Inhibition of Myeloid Differentiation by the Helix-Loop-Helix Protein Id

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Id is a helix-loop-helix (HLH) protein that represses activity of several basic helix-loop-helix (bHLH) proteins involved in cell type–specific transcription and cell lineage commitment. The myeloid precursor cell line 32DCl3(G) expressed Id messenger RNA, which was transiently decreased when cells were induced to terminally differentiate with granulocyte–colony-stimulating factor. Concomitant with the decrease of Id messenger RNA was the appearance in nuclear extracts of DNA binding proteins that recognized a canonical E-box motif, a DNA binding site for some bHLH proteins. Constitutive expression of an Id complementary DNA in 32DCl3(G) cells blocked their ability to differentiate and to induce E-box–binding activity. These results suggest that Id and, hence, bHLH proteins function in the process of myeloid differentiation.

THE HELIX-LOOP-HELIX (HLH) proteins define a class of transcription factors whose dimerization surfaces consist of two conserved amphipathic α helices separated by a loop of variable length and sequence. Members of a subclass of these proteins, designated bHLH, contain a region of basic amino acids adjacent to the HLH domain. The bHLH proteins activate genes associated with a variety of specific cell lineages, including muscle-specific genes (*1, 2*), immunoglobulin genes (*2, 3*), insulin genes (*4, 5*), and neuronal-specific genes (*6*). Binding sites for these proteins are represented by the palindromic E-box motif, CANNTG (N = any nucleotide) (7). Four HLH proteins, Id, Id-2, HLH462, and the product of the *extramacrochaetae* locus of *Drosophila* (Emc), lack the basic amino acids that are necessary for DNA binding and can inhibit other bHLH proteins (*8, 9, 10*). For example, heterodimerization of Id with MyoD, E12, or E47 renders these bHLH proteins unable to bind DNA in vitro, and expression of Id interferes with the ability of MyoD to activate transcription in transfected cells (*8*). Id expression also inhibits transcriptional activation by the immunoglobulin (*11*) and by the insulin enhancers (*5*). Id mRNA decreases in a variety of cell lines when they are induced to differentiate. Thus, it has been suggested that Id may serve as a general inhibitor of the differentiated state by blocking the activity of bHLH proteins that are required for specific differentiation programs (for example, MyoD).

To determine if Id functions in myeloid differentiation, we used the 32DCl3(G) cell line as a model. The 32DCl3(G) cell line is an interleukin-3 (IL-3)–dependent, hematopoietic precursor cell line that terminally differentiates to neutrophilic granulocytes over a 10- to 12-day period after treatment with G-CSF (granulocyte–colony-stimulating factor) (*12*). We measured Id mRNA concentrations in 32DCl3(G) cells that were induced to differentiate with G-CSF (Fig. 1). The concentration of Id mRNA rapidly decreased in cells treated with G-CSF, remained low for several hours and then increased to near preinduction concentrations for the duration of the differentiation process (10 to 12 days). The concentration of Id mRNA in cells growing in IL-3 was on average tenfold higher than in cells treated with G-CSF for 6 hours.

Various bHLH proteins bind promiscuously in vitro to the E-box motifs present in the enhancers associated with the immunoglobulin κ light chain, immunoglobulin heavy chain, insulin, and muscle creatine kinase genes (*5, 7*). We determined whether such bHLH proteins were present in differentiating myeloid cells with an electrophoretic mobility shift assay (EMSA) and an oligonucleotide probe that carried an E-box from the muscle creatine kinase enhancer, the muscle enhancer factor–1 (MEF-1) binding site (Fig. 2, upper panel). Nuclear extracts from uninduced 32DCl3(G) cells produced only weak E-box–binding activity (Fig. 2, basal), and the intensity of the complexes observed varied somewhat among different preparations (Fig. 3). Within 6 hours of G-CSF treatment, additional complexes were detected with the predominant one reaching a maximum level between 16 and 24 hours. All of these new complexes disappeared by 36 to 40 hours of G-CSF treatment. These complexes were not formed with, nor was formation inhibited by, an oligonucleotide that contained a mutated MEF-1 site, indicating that they were specific for the E-box (*13*). The complexes have mobilities distinct from those observed in B cells, and initial experiments indicated that they were unaltered in the presence of antisera directed against bHLH proteins encoded by the E2A gene (*13*). Hence, it is possible that they represent myeloid-specific bHLH proteins. EMSAs carried out with an oligonucleotide that contained an upstream stimulatory factor (USF)–binding site confirmed the integrity of the extracts (Fig. 2, lower panel), although the precise pattern obtained varied among different extracts and experiments (Fig. 3). The USF is a member of a different class of bHLH proteins (bHLH-zip) and is not affected by Id (*10, 14*).

If the decrease in Id mRNA is necessary for the subsequent differentiation of 32DCl3(G) cells, then artificially elevating the Id concentration should alter their developmental phenotype. To test this, we subjected cells to electroporation in the presence of pSV2neo and an expression vector that contained the Id gene under control of the Moloney murine sarcoma virus long terminal repeat (EMSV-Id). Transfected cells were selected in geneticin (G418), and eight resistant clones were isolated. Of these, three contained the Id expression vector (*13*). Two of these clones (32D/Id #6 and 32D/Id #7) and a clone that contained only the pSV2neo vector (32D/neo) were then characterized with respect to

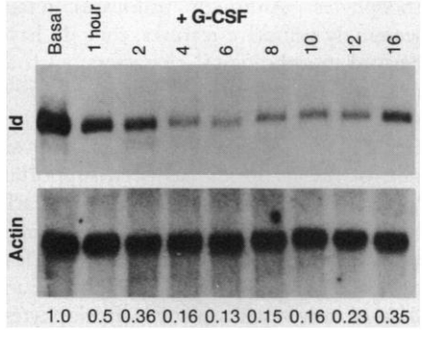

Fig. 1. Transient decrease in Id mRNA in 32DCl3(G) cells after exposure to G-CSF. The 32DCl3(G) cells were treated with G-CSF for the number of hours indicated above the lanes (*16*), and total RNA was isolated and subjected to Northern RNA analysis (*17*). "Basal" refers to RNA isolated from cells growing exponentially in the presence of IL-3. The Northern (RNA) blot was hybridized to an Id cDNA probe (*8*), stripped, and hybridized to an actin cDNA probe (*18*). Given below each lane are the relative intensities of the Id signals (basal = 1.0), deduced on the basis of densitometric scanning relative to the actin signal.

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growth in interleukin-3 (IL-3) and to the ability to differentiate in response to G-CSF.

Although all three clones grew in IL-3, their growth rates and their responses to G-CSF differed (Fig. 4). In IL-3 alone, 32DCl3(G) parent cells and clone 32D/neo grew slightly faster than the two clones that harbored the Id expression vector. When treated with G-CSF, only the 32DCl3(G) cells and clone 32D/neo survived and differentiated normally (Fig. 4). Clones that contained the EMSV-Id vector (32D/Id #6 and 32D/Id #7) did not respond to G-CSF and were less than 5% viable within 2 to 4 days of G-CSF treatment. To confirm that this correlated with elevated expression of Id, we analyzed Id mRNA in clone 32D/Id #6 after G-CSF treatment. The basal concentration of Id mRNA was about the same as that found in 32DCl3(G) cells. However, the amount of Id mRNA in clone 32D/Id #6 did not decrease as much after 6 hours of treatment with G-CSF; this three to four times higher residual expression was likely due to transcription of the transfected Id cDNA (*13*). Transformants that expressed Id from an MMTV promoter also died in the presence of G-CSF (*13*). As myeloid precursors, including 32DCl3(G) cells, undergo apoptosis at a similarly rapid rate on removal of growth factors or differentiative agents (*15*), these results suggested that these clones had lost their ability to respond to G-CSF. However, this was not due to loss of the G-CSF receptor because G-CSF was able to induce transcription of fos and c-*jun* in both the parental cells and in the Id transformants (*13*).

Although E-box–binding activity peaked a few hours after Id mRNA levels reached their lowest levels and began to increase, the appearance of E-box–binding activity may have been a direct consequence of Id down-regulation, as might be expected if the activity was composed of bHLH proteins. Clones 32D/Id #6 and 32D/Id #7 did not induce E-box–binding activity on G-CSF treatment, whereas clone 32D/neo did (Fig. 3, upper panels). The pattern of bands obtained from the clone 32D/neo cells was similar to that seen with the 32DCl3(G) parent cells (Fig. 2), although the induced complexes were not as abundant.

We suggest the following function for Id in differentiating 32DCl3(G) cells. In exponentially growing cells, Id forms heterodimers with bHLH proteins. On G-CSF treatment, the Id protein concentration decreases as a result of a decrease in steady-state mRNA. When Id expression drops below a critical threshold, newly synthesized, and perhaps preexisting, bHLH proteins are then free to form functional homodimers or heterodimers that bind to and stimulate transcription of a set of genes required for further differentiation. Id protein then reappears on mRNA up-regulation, and the bHLH proteins are again sequestered as nonfunctional Id heterodimers. The reappearance of Id mRNA implies that the activation of target genes by the bHLH proteins may be transient. In at least three other mature hematopoietic–cell types (B cells, T cells, and erythroid cells), Id mRNA is not present. Hence, the reappearance of Id and the disappearance of E-box–binding activity in terminally differentiating 32DCl3(G) cells may reflect a unique prop-

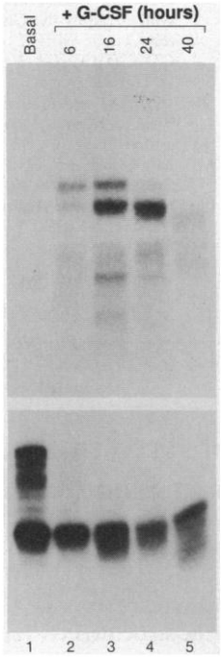


Fig. 2. Transient induction of E-box binding complexes on exposure to G-CSF. EMSAs (*19*) were performed with a MEF-1 oligonucleotide (upper panel) or a USF oligonucleotide (lower panel). Nuclear extracts were made from 32DCl3(G) cells growing in IL-3 (basal) or incubated with G-CSF for the number of hours indicated above the lanes.

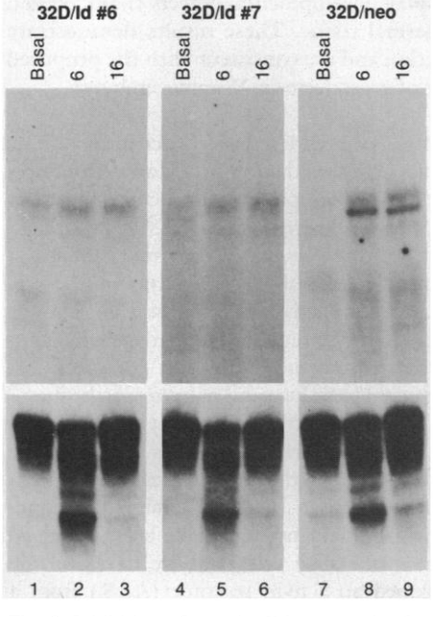
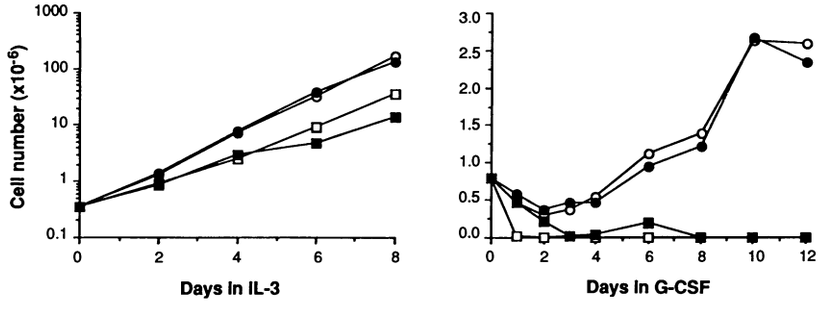


Fig. 3. Inhibition of protein–E-box complex formation by deregulated expression of Id. EMSAs were carried out with an MEF-1 probe (upper panel) or a USF probe (lower panel), and extracts that were derived from clones 32D/Id #6, 32D/Id #7, and 32D/neo grown in IL-3 (basal) or treated with G-CSF for 6 or 16 hours as indicated above lanes.



Cell Line	Cells in IL-3			Cells in G-CSF		
	Myeloblast	Pro/Myelo	Meta/Gran	Myeloblast	Pro/Myelo	Meta/Gran
32DCl3(G)	94	6	0	0	0	100
32D/Id #6	95	5	0	0	0	0
32D/Id #7	96	4	0	0	0	0
32D/neo	94	6	0	0	0	100

Fig. 4. Response of 32DCl3(G) and 32D/Id clones to IL-3 and G-CSF. 32DCl3(G) parent cells (closed circles), 32D/Id clone #6 (open squares), 32D/Id clone #7 (closed squares), and 32D/neo (open circles) cells were seeded in medium containing IL-3 (left panel) or G-CSF (right panel) and cell counts were determined every 1 to 2 days (*16*). On day 8 of culture in IL-3 and on day 10 of culture in G-CSF, cells were stained for morphological characteristics. After 8 days in IL-3, >90% of all clones displayed myeloblast characteristics and <10% displayed promyelocyte (Pro) or myelocyte (Myelo) characteristics. After 10 days in G-CSF, 100% of the 32DCl3(G) cells and 100% of the 32D/neo cells had the morphological characteristics of metamyelocytes (Meta) or granulocytes (Gran). Less than 1% of the 32D/Id #6 and #7 cells were viable after 10 days in G-CSF.

erty or determinant of myeloid cells or of the neutrophil lineage. Moreover, whereas the bHLH proteins active in muscle cells and B cells are thought to both stimulate differentiation and to transcriptionally define a cellular phenotype, the bHLH proteins of myeloid cells may serve only to promote a specific, transient phase of differentiation.

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16. The murine IL-3–dependent cell line 32DCl3(G) was maintained in IL-3 and treated with recombinant human G-CSF [as described (*17*)]. For G-CSF treatment, cells were washed and reseeded at a density of 5×10^5 cells per milliliter in Iscove's modified Dulbecco's medium (IMDM) containing fetal bovine serum (10%) and rhG-CSF–conditioned medium (10%). For the generation of stable transfectants, the EMSV-Id (*9*) and pSV2neo plasmids (10:1 ratio) were electroporated into 32DCl3(G) cells with a Gene Pulser (Bio-Rad). The cells and DNA were incubated on ice for 10 min, electroporated at 600-V and 25-μF capacitance, incubated on ice again for 10 min, and then seeded at a density of 2.5×10^5 cells per milliliter in complete medium. Selection in G418-containing medium [geneticin (600 μg/ml), Gibco, Grand Island, NY] was started 48 hours later and positive clones began to appear within 3 to 4 weeks. For growth curves, cells were washed as described and resuspended at a density of 4×10^5 cells per milliliter in either IL-3– or G-CSF–containing medium (*17*). Cell counts were performed every 2 to 3 days, and cultures were adjusted according to their densities. For morphological characterization, cells were harvested and concentrated on slides by cytocentrifugation (Shandon Southern Products, Sewickly, PA) and then fixed and stained with May Grunwald-Giemsa (*12*).
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19. Cells were induced with G-CSF [as described (*17*)] and mininuclear extracts were prepared. Cells (5×10^6 to 50×10^6) were harvested, were washed once with ice-cold, phosphate-buffered saline (PBS) [10 mM $NaPO_4$ (pH 7.4), 154 mM NaCl] and were washed twice with ice-cold wash buffer [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were then resuspended in ice-cold wash buffer (1 ml), transferred to a microfuge tube, and centrifuged briefly to pellet the cells. The pellet was then resuspended in wash buffer with NP-40 (0.1%) (15 μl per 10^7 cells), incubated on ice for 5 min, and centrifuged in a microfuge for 15 min at 4°C. The nuclear pellet was then resuspended in lysis buffer [20 mM Hepes (pH 7.9), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% v/v glycerol; 10 μl per 10^7 cells] and incubated on ice for 15 min with frequent, gentle vortexing. We pelleted the nuclear debris by centrifugation for 15 min at 4°C, and we diluted the supernatant 1:6 with storage buffer [20 mM Hepes (pH 7.9), 0.05 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 20% v/v glycerol]. Extracts were dialyzed against binding buffer [10 mM tris-HCl (pH 7.9), 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 5% v/v glycerol, 5% w/v sucrose, 1 mM PMSF] overnight at 4°C. An aliquot was removed for protein determination with the Bio-Rad protein assay dye reagent concentrate, and the extracts were aliquoted and stored at −70°C. For EMSAs, extracts (10 μg of protein) were incubated with poly(dI-dC) (2 μg) for 15 min on ice. A ^{32}P end-labeled probe (1×10^5 to 2×10^5 cpm) was added, and the mixture was incubated for 15 min. Binding complexes were then resolved on a polyacrylamide gel (5%) in 0.5 × TBE [45 mM tris-borate (pH 8.3), 45 mM boric acid, 1 mM EDTA]. The gel was fixed in acetic acid (10%) for 5 min, dried, and exposed at −70°C. The following double-stranded oligonucleotides were synthesized by the University of Pennsylvania Cancer Center: MEF-1, 5′-GATCCCCCCCCAACACCTGCTGCCTGA-3′; USF, 5′-GATCTGGTCACGTGGCCTACACCTATAAG-3′.
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Cloning of a Second Type of Activin Receptor and Functional Characterization in *Xenopus* Embryos

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A complementary DNA coding for a second type of activin receptor (ActRIIB) has been cloned from *Xenopus laevis* that fulfills the structural criteria of a transmembrane protein serine kinase. Ectodermal explants from embryos injected with activin receptor RNA show increased sensitivity to activin, as measured by the induction of muscle actin RNA. In addition, injected embryos display developmental defects characterized by inappropriate formation of dorsal mesodermal tissue. These results demonstrate that this receptor is involved in signal transduction and are consistent with the proposed role of activin in the induction and patterning of mesoderm in *Xenopus* embryos.

POLYPEPTIDE GROWTH FACTORS AND their receptors are believed to mediate the induction and patterning of mesodermal tissues in early *Xenopus* embryos. The inducing activities of these factors have been characterized in part by their effects on animal cap tissue from blastula embryos. Untreated caps form ectodermal tissues such as skin, whereas caps treated with different members of the fibroblast growth factor (FGF) and transforming growth factor (TGF)-β families form a variety of mesodermal tissues (*1*). Among the members of the TGF-β family, the activins are particularly potent in this assay, inducing a full complement of mesodermal tissues in a concentration-dependent manner (*2, 3*). The inducing activity of activin has also been measured by injecting activin RNA into embryos that have been rendered axis-deficient by ultraviolet (UV) irradiation. Activin RNA induces only partial, posterior structures in these embryos, whereas RNA for *Xwnt-8*, a member of the *wnt* oncogene family, induces a complete dorsal axis (*4*). Although the precise role of activin in embryos is not known, these results suggest that activin acts downstream from the creation of Spemann's organizer to induce and pattern specific mesodermal derivatives. One way to determine the role of activin in embryos is to study the receptors for these molecules, as has been done for FGF (*5*). Several high-affinity activin-binding proteins have been identified in mammalian cells, and we have recently cloned an activin receptor (ActR) from a mouse corticotroph cell line, AtT20, which is predicted to be a transmembrane protein serine kinase (*6*). To determine if a similar receptor mediates mesodermal induction in early embryos, we cloned a *Xenopus laevis* activin receptor. Introduction of this second type of ActR into early embryos causes

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