The amino terminus of the mixed lineage leukemia protein (MLL) promotes cell cycle arrest and monocytic differentiation


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Several lines of evidence suggest that the mixed lineage leukemia protein (MLL, ALL-1, HRX) plays a role in regulating myelomonocytic differentiation. In this study we examined the effect of expression of MLL-AF9 on differentiation of the monoblastic U937 cell line by using a tetracycline-inducible expression system. MLL-AF9 arrested growth of U937 cells and induced these cells to differentiate into macrophages; induction was accompanied by expression of CD11b and CD14 and ultimately cell death. Deletion mutants of MLL-AF9 were used to map the sequences responsible for this effect. The amino-terminal half of MLL was sufficient for both cell cycle arrest and macrophage differentiation, whereas the carboxyl terminus of MLL or AF9 was found to be dispensable for this effect. Further deletions showed that a 35-kDa amino-terminal fragment spanning two AT hook motifs was sufficient for cell cycle arrest, up-regulation of p21\(^{CIP1}\) and p27\(^{KIP1}\), and partial differentiation toward macrophages. These findings suggest a possible role for the MLL AT hook-containing region in regulating myelomonocytic differentiation.

Recent rearrangements of the mixed lineage leukemia gene (MLL, ALL-1, HRX) are common in infant acute lymphocytic leukemia, myelomonocytic leukemias, and in leukemias that arise secondary to DNA topoisomerase II inhibitor therapy (1–4). The mixed lineage leukemia protein (MLL) is homologous to the Drosophila protein Trithorax, and like Trithorax has been shown to be a positive regulator of homeobox gene expression (5–12). The main regions of homology with Trithorax include two small regions in the amino-terminal half of the protein, a central zinc finger (PHD) domain, and a carboxy-terminal SET domain, a motif that is also found in some mammalian Polycomb homologues, which are negative regulators of Hox gene expression. Translocations involving MLL delete these latter two domains and replace them with one of many different translocation partners that in general share little sequence homology (2).

Defining the role of motifs retained in MLL fusion proteins is critical for understanding MLL-mediated leukemogenesis. To date two motifs have been identified that are necessary for leukemic transformation (13). The first of these are three AT hook motifs homologous to those in the high mobility group proteins HMGIC-C and HMGIC(Y) that are not present in Drosophila Trithorax. These motifs appear to activate transcription by binding to A+T-rich regions of DNA. Interestingly, translocations involving both HMGIC-C and HMGIC(Y) have been identified in a variety of benign mesenchymal tumors (14). The involvement of HMG proteins in tumorigenesis may be relevant to the function of MLL fusion proteins, because in all of these translocations the AT hook motifs are retained and are separated from the carboxy-terminal portion of the protein (15). The second region important for transformation, which is located just amino-terminal to the translocation breakpoint, is homologous to the noncatalytic region of DNA methyltransferase (16).

Several lines of evidence suggest that MLL plays a role in regulating myelomonocytic differentiation. First, the t(9;11) translocation, which fuses MLL to AF9 (LTG3), is one of a group of MLL translocations that is associated with a distinctive subtype of acute leukemia showing prominent monocytic differentiation (17–19). MLL is expressed at high levels in more differentiated myeloid cells and macrophages, is expressed at lower levels in earlier hematopoietic progenitor cells and T and B lymphocytes, and is not expressed in erythroid cells (20, 21). This pattern of expression raises the possibility that MLL skews hematopoietic differentiation away from erythroid differentiation and toward myelomonocytic differentiation. Further support for this concept comes from gene targeting experiments, which showed that loss of functional Mll (the mouse homologue of MLL) results in marked reductions in the number of myeloid and macrophage colonies in yolk sac cultures (20).

These same studies suggested that MLL has effects on proliferation or possibly survival of hematopoietic cells. The hematopoietic colonies in yolk sac cultures lacking functional Mll were smaller and took longer to develop than wild-type colonies. Similar findings have been reported in cultures of Mll-null fetal liver cells (22). It is also noteworthy that mice that are haploinsufficient for functional Mll are markedly runted, to the same extent as “pygmy” mice, which show homozygous mutation of HMGIC(Y), which is also associated with growth control (23). Finally, expression of MLL in both T and B lymphocytes is dramatically increased upon treatment with mitogens (J.L.H., unpublished observations) (24). All of these observations suggest that MLL may play a role not only in myelomonocytic differentiation but also in cell cycle regulation.

In this study we have examined the effect of expression of MLL-AF9 on cell cycle progression and differentiation in the monocytic lineage by using U937 cells. This well characterized human monoblastic cell line can be induced to differentiate into macrophages by using phorbol esters or vitamin D3 (25–27). Previous experiments in our laboratory and others (28) suggested that constitutive expression of MLL fusion proteins in many cell lines is difficult or impossible, so a tetracycline-inducible expression system was employed. Expression of MLL-AF9 arrested the growth of these cells and induced them to differentiate into macrophages and ultimately to die. This in-

Abbreviations: MLL, mixed lineage leukemia protein; HMG, high mobility group.

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ducible expression system in U937 cells was then used to map the regions of MLL-AF9 responsible for its effects on cell cycle regulation and differentiation.

Materials and Methods

Expression Plasmids. A cDNA corresponding to the amino-terminal half of MLL was constructed from cDNAs of human MLL obtained from Masao Seto (Aichi Cancer Center) and Stanley Korsmeyer (Dana–Farber Cancer Institute) (8, 29). This cDNA was modified by the addition of a double-stranded oligonucleotide encoding an amino-terminal FLAG epitope tag (DYKDDDDK) beginning with an ATG codon within a Kozak consensus sequence. For construction of MLL-AF9 (encoding 1551 amino acids), a 270-bp fragment representing the carboxyl-terminal 90 amino acids of AF9 was cloned from human bone marrow by reverse transcription-PCR, sequenced, and fused in-frame to MLL at base pair 4383. MLLAT (encoding 1436 amino acids), and MLL2AT (encoding 312 amino acids) were generated by digestion of MLL-AF9 with either PstI or HindIII, respectively, filling in with Klenow enzyme, ligating on XhoI linkers, digestion with XhoI, and purification and religation of the vector (Fig. 1). The constructs were confirmed by sequencing across cloned regions. MLL3AT was generated from an MLL cDNA that contained a stop codon at amino acid 410 as a result of a nucleotide substitution that apparently arose during cloning (8). cDNAs were subcloned into the pUHD10S expression vector (30).

Establishment of Cell Lines Expressing MLL and MLL Fusion Proteins. U937 cells expressing the tetracycline-controlled transactivator (tTA) protein (30) were electroporated with 45 μg of linearized expression plasmid along with 5 μg of a hygromycin resistance plasmid, either pGKHygro (MLL-AF9, MLL2AT, MLLAT) or pGEM-Hygro (MLL3AT). Hygromycin-resistant clones were obtained by limiting dilution in 96-well plates. Those showing inducible expression were identified by immunofluorescent staining of paraformaldehyde-fixed cells induced to adhere to chamber slides by phorbol 12-myristate 13-acetate. Briefly, cells were permeabilized with 0.2% Triton X-100/PBS, blocked with 2% BSA/PBS, and stained with M2 FLAG monoclonal antibody (10 μg/ml) followed by staining with goat anti-mouse IgG conjugated to Alexa 488 (Molecular Probes). For Western blotting analysis of MLL expression, cells were lysed in lysis buffer [10% (vol/vol) glycerol/40 mM Hepes, pH 7.9/300 mM (NH₄)₂SO₄/100 mM KCl] containing a mixture of protease inhibitors, and extracts were electrophoresed on either 6% or 12% polyacrylamide gels. Gels were blotted to nitrocellulose, which was blocked with skim milk and incubated with M2 FLAG primary antibody (10 μg/ml) followed by detection with goat anti-mouse-horseradish peroxidase (Sigma) and enhanced chemiluminescence (ECL) detection (Amersham Life Science). For Western analysis of p21Cip1 and p27Kip1 expression, cells were harvested at various times and lysed in 150 mM NaCl/1% sodium deoxycholate/50 mM Tris-HCl/1% Nonidet P-40, pH 8.0, containing a mixture of protease inhibitors. Protein concentration was measured by Bradford assay, and 50-μg samples were electrophoresed on SDS/15% polyacrylamide gels and blotted to poly(vinylidene difluoride) (PVDF) filters. Filters were blocked with skim milk and were incubated with monoclonal mouse anti-p21 or polyclonal rabbit anti-p27 (Santa Cruz Biotechnology) with detection with horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit Ig (Amersham Life Science) and ECL detection as above.

Cell Growth Analysis. For growth rate analysis, cells were plated at a density of 3 × 10⁵ cells per ml in 1 ml of RPMI medium 1640 containing 10% fetal bovine serum in triplicate wells in 24-well cluster plates with or without 1 μg/ml tetracycline. Viable and dead cells were assessed by counting with trypan blue exclusion at 24, 48, 72, 96, and 120 hr.

Flow Cytometric Analysis. For surface immunostaining, cells were cultured in tetracycline-free medium for various lengths of time (48–72 hr) and were then harvested, stained with either CD11b or CD14 (PharMingen), and analyzed on a FACsVantage flow cytometer (Becton Dickinson). For cell cycle analysis, cells were washed in phosphate-buffered saline and were resuspended in Krishan’s reagent (30) for 30 min on ice. Fluorescence of stained nuclei was analyzed at wavelengths of 563–607 nm by using CellQuest software, and proportions of cells in G₀/G₁, S, and G₂/M and the number of apoptotic cells were quantitated by using ModFit.

Fig. 2. Immunofluorescent staining of U937T cells expressing MLL-AF9 and deletion mutants MLL3AT and MLL2AT, as well as the U937T parent. Cells were induced by tetracycline withdrawal for 48 hr and were stained with M2 FLAG monoclonal antibody followed by goat anti-mouse Alexa 488 conjugate. MLL-AF9 and MLL3AT have a predominantly punctate subnuclear distribution (green background with bright green/white spots), whereas MLL2AT is uniformly distributed in the nucleus (uniform green nuclear staining). (×1,000.)
Results

Induction of Cell Death and Monocytic Differentiation by MLL-AF9.

Initially U937 cell lines were established that inducibly expressed MLL-AF9 (Fig. 1). In multiple independent clones examined, the fusion protein had a punctate subnuclear localization, occasionally forming ring-like arrangements of spots apparently surrounding nucleoli (Fig. 2). Western blotting revealed that the cells expressed the appropriately sized fusion protein (170 kDa) as detected by antibodies directed against the amino-terminal FLAG epitope when tetracycline was withdrawn from the culture medium (Fig. 3A). Three independent high-expressing clones were selected for analysis of growth rate by counting cells and using trypan blue exclusion. The growth rate of a representative clone is shown in Fig. 4. Surprisingly, induction of MLL-AF9 expression in all of these clones resulted in complete inhibition of cell growth within 24 hr of tetracycline withdrawal that was the result of rapid cell death (Figs. 4 and 5 and Table 1). Although the percent of cells in S phase remained the same or increased, by 24 hr, 33% of cells were apoptotic (Table 1), and by 72 hr more than 80% of the cells were nonviable by trypan blue exclusion and propidium iodide staining. Examination of Wright–Giemsa-stained Cytospin preparations showed that the uninduced U937 cells and the parent U937T cells grown either in the presence or in the absence of tetracycline all showed identical morphology (Fig. 6). These cells had small amounts of cytoplasm and nuclei with prominent blast-like nucleoli. In contrast, cells induced to express MLL-AF9 uniformly differentiated into macrophages with abundant, often vacuolated, cytoplasm and nuclei with finely dispersed chromatin and inconspicuous nucleoli intermixed with frequent karyorrhectic cells. Flow cytometric examination of these clones showed a dramatic increase in both the size and cytoplasmic granularity in the MLL-AF9-expressing clones as evidenced by forward and side light scattering as well as by the appearance of CD11b and CD14 expression compatible with differentiation of these cells into macrophages (Fig. 7).

Role of Amino-Terminal MLL Sequences in Cell Cycle Control and Induction of Differentiation.

To examine the role of AF9 on cell cycle progression and differentiation, we generated additional cell lines that expressed a form of MLL that was truncated in exon 7 and lacks a translocation partner (MLL ΔT). Like MLL-AF9, this mutant showed a punctate subnuclear localization (data not shown). Induction of expression of this truncated product also resulted in growth arrest, cell death, and induction of macrophage differentiation in two independent cell lines. Additional MLL mutants composed of progressively smaller amino-terminal fragments of MLL were then generated to further map the sequences responsible for the growth-inhibitory and differentiation-promoting effects. U937T lines were generated that expressed high levels of the expected-sized proteins. In particular, two mutants, MLL2AT and MLL3AT, were generated that contained either 2 or 3 AT hook motifs in proteins of approximately 35 kDa and 50 kDa, respectively (Fig. 3B). Cells expressing MLL3AT showed coarse punctate spots, some of which were associated with the nuclear envelope, while others apparently surrounded nucleoli (Fig. 2). Cells expressing MLL2AT showed a uniform distribution of protein within the nucleus.

Like cells expressing MLL-AF9, multiple independent isolates...
of MLL2AT and MLL3AT were capable of arresting cell growth within 48 hr of induction of expression, and this arrest was accompanied by up-regulation of p21Cip1 and p27Kip1 (Fig. 8). However, this growth arrest was not accompanied by the degree of cell death observed for MLL-AF9 or MLL$D$ despite the expression of much higher levels of protein as judged by Western blotting and immunofluorescent staining. For example, U937T cells induced to express MLL2AT or MLL3AT for 96 hr showed greater than 80% viability as judged by trypan blue staining despite complete arrest in cell growth, compared with 20–25% viability for MLL-AF9 and 50% viability for MLL$D$. Morphologic examination of these latter cultures revealed frequent karyorrhectic cells.

U937T cells expressing the amino-terminal region of MLL showed partial differentiation toward macrophages (Figs. 6 and 7).

**Discussion**

Although the molecular mechanisms underlying transformation by MLL fusion proteins are unknown, in vivo models suggest that the fusion proteins convey a proliferative or survival advantage to myeloid progenitor cells but that additional genetic hits are required for leukemogenesis. Experiments using retroviral transduction of MLL-ENL in murine bone marrow cells have shown that the fusion protein increases the replating efficiency of hematopoietic colonies and transforms progenitor cells so that they can be propagated in liquid culture (31). Mice transplanted with transduced bone marrow develop acute myeloid leukemias with a latency period averaging 3–5 months, which can be
shortened by passage in vitro (31). Knock-in mice that express mll-AF9 also have been shown to develop leukemia after a relatively long latency period of 3–7 months (21). A recent study of mice with germ-line transmission of the mll-AF9 allele showed these mice have a selective expansion of CD11b/Gr-1-positive myeloid cells as early as 6 days after birth, compared with mice expressing only a truncated form of MLL (32). These data suggest that the MLL fusion proteins convey a selective proliferation or survival advantage on myeloid progenitors and that it is within this expanded hematopoietic compartment that additional genetic “hits” occur that result in transformation to acute leukemia.

An alternative approach to study the effects of expression of MLL fusion proteins has been to inducibly express them in hematopoietic cell lines in vitro. A recent study by Joh et al. (28) reported that expression of MLL-AF9 in 32D cells, a murine myelomonocytic cell line, blocked granulocyte colony-stimulating factor (G-CSF)-induced granulocytic differentiation as well as expression of Hox genes (Hoxa7, -b7, -e9). The sequences required for this effect were contained within the amino-terminal half of MLL and did not require the translocation partner. This result suggested that amino-terminal MLL sequences were capable of interfering with transcription of genes such as Hox genes that regulate granulocytic differentiation.

Given the unique association between MLL-AF9 and monocytic leukemias, we used U937 cells to develop a model system for studying the effects of MLL fusion proteins in the monocytic cells. MLL-AF9 was found to be a potent inducer of macrophage differentiation and cell death. This induction was not accompa-

ned by G0/G1 arrest, and the translocation partner was not necessary for this effect. Analysis of deletion mutants showed that a small amino-terminal portion of MLL containing two AT hook motifs was sufficient both for growth arrest and for promotion of macrophage differentiation. Cell cycle analysis revealed that, in contrast to the death induction by MLL-AF9, the primary mechanism of growth inhibition was G0/G1 arrest. Organization of MLL AT hook motifs into punctate nuclear spots was not necessary for their effects on the cell cycle and differentiation. MLL-3AT had a punctate subnuclear organization, whereas the smaller amino-terminal portion of the protein (MLL2AT) was uniformly distributed in the nucleus.

The localization differences between MLL3AT (coarse punctate distribution) and MLL2AT (diffuse) suggest that sequences immediately carboxyl-terminal to the AT hooks are important for subnuclear localization of MLL. This concept is in good agreement with the work of Yano et al. (33), who linked short segments of MLL to pyruvate kinase and studied their effects on subnuclear localization. These workers identify a domain within amino acids 322 and 480 of MLL termed SNL-1 that conferred both nuclear localization and a punctate subnuclear distribution when linked to pyruvate kinase. Interestingly, this SNL-1 domain spans one of only two short regions in MLL homologous to Trithorax which are retained in MLL fusion proteins. Although important for subnuclear localization, the SNL-1 region and the Trithorax homology region are dispensable for both cell cycle arrest and induction of differentiation.

The results suggest that the AT hook region of MLL interacts with proteins involved in cell cycle regulation that remain to be defined. Sequences flanking the AT hook motifs have been shown to bind the protein SET and protein phosphatase 2A (34). One binding site for SET is retained in MLL2AT, so it is possible that expression of this mutant protein interferes with the normal inhibition of P2PA by SET, thereby resulting in cell cycle arrest. This cell cycle arrest could then lead to macrophage differentiation, as it appears that cell cycle arrest in U937 cells is sufficient for induction of differentiation (35). In particular, transient expression of the Cdk inhibitor p21Cip1 in U937 cells has been shown to be sufficient for induction of differentiation into macrophages (36). Inhibition of cell cycle progression appears to be cell type specific, as expression of high levels of the amino terminus of MLL (MLL3AT) in HeLa cells had no effect on the cell cycle (data not shown).

The AT hooks of MLL have been previously shown to bind to cruciate and scaffold attachment region DNA, so an alternative possibility is that the differentiation-promoting activity of MLL is mediated by the AT hook motifs of MLL binding directly to DNA, rather than primarily through protein–protein interactions (36). In the HMG proteins, binding of the AT hook motifs to A+T-rich regions of DNA, including scaffold attachment regions, has been shown to enhance or in some cases repress the binding of other transcriptional factors (37). For example, HMG1(Y) has been shown to be required for the activation of the β-interferon promoter by NF-κB and ATF-2 (38, 39) and synergizes with Stat5, Elf-1, NF-κB, and a GATA family protein to regulate the interleukin-2α receptor (40, 41). One possibility is that the AT hook motifs facilitate transcription from promoters of macrophage-specific genes such as CD11b, or genes that inhibit cell cycle progression and induce macrophage differentiation such as p21Cip1. In support of this concept is the finding that MLL-null yolk sac cultures lacking functional MI were strikingly deficient in mature macrophages and showed an absence of expression of CD11b (20) and the MLL-induced up-regulation of p21Cip1 and p27Kip1 in these experiments.

Our data also indicate that more carboxyl-terminal sequences in MLL also contribute to induction of monocytic differentiation and rapid induction of cell death. It is noteworthy that when these additional MLL sequences are present the cells do not
arrest in G0/G1 but that a portion remain in S phase at the same time cell death is occurring. In our experiments, complete morphologic differentiation and induction of CD14 expression was not seen with expression of the amino terminus of MLL alone, but required more downstream sequences, including the DNA methyltransferase homology region. Additional MLL mutants would need to be examined both to confirm the role of the AT hooks and to further localize the sequences involved in CD14 induction, cell cycle regulation, and induction of cell death. The finding that MLL-AF9 actually promoted cell differentiation and cell death in U937 cells was surprising, given its role in inducing leukemia.

This finding likely reflects differences between U937 cells and the susceptible progenitor cell type in vivo, or possibly is the result of expression of the fusion protein at greater than physiologic levels. The findings are reminiscent of the effect of other oncogenes such as c-Myc, which when expressed in the absence of an appropriate collaborating oncogene or under growth-limiting conditions can trigger apoptosis (42). Having an inducible expression system in the monocytic lineage will now make it possible to examine in detail the effects of MLL fusion proteins on target genes, including Hox genes, and their effect on signal transduction pathways regulating proliferation, differentiation, and survival.

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