A Subset of Mixed Lineage Leukemia Proteins Has Plant Homeodomain (PHD)-mediated E3 Ligase Activity*

Jingya Wang, Andrew G. Muntean, Laura Wu, and Jay L. Hess

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109

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1 To whom correspondence should be addressed: Dept. of Pathology, University of Michigan Medical School, MS240 Medical Sciences I, 1301 Catherine Ave., Ann Arbor, MI 48109-0602. Tel.: 734-763-6384; Fax: 734-763-4782; E-mail: jayhess@umich.edu.
2 The abbreviations used are: MLL1, mixed lineage leukemia protein; PHD, plant homeodomain; MBP, myelin basic protein; qPCR, quantitative PCR; LUC, luciferase.

The mixed lineage leukemia protein MLL1 contains four highly deleted plant homeodomain (PHD) fingers, which are invariably deleted in oncogenic MLL1 fusion proteins in human leukemia. Here we show that the second PHD finger (PHD2) of MLL1 is an E3 ubiquitin ligase in the presence of the E2-conjugating enzyme CDC34. This activity is conserved in the second PHD finger of MLL4, the closest homolog to MLL1 but not in MLL2 or MLL3. Mutation of PHD2 leads to MLL1 stabilization, as well as increased transactivation ability and MLL1 recruitment to the target gene loci, suggesting that PHD2 negatively regulates MLL1 activity.

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Background: MLL1 contains four PHD fingers that are deleted in leukemogenic MLL1 fusion proteins.
Results: The second PHD finger of MLL1 exhibits E3 ubiquitin ligase activity, and this activity is conserved in MLL4.
Conclusion: PHD2 ligase activity potentially regulates MLL1 levels and activity.
Significance: This study reveals a novel activity of MLL PHD fingers that may have important roles in gene regulation and carcinogenesis.
MLL1 is associated with increased transactivation ability and MLL1 recruitment to target gene promoters.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—cDNA encoding the MLL PHD fingers were synthesized (GenScript) and cloned into the pMCSG9 vector that contains His-MBP tag. Expression plasmids were transformed into the AI bacteria strain and screened at the University of Michigan High-Throughput Protein Lab. Bacteria were grown at 37 °C in Terrific Broth (TB) medium at 250 rpm with 50 μg/ml spectinomycin and 100 μg/ml ampicillin to an A600 of 0.7, and protein expression was induced with 200 μM isopropyl-1-thio-D-galactopyranoside overnight at 20 °C. Bacteria were lysed by sonication in sodium phosphate buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 10 mM imidazole, pH 8.0, supplemented with 0.4 mg/ml lysozyme). Proteins were batch-purified using nickel-nitrilotriacetic acid-agarose (Qiagen) according to the manufacturer’s instructions and visualized by Coomassie Blue staining. The sequences of the PHD fingers purified are: MLL1-PHD1, Ile-1393–Phe-1481; PHD2, Lys-1480–Gly-1544; PHD3, His-1551–Pro-1629; PHD4, Asp-1914–Leu-1980; MLL2-PHD1, Glu-225–Arg-280; PHD2, Glu-272–Ser-330; PHD3, Thr-1359–Ser-1437; PHD4, Gly-1421–Ser-1484; PHD5, Ala-1502–Val-1560; PHD6, Thr-5090–Gly-5140; MLL3-PHD5, Ser-990–Ser-1064; MLL4-PHD6, Gln-1239–Asn-1313.

**In Vitro Ubiquitination Assay**—Purified His-MBP-tagged PHD fingers (2 μg) were incubated with 180 ng of UBE1 (E1), 560 ng of E2, 1 μg of HA-ubiquitin, and 2 mM ATP in 30 μl of reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM NaF, 0.5 mM DTT) at room temperature for 3 h. Reactions were terminated by adding 30 μl of 2 × SDS sample buffer (Invitrogen) and boiling at 95 °C for 5–10 min. Western blotting was then performed to detect ubiquitin conjugates using HA antibody (Abcam). For the ubiquitination assays using histones as substrates, 1 μg of recombinant histone H2A, H2B, H3, or H4 (Boston Biochem) was added to the reactions, respectively. Human recombinant UBE1, CDC34, RAD6, UBCH5c, and HA-tagged ubiquitin were purchased from Boston Biochem. Human recombinant UBCH13/Uev1a was kindly provided by Dr. Yali Dou (University of Michigan).

**In Vitro Binding Assay**—Equal amounts of purified His-MBP-tagged PHD fingers (2 μg) were incubated with 180 ng of UBE1 (E1), 560 ng of E2, 1 μg of HA-ubiquitin, and 2 mM ATP in 30 μl of reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM NaF, 0.5 mM DTT) at room temperature for 3 h. Reactions were terminated by adding 30 μl of 2 × SDS sample buffer (Invitrogen) and boiling at 95 °C for 5–10 min. Western blotting was then performed to detect ubiquitin conjugates using HA antibody (Abcam). For the ubiquitination assays using histones as substrates, 1 μg of recombinant histone H2A, H2B, H3, or H4 (Boston Biochem) was added to the reactions, respectively. Human recombinant UBE1, CDC34, RAD6, UBCH5c, and HA-tagged ubiquitin were purchased from Boston Biochem. Human recombinant UBCH13/Uev1a was kindly provided by Dr. Yali Dou (University of Michigan).

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In **Vitro Binding Assay**—Equal amounts of purified His-MBP/His-MBP-PHD2 and recombinant CDC34 (1 μg) were incubated in 500 μl of binding buffer (50 mM Tris-HCl, pH 7.9, 300 mM KCl, 0.05% Nonidet P-40, 0.1% BSA) for 2 h at 4 °C. 20 μl of amylose resin (New England Biolabs) was then added and incubated for 2 h at 4 °C. Resins were washed with binding buffer and 50 mM sodium phosphate buffer (pH 7.5, 100 mM NaCl, 1 mM DTT) and then boiled at 95 °C for 5 min. Proteins were released from the resin and analyzed by SDS–PAGE and Coomassie Blue staining.
added to the reaction and incubated for 1 h at 4 °C. Bound material was washed with wash buffer (50 mM Tris-HCl, pH 7.9, 300 mM KCl, 0.3% Nonidet P-40) three times and eluted from beads by boiling in SDS sample buffer. Eluted material was detected by Western blotting and Coomassie Blue staining.

**Immunoprecipitation and Western Blotting**—Immunoprecipitation and Western blotting were performed as described previously (5). 293 cells were lysed with BC-300 lysis buffer (20 mM Tris-HCl (pH 7.4), 10% glycerol, 300 mM KCl, 0.1% Nonidet P-40) and incubated with protein-G-agarose beads (Roche Applied Science) and MLL1 antibody or normal IgG overnight at 4 °C. Bound material was washed three times with BC-300 buffer. Proteins were eluted by boiling in SDS loading buffer, resolved by SDS-PAGE, and detected by Western blotting. Primary antibodies against MLL1 and CDC34 were obtained from

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**FIGURE 2.** The second PHD finger of MLL4 shows highest similarity to PHD2 of MLL1. A, phylogenetic tree using CLUSTAL W (35) with neighbor-joining method based on sequence alignment of the PHD fingers of MLL1 through MLL4. Dashed circle indicates the PHD fingers that are similar to PHD2 of MLL1. B, sequence alignment of PHD4 of MLL2, PHD5 of MLL3, and PHD2 of MLL4 with PHD2 of MLL1, respectively. Black background indicates conserved cysteines and histidines. Gray background indicates other conserved amino acids (aa). C, schematic diagram showing the structure of MLL methyltransferase complexes. Black indicates MLL proteins. Dark gray indicates common subunits. Light gray indicates unique subunits. Star indicates E3 ligase activity. HCF1: host cell factor 1. ASH2: absent, small, or homeotic-like 2. hDYP30, dpy-30 homolog; RBBP5, retinoblastoma binding protein 5; WDR5, WD repeat domain 5; PTIP, PAX interacting protein; PA1, PTIP associated protein 1; ASC-2, activating signal co-integrator-2.

**FIGURE 3.** The E3 ligase activity is conserved in the second PHD finger of MLL4. A, in vitro ubiquitination assays with PHD2 of MLL1, PHD4 of MLL2, PHD5 of MLL3, and PHD2 of MLL4. Western blotting shows that the PHD finger of MLL1 and MLL4 contain ubiquitinating activity. B, in vitro ubiquitination assays with PHD2 of MLL1 and the PHD fingers of MLL2. None of the MLL2 PHD fingers show ubiquitinating activity.
Vector Construction and Dual-Luciferase Assay—pCXN2-FLAG-MLL1 has been previously described (24). pCXN2-FLAG-MLL1C1509A and pCXN2-FLAG-MLL1/H9004P2 were cloned from pCXN2-FLAG-MLL1 using the QuikChange site-directed mutagenesis kit (Agilent). 293 cells were transfected with MLL1 or MLL1 mutants, Renilla luciferase reporter (serves as an internal control), and Hoxa9-LUC or Myc-LUC reporter with FuGENE 6 according to the manufacturer’s instructions. The amount of MLL1 and MLL1 mutant constructs transfected was adjusted to yield equal protein levels (MLL1:MLL1C1509A:MLL1/H9004P2 = 1:1.2:1). Vector DNA was added to ensure that the total amount of DNA transfected was equal among all samples. Cells were serum-starved in 0.5% FBS in OPTI-MEM medium for 48 h. Luciferase assays were performed using the Dual-Luciferase assay kit (Promega) according to the manufacturer’s instructions. Emission was detected using a Monolight 3010 luminometer (BD Biosciences).

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed as described previously (25). Antibody against FLAG tag was obtained from Sigma-Aldrich. qPCR was performed on the precipitated DNAs with TaqMan primers and probes from Applied Biosystems. Binding was quantitated as follows: $\Delta C_T = C_T(\text{input}) - C_T(\text{chromatin immunoprecipitation})$, % of total = $2^{\Delta C_T}$. Primer and probe sequences were described previously (26).

RESULTS

The Second PHD Finger of MLL1 Has E3 Ligase Activity—The MLL1 protein contains four PHD fingers (PHD1–PHD4) with conserved Zn$^{2+}$-coordinating cysteine and histidine amino acids and variable intervening sequences (Fig. 1A). Recent publications have shown that PHD fingers can function as an H3K4me3 “reader” (27). Although the third PHD finger of MLL1 shares this high affinity recognition specifically for H3K4me3, this activity is notably absent from PHD1, PHD2, and PHD4 of MLL1 (28). As several PHD fingers function as E3 ligases, we investigated whether any of the MLL1 PHD fingers have this activity. To this end, we bacterially expressed and purified His-MBP-tagged individual PHD fingers of MLL1. In vitro ubiquitination assays were performed in the presence of E1-activating enzyme UBE1, E2-conjugating enzyme CDC34, HA-tagged ubiquitin, and ATP. High molecular weight ubiquitin conjugates were observed only in the presence of PHD2 (Fig. 1B). This activity is ATP-dependent as no ubiquitination was observed without ATP (Fig. 1B, lane 6). To confirm this enzymatic activity, the sixth cysteine of PHD2 was mutated to alanine to disrupt the cross-brace structure of the domain. No ubiquitination activity was observed with the mutant, indicating that the observed E3 ubiquitin ligase activity is intrinsic to PHD2 (Fig. 1C). To test whether PHD2 can ubiquitinate other substrates, ubiquitination assays were performed with free histones, including H2A, H2B, H3, and H4. Interestingly, PHD2 showed specific activity for histone H3 and H4, but not H2A or H2B (Fig. 1D).

PHD E3 Ligase Activity Is Conserved in MLL4—The MLL protein family consists of five members (MLL1–MLL5), with MLL4 showing the greatest structural similarity to MLL1. To test whether the E3 ligase activity is conserved among the MLL family members, a sequence alignment of all predicted PHD fingers in MLL1 through MLL4 was performed, which revealed the highest homology between the second PHD finger (PHD2) of MLL4 and PHD2 of MLL1. The fourth PHD finger (PHD4) of MLL2 and the fifth PHD finger (PHD5) of MLL3 are the next most similar to PHD2 of MLL1 (Fig. 2, A and B). Further, the alignment shows that each PHD finger in MLL4 clusters together with the corresponding PHD fingers in MLL1, whereas five of the six PHD fingers in MLL2 cluster together.
with the corresponding PHD fingers in MLL3 (Fig. 2A). Notably, previous studies have shown that MLL1 and MLL4 are functionally distinct from MLL2 and MLL3, which form unique macromolecular protein complexes and target distinct genes (Fig. 2C) (10, 29, 30). To test for conservation of E3 ligase activity, His-MBP-tagged PHD2 of MLL4, PHD5 of MLL3, and all PHD fingers of MLL2 were bacterially expressed, purified, and tested in vitro ubiquitination assays. These showed that only the most homologous PHD finger (PHD2 of MLL4) has conserved E3 ligase activity, whereas no activity was observed in other MLL family member PHD fingers under the same conditions (Fig. 3, A and B). These data demonstrate a novel E3 ubiquitination activity.

FIGURE 5. Mutation of PHD2 in MLL1 leads to increased MLL1 transactivation ability and prolonged protein degradation. A, schematic diagram of the Hoxa9-LUC reporter. Black bars indicate the position of the probes used in ChIP-qPCR experiments. B, expression of wild type MLL1 and MLL1 mutants in 293 cells. β-Actin (β-ACT) blot shows equal loading. C, Dual-Luciferase assays were performed in 293 cells with Hoxa9-LUC reporter showing that mutations of PHD2 in full-length MLL1 lead to higher activation of Hoxa9 reporter when compared with wild type MLL1. All changes are normalized to lane 1, which contains Hoxa9-LUC and an empty expression vector. Error bars indicate S.D. D, Dual-Luciferase assay was performed in 293 cells with Myc-LUC reporter. E, ChIP-qPCR assays were performed in 293 cells transfected with Hoxa9-LUC, MLL1, or MLL1 mutants. The x axis labels the distance of the probes relative to the transcription start site. Gray line: vector control. Solid line with squares: wild type MLL1. Long dashed line with triangles: MLL1C1509A. Short dashed line with crossings: MLL1ΔP2. TSS, transcription start site. F, 293 cells transfected with MLL1 or MLL1C1509A were treated with cycloheximide for different time periods as indicated to block translation. The turnover of MLL1 or MLL1C1509A was monitored by Western blotting using whole cell lysate. MLL1C1509A shows prolonged protein degradation when compared with wild type MLL1.
uitin ligase activity associated with PHD2 of MLL1 that is evolutionarily conserved in PHD2 of MLL4.

CDC34 facilitates the E3 Ligase Activity of PHD2 and Interacts with MLL1—The interaction between E2-conjugating enzymes and E3 ligases plays a central role in ubiquitination as it provides specificity to the reaction. We sought to identify the E2-conjugating enzyme required for PHD2 activity by performing ubiquitination assays with a panel of E2 enzymes, including Cdc34, Rad6, Ubch5c, and Ubch13. Ubiquitin conjugates were only observed in the presence of CDC34, demonstrating specificity for CDC34 in PHD2-mediated ubiquitination (Fig. 4A). To test for a direct interaction, in vitro binding assays were performed using purified His-MBP-PHD2 and recombinant CDC34. Immunoprecipitation of PHD2 but not the tag control leads to co-precipitation of CDC34 (Fig. 4B). We then tested for an endogenous interaction between full-length MLL1 and CDC34 in 293 cells. Endogenous MLL1 was immunoprecipitated using anti-MLL1 antibody, and Western blotting using anti-CDC34 antibody showed that endogenous CDC34 co-precipitates with full-length MLL1 (Fig. 4C).

Transcriptional Activity of MLL1 Is Augmented by Mutation of PHD2—MLL1 is a positive regulator of gene expression. To test the effect of PHD2 on MLL1 transactivation ability, two mutants were made in the context of full-length MLL1. MLL1C1509A replaces the sixth cysteine of PHD2 with alanine, which resembles the PHD2 mutant used in the in vitro ubiquitination assay, and MLL1ΔP2 encodes full-length MLL1 with a deletion of the entire PHD2. Wild type MLL1 or MLL1 mutants were expressed in 293 cells with a luciferase reporter driven by the promoter of Hoxa9 (Hoxa9-LUC), an important target gene of MLL. Dual-Luciferase assays were performed to test the activation of the reporter, which reflects the transactivation ability of MLL1 (Fig. 5A). Both MLL1C1509A and MLL1ΔP2 showed higher transactivation when compared with wild type MLL1 (Fig. 5, B and C). Similar results were observed when using a luciferase reporter driven by the Myc promoter, another target of MLL1 (Fig. 5D). Further, ChIP-qPCR experiments were performed to test the recruitment of MLL1 or MLL1 mutants to the promoter region of the Hoxa9 reporter. 293 cells were transfected with MLL1 or MLL1 mutant expression vectors and Hoxa9-LUC reporter at the same ratio as used in the Dual-Luciferase assays. This yielded comparable levels of MLL1 and MLL1 mutant expression (data not shown). ChIP-qPCR revealed a more robust recruitment of both MLL1 mutants when compared with wild type MLL1 (Fig. 5E). To test the effect of PHD2 on MLL1 stability, the turnover of MLL1 and MLL1C1509A was monitored following treatment with cycloheximide. In these experiments, MLL1C1509A displayed prolonged degradation and greater stability than wild type MLL1 (Fig. 5F).

**DISCUSSION**

MLL1 contains four PHD fingers, which are invariably deleted in leukemogenic MLL1 fusion proteins. Moreover, insertion of the PHD fingers into MLL1 fusion proteins abolishes their transformation ability (4, 5). Sequence variation between the MLL1 PHD fingers suggests that they have different functions. Indeed, the third PHD finger of MLL1 has been reported to bind di/trimethylated histone H3K4 and the cyclophepin Cyp33 simultaneously, whereas the first and fourth PHD fingers are involved in mediating intramolecular interactions between the N-terminal and C-terminal fragments of MLL1 (28, 31, 32). Here we report that MLL1 contains a second enzymatic activity in addition to the methyltransferase activity intrinsic to the SET domain. The second PHD finger functions as an E3 ligase in conjunction with the E2 enzyme CDC34. Mutation of PHD2 leads to increased transactivation ability of MLL1 and its recruitment to target genes, which is likely due to increased protein stability (Fig. 5). However, PHD2 may also ubiquitinate other substrates that contribute to its inhibitory effect. Interestingly, we observed that PHD2 ubiquitinates histone H3 and H4 in vitro. Further experiments are required to determine whether histone H3 and H4 are bona fide substrates in vivo. Three E3 ligases have been shown to ubiquitinate MLL1, including SctSkp2, APC/Cdc20, and EcsAsnb2 (26, 33). Our findings indicate that the second PHD finger also plays a role in MLL1 ubiquitination. CDC34 is an important regulator of cell cycle progression (34), raising the possibility that PHD2 plays a role in regulating MLL1 degradation during cell cycle.

The MLL protein family contains five members, with MLL1 and MLL4 being functionally distinct from MLL2 and MLL3 (6–10). This is reflected from the sequence alignment of the MLL PHD fingers as the PHD fingers of MLL1 and MLL4 cluster together, whereas the PHD fingers of MLL2 and MLL3 cluster together (Fig. 2C). Further, the PHD2 of MLL4 also has E3 ligase activity (Fig. 3A). Like MLL1, MLL4 also undergoes cancer-associated gene rearrangements. In one study, intrinsic insertions of hepatitis B virus genomic sequences or chromosomal translocations of MLL4 were found in 26 out of 42 human hepatocellular carcinoma cases, suggesting that MLL4 overexpression or alteration plays a key role in the pathogenesis of hepatocellular carcinoma (13). Given the central role of MLL family members and their frequent rearrangements and mutations in both leukemia and solid tumors, additional studies appear warranted to define the role of PHD finger-mediated E3 ligase activity in normal gene regulation and carcinogenesis.

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MLL E3 Ligase Activity

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