. The antisense clones showed a higher percentage of benzidine positive cells at all concentrations of hemin. Moreover, the hemin dose response curve was shifted to the left (Fig. 4B), consistent with the antisense clones being more responsive to hemin than clones containing sense Notch or the empty vector.

Expression of NIC-1 does not Downregulate GATA-1 and TAL-1 - The inhibition of erythroid maturation by NIC-1 may result from downregulation or inhibition of a known factor required for erythroid maturation. Erythroid differentiation requires the erythroid cell- and megakaryocyte-specific transcription factor GATA-1 (51). In addition, the hematopoietic-specific
transcription factor TAL-1 is required for differentiation of all blood cell lineages (52). Thus, reduced levels of these factors may interfere with erythroid maturation of K562 cells. To test whether NIC-1 expression downregulates GATA-1, we measured the GATA-1 DNA binding activity of nuclear extracts from K562-Babe and K562-NIC-1 cells. EMSA analysis of GATA-1 DNA binding revealed a single activity that bound specifically to the GATA-1 oligonucleotide (Fig. 5A). As the complex competed specifically with a GATA-1 oligonucleotide, and the protein-DNA complex was supershifted with an antibody specific for GATA-1 (data not shown), the complex contained GATA-1. Similar levels of GATA-1 DNA binding activity were present in extracts from K562-Babe and K562-NIC-1 cells, inconsistent with the downregulation of GATA-1 by NIC-1.

Previously, we were unable to detect TAL1 DNA binding activity in K562 nuclear extracts by EMSA with an oligonucleotide probe containing an E-box from the β-globin LCR (39). However, TAL1 is expressed in K562 cells and can be detected by Western blot analysis (40). We measured the levels of TAL-1 in nuclear extracts from K562-Babe and K562-NIC-1 cells by Western blot analysis. A major immunoreactive 39-kDa band was detected in the extracts (Fig. 5B). Similar levels of TAL-1 were present in extracts from K562-Babe and K562-NIC-1 cells, inconsistent with the downregulation of TAL-1 by NIC-1.

Expression of NIC-1 does not Influence Megakaryocytic Differentiation of K562 cells - To determine whether NIC-1 has a general inhibitory effect on K562 cell differentiation, we tested whether megakaryocytic differentiation was suppressed in pools (Fig. 6) and clones (data not shown) of K562-Babe and K562-NIC-1 cells. Pools of each cell type were treated with TPA to determine if they were competent to undergo megakaryocytic differentiation. TPA treatment resulted in enlargement of the majority of cells (Fig. 6), consistent with previous reports (45). Cells showed an identical altered morphology, regardless of whether they were derived by stable transfection with pBabe or pBabe-NIC-1. RT-PCR was used to measure the expression of the megakaryocytic marker Integrin β3. As shown in the representative gel of Fig. 6, TPA treatment induced a comparable level of Integrin β3 transcripts in K562-Babe and K562-NIC-1 cells. Similar results were seen with a second megakaryocytic marker, Integrin αIIb (data not shown). Thus,
NIC-1 expression does not inhibit megakaryocytic differentiation of the majority of K562 cells in the culture.

**Identification of Hemin-induced Genes by Microarray Gene Expression Analysis and Disruption of Maturation-dependent Changes in Gene Expression by NIC-1** - The activity of NIC-1 to inhibit hemin-mediated induction of hemoglobin may reflect a specific inhibitory effect of NIC-1 on hemoglobin biosynthesis. Alternatively, the failure to accumulate hemoglobin may result from a differentiation block, which would impair the induction of all proteins associated with erythroid maturation. To distinguish between these possibilities, we asked whether NIC-1 inhibits maturation-dependent changes in the expression of genes distinct from globin or enzymes that mediate hemoglobin biosynthesis.

We used microarray analysis to identify genes whose expression changed upon hemin-induced erythroid differentiation of K562 cells. Of 9844 human cDNA fragments immobilized on the UniGEM1 microarray, the expression of only 21 genes increased and 23 genes decreased 2.5-fold or greater. As a control, we compared gene expression between two identical RNA samples from hemin treated K562 cells; no differential expression greater than 2.5-fold was measured. The largest hemin-induced change in expression was a 10.1-fold induction of a novel expressed sequence tag. The next largest changes were represented by interleukin 8 (*IL-8*), heat shock protein 70 (*HSP70*), and dihydriodiol dehydrogenase (human bile acid binding protein DD2) (*DD*), which were induced 8.2-, 8.1-, and 7.4-fold, respectively. The complete microarray data set is available as supplementary material. HSP70 levels had been shown to increase upon hemin-induced erythroid maturation of K562 cells (53). Although ELISA has been used to detect IL-8 in culture supernatants from K562 cells (54), nothing is known about *IL-8* expression during erythroid differentiation. *DD* is a member of a gene family, which encodes enzymes mediating steroid metabolism and the biotransformation of certain chemical carcinogens (55). However, the physiological role of these enzymes is unclear, and nothing is known about their expression in blood cells.
The hemin-mediated induction of HSP70, DD, and IL-8 in K562 (data not shown) and K562-Babe cells (Fig. 7A) was verified by Northern blotting. We then tested whether NIC-1 influences the basal or hemin-induced expression of HSP70, DD, and IL-8. We also measured the levels of α-globin transcripts, which are known to increase upon erythroid maturation of K562 cells, and GAPDH transcripts as a control. A 2.6-fold increase in α-globin expression was measured by the microarray analysis. The levels of α-globin, HSP70, IL-8, and DD transcripts increased upon hemin treatment of K562-Babe cells (Fig. 7B), consistent with the microarray analysis. NIC-1 reduced basal α-globin expression, but did not affect basal DD expression. NIC-1 weakly inhibited the low basal expression of HSP70 and IL-8. The hemin-induced level of expression of all four genes was considerably lower in K562-NIC-1 versus K562-Babe cells. In contrast, NIC-1 had little effect on GAPDH expression. In certain pools of K562-NIC-1 cells, no hemin induction of HSP70 was evident. The expression of IL-8 was particularly sensitive to NIC-1, as almost no IL-8 expression was observed before or after hemin treatment. As an additional control, we measured the levels of CBF1 transcripts in control and hemin treated K562-Babe and K562-NIC-1 cells. Hemin did not alter CBF1 expression. CBF1 transcript levels were nearly identical in all conditions, resembling the ribosomal RNA from the ethidium bromide stained gel (data not shown). However, normalization to GAPDH transcript levels, which were approximately 2-fold lower in K562-NIC-1 versus K562-Babe cells, revealed that the CBF1/GAPDH ratio was 2.9-fold higher in K562-NIC-1 versus K562-Babe cells. Thus, the microarray analysis identified new marker genes associated with hemin-induced erythroid maturation of K562 cells distinct from globin or enzymes mediating hemoglobin biosynthesis. Expression of NIC-1 deregulated maturation-dependent changes in the expression of these genes, consistent with NIC-1 establishing a true maturation block rather than specifically inhibiting hemoglobin biosynthesis.
DISCUSSION

The Notch signaling paradigm has emerged largely from studies of simple organisms such as *Drosophila* and *C. elegans* (8). Analysis of these systems and complex mammalian systems has revealed a regulatory role for Notch signaling in multiple developmental processes. In addition, differentiated cells can express Notch receptors, suggesting that Notch signaling may also control aspects of cell function unrelated to differentiation, such as proliferation and apoptosis (8). Although components of the Notch pathway were found to be expressed in hematopoietic progenitors several years ago (25,56), the importance of Notch signaling in hematopoiesis and in the function of blood cells is not well understood. An exception is the regulation of T-cell development, in which a role for Notch signaling has been firmly established (57). The slow progress in defining the involvement of Notch signaling in the differentiation of other lineages and hematopoietic stem cell function is likely related to several factors. First, there is considerable complexity to Notch signaling as described earlier. Thus, the interpretations of knockouts of components of the Notch pathway may be complicated by functional redundancies. Secondly, it is difficult to manipulate the Notch pathway in primary hematopoietic cultures. Small peptides from the DSL region of Jagged-1 influence the differentiation of 32D myeloid progenitor cells (24). The generation of potent and efficacious ligands that activate and repress Notch may facilitate the analysis of Notch signaling with primary cells. Lastly, mammalian systems lack the facile genetic analysis allowed in *Drosophila* and *C. elegans*, and therefore additional approaches are required to analyze Notch signaling in mammals.

Our establishment of the erythroleukemia cell system should allow molecular and biochemical analyses of the mechanism by which Notch controls hematopoietic cell fate, which will be considerably more complex with primary hematopoietic cultures. To our knowledge, this is the only cell culture system in which Notch selectively impairs one of the two developmental fates. Thus, the actions of Notch on K562 cells resemble the physiological role of Notch to control cell fate via lateral inhibition in the *Drosophila* embryo (18).
Considering that Notch signaling influences cell function via changes in gene expression, the inhibition of erythroid maturation by NIC-1 may result from increased expression of a gene that opposes erythroid differentiation. Alternatively, NIC-1 may downregulate factors required for erythroid maturation. The levels of GATA-1 and TAL1 were not decreased by NIC-1, inconsistent with the downregulation of these proteins, which are required for erythropoiesis. It is unlikely that NIC-1 inhibits erythroid differentiation by interfering with erythropoietin signaling, as K562 cells are poorly responsive to erythropoietin (58). As NIC-1 interfered with hemin- and butyrate-induced erythroid maturation, the inhibition appears to reflect the disruption of a fundamental step of erythroid maturation, rather than disruption of heme signaling or a specific inhibition of hemoglobin biosynthesis. This is further supported by the microarray analysis, which identified hemin-induced genes unrelated to globin or enzymes mediating hemoglobin biosynthesis. NIC-1 disrupted the maturation-dependent expression of these genes, consistent with a maturation block rather than a specific disruption of globin biosynthesis. The physiological relevance of our results showing that NIC-1 blocks erythroid maturation is supported by the recent analysis of erythroid differentiation in Notch-1-/- murine ES cells (59). These mutant cells were more efficient in differentiating into primitive erythroid colonies than wild-type ES cells. It will be important to define whether physiological cross-talk exists between the Notch pathway and other signaling pathways that control hematopoiesis.

Notch activation in a physiological context has a requirement for ligand binding, which is bypassed by NIC-1. Components of the hematopoietic microenvironment expressing Notch ligands may inhibit erythroid differentiation of HSC/progenitor cells expressing Notch receptors. This would allow HSC/progenitor cell expansion and megakaryocytic differentiation in response to the appropriate physiological cues. Survival and differentiation of HSC/progenitor cells are dependent upon physical interactions with components of the hematopoietic microenvironment. However, the molecular basis for this intercellular communication is unresolved (28). The Notch pathway may represent an important constituent of the circuitry that mediates communication between HSC/progenitor and stromal cells. This intercellular communication is likely to involve
crosstalk between distinct pathways, yielding composite signals that uniquely control the developmental fate or function of HSC/progenitor cells.

Notch has been shown to interact functionally with Ras and c-Jun N-terminal kinase (JNK) signaling pathways. Zecchini et al. (60) reported that Notch downregulates the JNK signaling pathway. Inhibition of JNK signaling was CBF1-independent and was important for dorsal closure in Drosophila. A potentially related observation showed that Notch inhibits transactivation mediated by the transcription factor E47 (61). This inhibition appeared to result from disruption of Ras signaling, in which JNK is a downstream kinase. Both positive and negative crosstalk between Notch and Wingless signaling pathways have been described (62, 63). It will be important to determine whether inhibitory signals conveyed by NIC-1 are influenced by other factors that control hematopoiesis. In this regard, stress- (64) and erythropoietin-induced (65) erythroid differentiation of SKT6 cells appears to require JNK activation. Thus, Notch-mediated inhibition of JNK may be relevant to the inhibition of erythroid differentiation by NIC-1.

Besides establishing whether crosstalk between Notch and other pathways is critical for hematopoiesis, questions remain about the Notch signaling mechanism that are critical for understanding how Notch controls hematopoiesis. For example, certain cells express Notch ligands and receptors (66), suggesting that ligands can signal through receptors without interactions between distinct cell types. Thus, the control of hematopoiesis by Notch might not be restricted to the paradigm involving interactions between distinct cell types. In this regard, Qi et al. (67) showed that the metalloprotease Kuzbanian proteolytically processes the ligand Delta in the extracellular space of Drosophila, liberating a soluble ligand. Soluble ligand generated from cells expressing ligand and receptor may be competent to activate Notch receptors on the same cell. The erythroleukemia cell system described herein should permit facile analyses to advance our understanding of Notch signaling and how Notch controls cell fate.
FIG. 1. Expression of Notch-1 and Jagged-1 in K562 and HEL cells. Notch-1, Jagged-1, and HPRT transcript levels were measured by RT-PCR. RT-PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Note that the signal was proportional to the RNA input. (A) RT-PCR was done using increasing amounts of total RNA from untreated (K562), hemin-treated (H-K562), and TPA-treated (T-K562) K562 cells, in the presence or absence of reverse transcriptase (RT). (B) RT-PCR was done with increasing amounts of total RNA from untreated (HEL), hemin-treated (H-HEL), and TPA-treated (T-HEL) HEL cells, in the presence or absence of reverse transcriptase. (C) RT-PCR was done with RNA from untreated K562 cells or cells treated with 50 nM TPA for one to five days. The first lane of the gel shows an RT-PCR analysis lacking RT of RNA from cells treated for five days with TPA.

FIG. 2. CBF1-dependent Notch signaling is functional in K562 cells. K562 cells were transiently transfected with reporter vectors containing four CBF1 (p4xCBF1luc) or five GAL4 (p5xGAL4luc) binding sites, with or without pBabe or pBabe-NIC-1. A constitutively-active β-galactosidase expression vector (pCH110) was included in all conditions to allow for normalization of transfection efficiency. The luciferase activity was also normalized by the protein content of the lysate. Note that strong luciferase activity was only apparent when pBabe-NIC-1 was cotransfected with the CBF1 reporter. The graph depicts averaged data from three independent transient transfection experiments.

FIG. 3. Expression of NIC-1 inhibits erythroid maturation of K562 cells. (A) Photomicrograph of uninduced and hemin-induced K562-Babe and K562-NIC-1 cells at 100× magnification. The inset in each micrograph shows a single cell at 1000x magnification. Cells were treated with 20 µM hemin for two days and then stained with benzidine. (B) Scatter plot of benzidine reactivity after treatment with 20 µM hemin. The solid symbols represent data from
individual pools of cells stably transfected (●) or retrovirally infected with pBabe (■). The open symbols represent data from individual pools of cells stably transfected (○) or retrovirally infected with pBabe-NIC-1 (□). (C) Transient transfection analysis of Notch-dependent reporter gene activity. A Notch-dependent luciferase reporter gene was transiently transfected into K562-Babe or K562-NIC-1 cells. The plot shows luciferase activity from K562-Babe and K562-NIC-1 cells, which were corrected for the background luciferase activity of untransfected K562 cells (1.4 RLU/s/µg). (D) Detection of stably expressed NIC-1-myc by Western blotting. Cell lysates were immunoprecipitated with anti-NIC-1 antibody, and bands were detected by Western blotting with anti-myc antibody. Lanes 1 and 2, lysates from two pools of cells containing the blank vector; lanes 3 and 4, lysates from two pools of cells containing the NIC-1-myc expression vector. (E) NIC-1-myc inhibits erythroid maturation of K562 cells. Pools of K562 cells stably transfected with the blank vector or the NIC-1-myc expression vector were treated with 20 µM hemin for two days and then stained with benzidine. The plot shows the mean percent benzidine positive cells from analysis of five pools of cells containing each construct.

FIG. 4. Expression of antisense Notch-1 induces erythroid maturation of K562 cells. K562 cell clones containing the empty vector (E1 - E7), sense Notch-1 (S1 - S7), and antisense Notch-1 (A1 - A7) were grown for five days and assayed each day for benzidine reactivity. (A) Scatter plot of benzidine reactivity. Closed circles represent the mean of the percentage of benzidine positive cells for each clone. Open circles represent the mean of all clones (p=0.042 and 0.012 for empty/antisense and sense/antisense, respectively). (B) Antisense Notch-1 confers hypersensitivity to hemin-induced erythroid differentiation. Two clonal lines containing either the empty vector (E3 and E4), sense Notch-1 (S2 and S5), or antisense Notch-1 (A4 and A6) were treated with increasing concentrations of hemin for two days. Benzidine reactivity was measured three independent times for each clone, and the averaged data is shown in the graph.
FIG. 5. **Expression of NIC-1 does not downregulate GATA-1 or TAL-1.** (A) GATA-1 DNA binding activity in nuclear extracts from untreated or hemin treated K562-Babe and K562-NIC-1 cells. GATA-1 DNA binding activity was measured by EMSA using a double stranded oligonucleotide spanning the GATA motif of the β-globin LCR. Lanes 1 - 6, pools of K562-Babe cells; lanes 7 - 12, pools of K562-NIC-1 cells. (B) Western blot analysis of TAL-1. Nuclear extracts from three pools of either K562-pBabe or K562-NIC-1 cells. Extracts were resolved on a 9% SDS-PAGE and analyzed by Western blotting with an anti-TAL1 polyclonal antibody.

FIG. 6. **Expression of NIC-1 does not inhibit megakaryocytic differentiation of K562 cells.** Untransfected K562 cells or pools of K562-Babe and K562-NIC-1 cells were treated with vehicle or TPA for 6 days. Note that TPA treated cells are considerably larger than control cells. The results are representative of three independent pools of K562-Babe and K562-NIC-1 cells. RT-PCR was carried out as described in Materials and Methods. RT-PCR was done using increasing amounts of total RNA from untreated or TPA-treated K562-Babe or K562-NIC-1 cells. Reactions were done with or without reverse transcriptase. After 35 cycles of amplification, Integrin β3 and HPRT products were electrophoresed through a 1.8% agarose gel, and bands were visualized by ethidium bromide staining. The intensity of the ethidium bromide signal was proportional to the RNA input in the RT-PCR reaction.

FIG. 7. **Expression of NIC-1 deregulates diverse genes in K562 cells.** (A) RNA from three and five pools of untreated and hemin-treated K562-Babe and K562-NIC-1 cells, respectively, was analyzed by Northern blotting with α-globin, HSP70, DD, IL-8, CBF1, and GAPDH probes. The blots are representative of results obtained from analysis of six and ten pools of K562-Babe and K562-NIC-1 cells, respectively. The solid bars on the left indicate the identical blots that were stripped and reprobed. (B) Quantitative analysis. The relative expression values were determined by analysis of Northern blots with a PhosphorImager. The levels of α-globin, HSP70, DD, IL-8, and CBF1 transcripts were normalized by the level of GAPDH transcripts to yield the relative
expression values. The quantitative data represents analysis of RNA from six and ten pools of K562-Babe and K562-NIC-1 cells, respectively, for all measurements except that of CBF1, in which three pools were analyzed (mean +/- SEM).
ACKNOWLEDGEMENTS

We thank Camilla Forsberg for a critical review of the manuscript. We thank Tatiana Zaboikina, Eric Mosser, Jason Norton, and Heather Christensen for excellent technical assistance. We also thank Gerd Blobel for providing the anti-GATA-1 antibody, Diane Hayward for the Notch reporter plasmid, David Denhardt for the IL-8 cDNA, and Rick Morimoto for the HSP-70 cDNA.

This work was supported by the Milwaukee Foundation, the Leukemia Society of America, the Pharmaceutical Research and Manufacturers of America Foundation, the Howard Hughes Medical Institute, and the National Institutes of Health (grant DK50107). Emery H. Bresnick is a Leukemia Society of America Scholar and a Shaw Scientist. Lloyd T. Lam is a predoctoral fellow of the Pharmaceutical Research and Manufacturers of America Foundation.
REFERENCES


Luciferase Activity (RLU/s/ g/ -Gal)

[Graph showing luciferase activity for different constructs: pBabe, pBabe-NIC-1, p4xCBF1luc, p5xGAL4luc]