The PHD fingers of MLL block MLL fusion protein–mediated transformation

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Chromosomal translocations involving the mixed lineage leukemia (MLL) gene are associated with aggressive acute lymphoid and myeloid leukemias. These translocations are restricted to an 8.3-kb breakpoint region resulting in fusion of amino terminal MLL sequences in frame to 1 of more than 60 different translocation partners. The translocations consistently delete the plant homeodomain (PHD) fingers and more carboxyl terminal MLL sequences. The function of the PHD fingers is obscure and their specific role in transformation has not been explored. Here we show that inclusion of the PHD fingers in the MLL fusion protein MLL-AF9 blocked immortalization of hematopoietic progenitors. Inclusion of 2 or more PHD fingers reduced association with the Hoxa9 locus and suppressed Hoxa9 up-regulation in hematopoietic progenitors. These data provide an explanation for why MLL translocation breakpoints exclude the PHD fingers and suggest a possible role for these domains in regulating the function of wild-type MLL. (Blood. 2008;112:4690-4693)

Introduction

Balanced translocations involving mixed lineage leukemia (MLL) are common in human acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL).1 MLL positively regulates Hox and other target gene expression through SET domain–dependent histone H3 lysine 4 methyltransferase activity.2,3 Like MLL, MLL fusion proteins bind directly to Hox loci and up-regulate their expression.2-5 Deregulated expression of Hox genes including Hoxa9 and the Hox cofactor Meis1 appears to be central for MLL fusion protein mediated transformation.6-8

Translocations in MLL occur within an 8.3-kb breakpoint cluster region (BCR) resulting in fusion of approximately 1400 amino acids of N-terminal MLL to 1 of more than 60 fusion partners.9 The BCR region has been extensively studied and contains several structural elements including Alu elements, topoisomerase II cleavage sites, and DNase I hypersensitive sites. These along with error-prone nonhomologous end joining have been proposed as mechanisms for MLL translocations.10 It is also possible that there is a functional reason for the location of the breaks. For example, sequences immediately 5’ to the BCR encode the CXXC domain, which must be retained intact for MLL-fusion protein transformation.11,12 Notably, sequences 3’ to the BCR, encoding the PHD fingers, are consistently deleted in leukemogenic fusion proteins. The function of PHD fingers in general is obscure. Some have been reported to mediate protein-protein interactions such as binding to methylated histones or other chromatin associated proteins.13,14 For example, HCF-1 binds to the HBM region between the bromodomain and PHD of MLL and modulates transcription through E2F interactions.15-17 In addition, the nuclear cyclophilin Cyp33 has been reported to bind the PHD fingers leading to enhanced binding of HPC2 and BMI-1 to the CXXC domain.18,19

The consistent deletion of the PHD fingers in balanced translocations, the reports of interactions of the PHD fingers with corepressors,20 the disrupted MLL architecture in MLL partial tandem duplications in AML21 and the finding that MLL partial deletion of exons 7 and 8, which encode the first PHD finger, is associated with T-ALL22 all raise the possibility that the domains play a negative regulatory role in MLL function. Understanding the contribution of the PHD domain would provide insights into not only the location of breaks in the BCR but also the possible functions of the enigmatic PHD domains in normal and leukemogenic MLL function. Here we assess the role of the PHD fingers in transcriptional regulation and test the significance of inclusion of the PHD fingers, located downstream of the BCR, on MLL-fusion protein mediated transformation.

Methods

All animal studies were approved by the University of Michigan Committee on Use and Care of Animals and Unit for Laboratory Medicine. For complete materials and methods information, see Document S1 (available on the Blood website; see the Supplemental Materials link at the top of the online article).

Results and discussion

The effect of the PHD fingers on MLL fusion protein–mediated transcriptional regulation and transformation was assessed by engineering MSCV-based MLL-AF9 fusion constructs with various combinations of the 4 MLL PHD fingers and bromodomain (Figure 1A). Equal expression of retroviral constructs was...
confirmed in transiently transfected 293 cells by quantitative polymerase chain reaction (qPCR; Figure S1). Western blotting showed the fusion proteins were expressed at the predicted molecular weights (Figure S2). As evidence of MLL protein complex integrity the MLL fusions were found to coimmunoprecipitate Menin (Figure S3). Retrovirus was packaged and used to transduce bone marrow from 6- to 8-week-old C57/BL6 mice after 5-fluorouracil treatment as previously described.23,24 Transduced cells were cultured in methylcellulose in the presence of IL-3, IL-6, GM-CSF, SCF, and G418. After 8 days in culture, methylcellulose cultures were harvested and reseeded for secondary and tertiary cultures. While colonies were observed in all infection conditions, only MLL-AF9 and MLL-PHD#1-AF9–transduced cells formed a significant number of colonies in tertiary cultures (Figure 1A,C). These colonies displayed a compact morphology characteristic of MLL fusion protein mediated immortalization (Figure 1C insets). Notably, transduction with an MLL-AF9 construct that contained only the second PHD finger successfully immortalized mouse bone marrow (Figure S4). Compact colonies were not seen in cells transduced with other constructs, which showed colonies of diffuse differentiating cells in primary and secondary cultures that were largely absent in tertiary cultures (Figure 1A,C). The few colonies present in tertiary plating, with the exception of MLL-AF9 and MLL-PHD#1-AF9, consisted of differentiated cells (Figure 1A). Finally, expression of MLL-fusion proteins was confirmed in the transduced bone marrow by real-time PCR using primers selective for the exogenous transcripts (Figure 1D).

Liquid cultures of MSCV transduced bone marrow cells maintained in the presence of IL-3, SCF, and G418 provided further support for the growth inhibitory effects of the PHD fingers. Bone marrow cells transduced with MLL-AF9 and MLL-PHD#1-AF9 displayed a marked proliferative advantage compared with transductions with MSCV constructs containing 2 or more PHD fingers (Figure 2A). Western blotting for the flag tagged proteins in cell lines established from bone marrow transduced with MLL-AF9 and MLL-PHD#1-AF9 confirmed fusion protein expression (Figure 2B). Cells transduced with MLL-AF9 fusion constructs containing 2 or more PHD fingers failed to proliferate and became quiescent after 2 weeks in culture. Longer incubation resulted in cell death. Wright-Giemsa staining of cells after 15 days in culture (when significant differences in proliferation were observed; Figure 2A) revealed a more blastlike morphology of cells transduced with MLL-AF9 and MLL-PHD#1-AF9 compared with the
other transductants, which showed full differentiation and were composed predominantly of macrophages (Figure 2C).

Inclusion of the PHD fingers appeared to inhibit the ability of MLL-AF9 to activate transcriptional targets. Analysis by qPCR of cells after the first round of replating revealed a robust activation of the endogenous \(\text{Hoxa9}\) locus by both MLL-AF9 and MLL-PHD1-AF9 fusion proteins but not in cells transduced with MLL-AF9 constructs containing 2 or more PHD fingers. Furthermore, luciferase assays performed using a \(\text{Hoxa9-LUC}\) construct for a direct analysis of transactivation potential of MLL fusion proteins showed that only MLL-AF9 and MLL-PHD1-AF9 could significantly activate the \(\text{Hoxa9}\) promoter (Figure 2E). Our experiments suggest that the differences between the activities of the fusion constructs are not simply the result of differences in protein stability (Figures S2,S3). One explanation for this would be that the PHD fingers have or recruit corepressor activity. However, experiments with tethering GAL4 fusions of the various PHD domains (not shown as well as Zeleznik-Le\textsuperscript{25}) show neither an activating nor repressing effect of the PHD domains in isolation. However, chromatin immunoprecipitation experiments did reveal that, despite comparable
levels of expression (Figures S2,S3), less MLL-PHD2-AF9 protein was bound to the HOX49 locus than MLL-AF9 and MLL-PHD1-AF9, suggesting that inclusion of 2 or more PHD fingers alters the efficiency or stability of target loci binding (Figure 2F).

While we do not have a complete understanding of the role of the MLL PHD fingers, our data along with previous work suggest that translocations occur within the BCR region so that the DNA binding activity of the DNA methyltransferase homology region is preserved and the transcriptional suppression activity of the PHD domain is deleted. The PHD fingers may mediate transcriptional suppression by interaction with corepressors that directly repress transcription or destabilizing MLL-PHD-AF9 binding to target loci. Of note, we cannot completely exclude a mechanism of transformation inhibition where the PHD fingers function in trans. Identification of PHD interacting proteins is likely to shed light on the mechanisms of transcription suppression and inhibition of transformation. This will provide insights into how the normal function of MLL is regulated and how this process is perturbed in acute leukemia.

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Authorship

Contribution: A.G.M. designed research, analyzed data, and wrote the manuscript; D.G. and A.M.U. generated reagents; and J.L.H. designed research, analyzed data, and edited the manuscript.

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