Structural Basis for WDR5 Interaction (Win) Motif Recognition in Human SET1 Family Histone Methyltransferases*

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Background: The WDR5 interaction (Win) motif of mixed lineage leukemia-1 (MLL1) is required for core complex assembly.

Results: Win motifs from human SET1 family methyltransferases differ 70-fold in their specificity for WDR5.

Conclusion: Residue differences around the conserved Win motif contribute to differences in affinity.

Significance: Knowledge of WDR5 recognition of SET1 Win motifs is crucial for understanding regulation of H3K4 methylation in cells.

Translocations and amplifications of the mixed lineage leukemia-1 (MLL1) gene are associated with aggressive myeloid and lymphocytic leukemias in humans. MLL1 is a member of the SET1 family of histone H3 lysine 4 (H3K4) methyltransferases, which are required for transcription of genes involved in hematopoiesis and development. MLL1 associates with a subcomplex containing WDR5, RbBP5, Ash2L, and DPY-30 (WRAD), which together form the MLL1 core complex that is required for sequential mono- and dimethylation of H3K4. We previously demonstrated that WDR5 binds the conserved WDR5 interaction (Win) motif of MLL1 in vitro, an interaction that is required for the H3K4 dimethylation activity of the MLL1 core complex. In this investigation, we demonstrate that arginine 3765 of the MLL1 Win motif is required to co-immunoprecipitate WRAD from mammalian cells, suggesting that the WDR5-Win motif interaction is important for the assembly of the MLL1 core complex in vivo. We also demonstrate that peptides that mimic SET1 family Win motif sequences inhibit H3K4 dimethylation by the MLL1 core complex with varying degrees of efficiency. To understand the structural basis for these differences, we determined structures of WDR5 bound to six different naturally occurring Win motif sequences at resolutions ranging from 1.9 to 1.2 Å. Our results reveal that binding energy differences result from interactions between non-conserved residues C-terminal to the Win motif and to a lesser extent from subtle variation of residues within the Win motif. These results highlight a new class of methylation inhibitors that may be useful for the treatment of MLL1-related malignancies.

The mixed lineage leukemia-1 (MLL1)² protein is a member of the SET1 family of lysine methyltransferases that regulate the degree of histone H3 lysine 4 (H3K4) methylation in eukaryotes (1). H3K4 methylation is an evolutionarily conserved epigenetic mark required for the recruitment of enzymes that heritably maintain transcriptionally permissible states of chromatin (2–6). Recent studies have shown that the Suppressor of Variegation, Enhancer of Zeste, and Trithorax (SET) domain of MLL1 catalyzes monomethylation of H3K4 (H3K4me1) (7, 8), a mark associated with nucleosomes in distal enhancer sequences and silenced genes (9–12). However, because H3K4 di- and trimethylation (H3K4me2,3) are associated with transcriptional competence (13, 14), the activity of MLL1 alone is insufficient to promote transcription. MLL1 interacts with an evolutionarily conserved subcomplex that includes WD repeat protein-5 (WDR5), retinoblastoma-binding protein-5 (RbBP5), absent-small-homeotic-2-like protein (Ash2L), and dumpy-30 (DPY-30) (15–23). This complex (called WRAD) catalyzes H3K4 methylation on its own (7, 8, 24, 25) and has been shown to catalyze H3K4me2 when in complex with MLL1 (8). These results suggest that the degree of H3K4 methylation is regulated in a sequential fashion by different enzymes within a multisubunit complex, together called the MLL1 core complex (7, 8).

MLL1 is one of six human SET1 family members that include MLL2, MLL3, MLL4, SETd1a, and SETd1b (16, 17, 22, 23).

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The atomic coordinates and structure factors (codes 4ESG, 4ERQ, 4ERY, 4ERZ, 4EWR, and 4ESO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: MLL, mixed lineage leukemia; H3K4, histone H3 lysine 4; WRAD, WDR5, RbBP5, Ash2L, and DPY-30; Win, WDR5 interaction; SET, Suppressor of Variegation, Enhancer of Zeste, and Trithorax; H3K4me2,3, H3K4 di- and trimethylation; H3K4me2,3, H3K4 dimethylation; WDR5, WD repeat protein-5; RbBP5, retinoblastoma-binding protein-5; Ash2L, absent-small-homeotic-2-like protein; DPY-30, dumpy-30; ITC, isothermal titration calorimetry.
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Each of these enzymes regulates H3K4 methylation levels and is assembled into large multisubunit complexes that include WRAD (16, 17, 26, 32). The presence of WRAD within each of these complexes suggests a common mechanism of complex assembly and enzymatic activity regulation among different SET1 family members. Indeed, although deletion of MLL1 affects methylation at only a subset of genes (33), deletion of WDR5 affects global H3K4me2,3 levels (26, 34), consistent with a more global role for WRAD in regulating H3K4 methylation. We have shown previously that the WDR5 component of WRAD specifically recognizes a conserved arginine (Arg-5340) in the MLL2 Win motif has been found to be among a spectrum of MLL2 missense amino acid mutations associated with a rare human multiple malformation disorder called Kabuki syndrome (37, 38). We have also demonstrated that peptides derived from the MLL1 Win motif specifically disrupt the formation of the MLL1 core complex in vitro and abolish the H3K4 dimethylation activity of the MLL1 core complex (36). These results suggest that Win motif peptides or related compounds may be useful for targeted therapies for treatment of malignancies that result from gain-of-function mutations in human SET1 family members (39). For example, amplifications of the MLL4 gene (previously known as MLL2) are associated with solid tumors (40, 41). In addition, a cytogenetically normal rearrangement of the MLL1 gene found in ~10% of acute myeloid leukemias results in a partial tandem duplication of N-terminal MLL1 sequences that retains the conserved SET domain (39, 42–44). These rearrangements display increased H3K4 methylation, lysine acetylation, and HOX gene expression and may be responsive to targeted inhibition (45–47). An understanding of how different human Win motif sequences interact with WDR5 will increase our knowledge of how SET1 family complexes are assembled and regulated and facilitate the rational design of novel targeted therapies for MLL1-related malignancies.

In this investigation, we show that the conserved arginine 3765 of the MLL1 Win motif is required for co-immunoprecipitation of WDR5, RbBP5, and Ash2L from mammalian cells, confirming its critical role in the assembly of the MLL1 core complex. However, we were also interested in understanding the role of the non-conserved residues flanking the six-residue Win motif in recognition by WDR5. A recent study by Zhang et al. (48) reports structures of WDR5 bound to six 11-residue consensus Win motif peptides containing the six-residue Win motif sequences flanked by five additional residues on the C terminus that were suggested to be important for affinity differences among the peptides. In this investigation, we tested this hypothesis by performing a systematic structural and functional analysis of the interaction between WDR5 and six different human SET1 family Win motif peptides containing the six-residue Win motif sequence flanked on both N and C termini by four additional naturally occurring amino acid residues.

Our results indicate that WDR5 interacts with different human SET1 family Win motif peptides with binding affinities ranging from 50 to 2800 nM with the MLL3 Win motif binding having the greatest affinity. Substitution of residues flanking the Win motif reveals that the amino acid four residues C-terminal to the conserved arginine (+4) accounts for the majority
of binding energy differences through the presence or absence of an additional hydrogen bond with WDR5 residues. However, our analysis reveals that subtle variation within the conserved Win motif sequence also contributes to binding energy differences, possibly through stabilization of the bound conformation when free in solution. We also observed that the residues N-terminal to the Win motif were ordered in five of six structures, the majority of which adopt a conformation that may further stabilize the bound conformation of the Win motif. In addition, we demonstrate that the other SET1 family Win motif peptides are 14–72-fold better inhibitors of the H3K4 dimethylation activity of MLL1 core complex than that of the MLL1 Win motif peptide. On the basis of these results, we suggest that the overall stability of different human SET1 family core complexes may substantially vary with the MLL1 core complex having the lowest stability. We propose that these differences may be exploited for development of Win motif-based peptide inhibitors that specifically target MLL1 over other human SET1 family complexes.

EXPERIMENTAL PROCEDURES

Co-immunoprecipitation and Immunoblotting—Human embryonic kidney (HEK293) cells were transiently transfected with pCMV-Myc-tagged MLL1-C180 constructs expressing either the wild type or mutant (R3765A) as described previously (16). After 48 h of transfection, nuclear extracts were prepared as described previously (16) and incubated with anti-Myc-agarose beads (Sigma) for 3 h. Bound proteins were eluted with SDS sample buffer after extensive washing and analyzed by Western blotting. The antisera used are as follows. Anti-Myc antibody was obtained from Santa Cruz Biotechnology, Inc. Antiserum directed against Ash2L and RbBP5 were obtained from Bethyl Laboratories. Anti-Wdr5 antiserum was described previously (17).

Protein Expression and Purification—Full-length WDR5 (residues 1–334) and an N-terminal truncated form of WDR5 (residues 23–334; ∆N-WDR5) were expressed and purified as described previously (35, 36). As a final step of purification, the protein was passed through a gel filtration column (Superdex 200™GE Healthcare) pre-equilibrated with the sample buffer after extensive washing and analyzed by Western blotting. The antisera used are as follows. Anti-Myc antibody was obtained from Santa Cruz Biotechnology, Inc. Anti-Wdr5 directed against Ash2L and RbBP5 were obtained from Bethyl Laboratories. Anti-Wdr5 antiserum was described previously (17).

Peptide Synthesis—All six human SET1 family Win motif peptides used in this study were synthesized by Genscript (refer to Table 1 for peptide sequences). MLL1H3769Y peptide was obtained from Pi-Proteomics. All peptides were synthesized with an acetyl- and amide-capping group at the N and C termini, respectively, to eliminate the contributions of unnatural N- and C-terminal charges to binding. It should be noted that the MLL1H3769Y peptide is insoluble in isothermal titration calorimetry (ITC) sample buffer, and therefore no ITC data could be collected with this peptide. However, the MLL1H3769Y peptide is soluble in methyltransferase assay buffer and was therefore used to determine inhibition constants.

Isothermal Titration Calorimetry—ITC experiments were carried out using a VP-ITC calorimeter (MicroCal). All ITC experiments were performed at 20 °C in a sample buffer containing 20 mM Tris (pH 7.5), 300 mM sodium chloride, 1 mM tris(2-carboxyethyl)phosphine, and 1 μM zinc chloride. Prior to ITC, all peptides and proteins were dialyzed against sample buffer to minimize variations in sample preparations. Individual ITC experiments for each of the six human SET1 family peptides were carried out by titrating a known concentration of Win motif peptide (determined by amino acid analysis at the Keck proteomics facility at Yale University) diluted in the sample buffer into a sample cell containing a known concentration of full-length WDR5 (0.037–0.050 mM) in the same buffer. For each ITC experiment, a 180-s delay at the start of the experiment was followed by 30 injections of 10 μl of the titrant solution spaced 300 s apart, and the sample was stirred at 300 rpm throughout the experiment. Binding stoichiometry (N), dissociation constant (Kd), standard enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) changes associated with Win peptide binding to WDR5 were derived by fitting the binding isotherm to a one-site binding model (Origin 7.0).

MLL1 Core Complex Inhibition Assays—MALDI-TOF mass spectrometry-based methyltransferase assays were performed as described previously (36). Methylation assays were carried out in the absence and presence of increasing concentrations of the different Win motif peptide inhibitors. 7.3 μM MLL1 core complex (MLL13745, WDR5, RbBP5, and Ash2L) was incubated with 250 μM S-adenosyl-methionine (Sigma-Aldrich), 10 μM histone H3 peptide (residues 1–20) (Global Peptides), and Win peptides (0–1 mM) at 15 °C in an assay buffer containing 50 mM Tris·Cl (pH 9.0), 200 mM sodium chloride, 3 mM dithiothreitol, 1 μM zinc chloride, and 5% glycerol. The reactions were quenched after 12 h by the addition of trifluoroacetic acid to a final concentration of 0.5%. The quenched samples were diluted 1:5 in deionized water and mixed 1:5 with α-cyano-4-hydroxycinnamic acid. MALDI-TOF mass spectrometry analysis was carried out on a Bruker AutoFlex mass spectrometer (State University of New York, College of Environment and Forestry, Syracuse, NY) operated in reflectron mode. Final spectra were averaged from 200 shots per position at 10 different positions.

Curve Fitting and IC50 Analysis—MALDI-TOF mass spectrometry was used to determine the relative distribution of unmodified, mono-, di-, and trimethylated species in each reaction as described previously (36). The IC50 value is defined as the concentration of the Win motif peptide that is required to inhibit the H3K4 dimethylation activity of the MLL1 core complex by 50% of the initial value. IC50 values were determined by plotting the percentage of dimethylation as a function of Win peptide concentration. The data were fitted using Sigma plot 11.0 to a four-parameter logistic model (Equation 1),

$$y = c + ((a - c)/(1 + ((x/IC_{50})^b)))$$  \hspace{1cm} (Equation 1)

where $a$ is percent inhibition at zero Win peptide concentration, $c$ is percent inhibition at infinitely high peptide concentration, $x$ is the concentration of inhibitor peptide, $y$ is percent dimethylation, and $b$ is the Hill slope. IC50 values derived from the fits were used to calculate the inhibition constants ($K_i$) utilizing the Cheng-Prusoff relation (49) (Equation 2),

$$K_i = IC_{50}/(1 + [S]/K_d)$$  \hspace{1cm} (Equation 2)

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where S is the concentration of the MLL1 (7.3 μM) used in the methylation assays and \( K_d \) is the dissociation constant for the interaction between MLL1 and WDR5 proteins, previously determined to be 120 nM (36).

Crystallization and Structure Determination—Crystals of the ∆N-WDR5-Win peptide binary complex were obtained using the hanging drop vapor diffusion method. Immediately before crystallization, a 13 mg/ml stock solution of ∆N-WDR5 in the sample buffer was mixed with a stock solution of each of the six Win motif peptides dissolved in the same buffer. The final concentrations of ∆N-WDR5 and the Win peptides were 11.7 mg/ml and 1 mM, respectively. The mother liquor used for crystallization contained 20–30 mM ammonium sulfate, 25–27% polyethylene glycol (PEG) 3350, and 100 mM HEPES (pH 7.3–7.5). The crystals were flash frozen in the mother liquor containing 40% PEG3350. Diffraction data for ∆N-WDR5-SETd1a Win peptide complex was collected at the National Synchrotron Light Source on the beamline X25 using an ADSC Quantum 270 CCD detector. All data sets were indexed, reduced, and scaled with HKL-2000 (50) and CCP4i (51). Data collection statistics are summarized in Table 2. Each of the six Win motif peptide-WDR5 binary complex structures were solved by molecular replacement with MOLREP (52) using as a search model the coordinates of the previously determined structure of apo-WDR5 (Protein Data Bank code 2H68) (53). After an initial rigid body refinement, the structures were further refined with rounds of simulated annealing, energy minimization, and individual B-factor refinement with a maximum likelihood target using CNS (54). CNS was used to calculate the initial difference Fourier maps \( (F_o - F_c) \) and to locate the electron density corresponding to each of the Win motif peptides, and the structures were built using O (55). Final refinements were carried out using the phenix.refine module within the PHENIX program (56), and the refinement statistics are reported in Table 3. MolProbity was used to analyze the protein geometry and steric clashes in final refined structures and the statistics are reported as MolProbity score in Table 3 (57, 58).

All structure figures were generated using PyMOL (59). All structure refinement programs used in this study were a part of the SBGrid Consortium.

RESULTS

Arg-3765 of the MLL1 Win Motif Is Required for the Co-immunoprecipitation of WDR5, RbBP5, and Ash2L from Mammalian Cells—Previous studies established that Arg-3765 of the MLL1 Win motif is required for the assembly and H3K4 dimethylation activity of MLL1 core complex in vitro (36). To determine whether Arg-3765 of MLL1 is required for the assembly of the MLL1 core complex in mammalian cells, we compared wild-type and R3765A MLL1 proteins for their ability to co-immunoprecipitate WDR5, RbBP5, and Ash2L from HEK293 cells. pCMV-Myc vectors encoding the 180-kDa C-terminal fragment of wild-type or R3765A MLL1 proteins were transfected into HEK293 cells, immunoprecipitated with antibodies against c-Myc, and probed by Western blotting for the presence of WDR5, RbBP5, and Ash2L. As shown in Fig. 2 (lane 5), whereas wild-type MLL1 co-immunoprecipitates the WDR5, RbBP5, and Ash2L subcomplex, co-immunoprecipitation is abolished when Arg-3765 of MLL1 is replaced with alanine (Fig. 2, lane 6). These results are consistent with the suggestion from our in vitro studies that Arg-3765 of the MLL1 Win motif is crucial for the assembly of the MLL1 core complex in cells.

Thermodynamic Binding Analysis of Human SET1 Family Win Motif Peptides—In our previous crystal structure of WDR5 bound to the 12-residue MLL1 Win motif peptide (GSARA-EVHLRKs, conserved amino acid residues highlighted in bold), we observed that the N terminus was well ordered, whereas the last three residues of the C terminus were disordered (35). The high quality of density at the N terminus suggested that additional interactions may be observed using peptides with additional N-terminal amino acids. However, ITC binding experiments with a longer 19-residue MLL1 Win motif peptide (EPPLNPHGSARA-EVHLRKs) show identical affinity, suggesting that our original Win motif peptide captured the majority of the salient interactions (35). To better understand SET1 family Win motif recognition by WDR5, we synthesized Win motif peptides derived from the six human SET1 family members: MLL1-1, SETd1a, and SETd1b. On the basis of our previous structural results, the length of each peptide was 14 residues and contained the conserved six-residue Win motif sequence with an additional four amino acid residues flanking both the N and C termini (Table 1). All peptides were synthesized with acetyl and amide capping groups to prevent unnatural charge-charge interactions from influencing the results.

Using these peptides, we first compared the 14-residue MLL1 Win motif peptide (LNP-HGSARA-EVHL) with that of the original 12- and 19-residue MLL1 Win motif peptides for their ability to bind WDR5 by ITC (Fig. 3). Although the 12- and 19-residue MLL1 peptides bind WDR5 with identical affinity.
The 14-residue MLL1 peptide binds 2-fold weaker with a dissociation constant of 2.8 \( \mu M \) (Fig. 3). It is likely that this difference is due to the absence of lysine and serine residues in the C terminus of the 14-residue peptide as it is present in both 12- and 19-residue peptides that bind WDR5 with identical affinity. A similar conclusion was reached upon deletion of lysine and serine residues.

**TABLE 1**

| SET1 family | Win motif sequence | Dissociation Constant (K_d) ± S.D., nM | IC_{50} ± S.E.M., \mu M | K_i ± S.E.M., \mu M |
|-------------|-------------------|----------------------------------------|--------------------------|-------------------|
| MLL1^{3758–3771} | AcLNPHGSARAEVHLR_{NH2} | 2762 ± 338* | 1782 ± 383 | 28.82 ± 6.19 |
| MLL2^{3333–5346} | AcINPTGCARSEPK_{NH2} | 75 ± 5 | 45 ± 0 | 0.73 ± 0 |
| MLL3^{4703–4716} | AcVNPTGCARSEPKMS_{NH2} | 54 ± 5 | 25 ± 12 | 0.40 ± 0.19 |
| MLL4^{2504–2517} | AcLNPHGAAEVVLK_{NH2} | 88 ± 16 | 103 ± 4 | 1.67 ± 0.07 |
| SET1α^{1488–1501} | AcEHQTGSARSEGYYP_{NH2} | 541 ± 46 | 132 ± 1 | 2.14 ± 0.02 |
| SET1b^{1698–1711} | AcEHVTCASEGFEYT_{NH2} | 103 ± 14 | 108 ± 5 | 1.75 ± 0.09 |

* Standard deviation of the fit from a single experiment.

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**FIGURE 3.** ITC data showing that peptides derived from the MLL1 Win motif have similar affinities for WDR5. α–d, ITC data for WDR5 binding to MLL1(3755–3773) (α), MLL1(3758–3771) (β), and MLL1(3762–3773) (γ) Win motif peptides. α–c, upper panels, show heat of binding plotted as a function of time. Lower panels show the binding isotherms fit to a one-site binding model. d, peptide sequence and dissociation constants (K_d ± S.E.) derived from the fits are indicated.

(1.7 \( \mu M \)), the 14-residue MLL1 peptide binds ~2-fold weaker with a dissociation constant of 2.8 \( \mu M \) (Fig. 3). It is likely that this difference is due to the absence of lysine and serine residues in the C terminus of the 14-residue peptide as it is present in both 12- and 19-residue peptides that bind WDR5 with identical affinity. A similar conclusion was reached upon deletion of
the RKS residues of a Win motif peptide in a related analysis (60). However, it is not clear how the RKS residues contribute to the binding affinity for WDR5 as they are disordered in all x-ray structures that contain these sequences (35, 61) and therefore were not included in the peptides used in this investigation. Because of the similarity in length to all other Win motif peptides in this study and the relatively small differences in binding affinities, the 14-residue MLL1 Win motif peptide was used as the basis for comparison for all other human SET1 family Win motif peptides.

We next compared the binding of WDR5 with the different 14-residue SET1 family Win motif peptides using ITC (Fig. 4 and Table 1). Despite the high sequence conservation of the six-residue Win motif within each peptide, a wide range of affinities for WDR5 was observed among different peptides. For example, although the 14-residue MLL1 Win motif peptide binds WDR5 with a dissociation constant ($K_d$) of $2,800 \text{ nM}$, the other human SET1 family Win motif peptides bind with 5–51-fold greater affinity (Table 1). These results suggest that the non-conserved residues flanking the Win motif contribute to the specificity of WDR5 for different SET1 family Win motifs. The MLL3 Win motif peptide binds with the greatest affinity at $54 \pm 5 \text{ nM}$ followed by MLL2 (75 nM), MLL4 (88 nM), SETd1a (103 nM), SETd1b (541 nM), and MLL1 (2,762 nM).

FIGURE 4. ITC data showing that Win motif peptides derived from human SET1 family members have differential affinities for WDR5. $a$–$f$, ITC data for WDR5 binding to MLL1 $a$, MLL2 $b$, MLL3 $c$, MLL4 $d$, SETd1a $e$, and SETd1b $f$ Win motif peptides. Upper panels show heat of binding plotted as a function of time. Lower panels show the binding isotherms fit to a one-site binding model. Peptide sequence and dissociation constants ($K_d \pm \text{S.E.}$) derived from the fits are indicated.
SET1 Family Win Motif Peptides Are Specific Inhibitors of the H3K4 Dimethylation Activity of the MLL1 Core Complex—We previously demonstrated that the 12-residue MLL1 Win motif peptide inhibits the H3K4 dimethylation activity of the MLL1 core complex in a concentration-dependent manner by competing with MLL1 for the arginine-binding pocket of WDR5 (36). To determine whether other human SET1 family Win motif peptides also inhibit the enzymatic activity of the MLL1 core complex, we compared inhibition constants among different Win motif peptides in enzymatic assays with the fully assembled MLL1 core complex using MALDI-TOF mass spectrometry. As a negative control, we performed the same assays with a peptide derived from the p53 tumor suppressor protein containing amino acids HSSHLKSKQGTSRHKK (p53(365–382)). The results show that all SET1 family Win motif peptides specifically inhibit the H3K4 dimethylation activity in a concentration-dependent manner (Fig. 5, a and b). Although we previously showed that the 12-residue MLL1 Win motif peptide has an IC_{50} of 400 μM (36), the 14-residue MLL1 Win motif peptide inhibits with an IC_{50} of 1.8 mM (Table 1). In contrast, all other 14-residue human SET1 family Win motif peptides inhibit with IC_{50} values that are between 14- and 72-fold lower than that of the 14-residue MLL1 Win motif peptide (Fig. 5a and Table 1). Little inhibition was observed with the p53 peptide (Fig. 5a and Table 1). The relative inhibitory efficiencies of the human SET1 family Win motif peptides correlate well with the differences in their binding affinities for WDR5 as measured by ITC (Table 1).

Structure Determination of WDR5 Bound to Human SET1 Family Win Motif Peptides—To begin to understand the structural basis for the observed differences in affinity for WDR5, we determined the three-dimensional x-ray structures of WDR5 bound to the 14-residue MLL2, MLL3, MLL4, SETd1a, and SETd1b Win motif peptides with resolutions ranging from 1.2 to 1.9 Å (Tables 2 and 3). In addition, we determined the structure of WDR5 bound to the 19-residue MLL1 Win motif peptide at 1.7-Å resolution. Crystals grown with the 14-residue MLL1 peptide did not show density for peptide.

The overall structure of WDR5 in each co-crystal structure is topologically similar to that of previously reported structures of WDR5 bound to MLL1 Win motif peptides or histone H3 peptides (35, 53, 61–64). The WDR5 structure consists of a seven-blade β-propeller with a central cavity that traverses the center of the protein from the top to the bottom (Fig. 6, a and b). Initial electron density difference maps revealed that each Win motif peptide binds in the central opening at the top of WDR5 with the conserved arginine residue in each structure (designated as position 0) inserted into the central tunnel (Fig. 6a and Table 1). The six conserved residues of the Win motif (which range from amino acid −3 to +2 relative to the conserved arginine) are ordered in each co-crystal structure and are all highly similar.
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TABLE 2

| Sequence | Space group | Cell dimensions (Å) | Resolution (Å) | Completeness (%) | Redundance | Rmerge |
|----------|-------------|---------------------|----------------|------------------|------------|--------|
| MLL1 | P2₁ | 38.606, 80.154, 83.517 | 6.7 (3.4) | 28.5 (2.61) | 99.4 (93.8) | 8.2 (47.8) |
| MLL2 | P2₁ | 139.78, 80.79, 88.12 | 7.5 (6.3) | 34.76 (4.42) | 100 (99.4) | 8.5 (54.3) |
| MLL3 | P2₁ | 195.12, 124–1.22 | 12.3 (10.0) | 58.9 (3.17) | 99.6 (96.9) | 7.9 (54.6) |
| MLL4 | P2₁ | 50.0 (1.55–1.50) | 13.6 (9.5) | 44.87 (6.07) | 99.8 (100) | 7.9 (54.6) |
| SETd1a | P2₁ | 78.49, 98.69, 80.280 | 13.6 (9.5) | 37.1 (3.25) | 99.7 (98.9) | 6.8 (52.1) |
| SETd1b | P2₁ | 78.254, 98.272, 79.879 | 13.2 (11.2) | 60.9 (12.06) | 98.3 (89.8) | 6.8 (52.1) |

*Values in parentheses are for the highest resolution shell.

Lys-259 (Fig. 9) lines the main chain hydrogen bond between the amide nitrogen of the +4 amino acid and the carbonyl oxygen of WDR5 residue Lys-259 (Fig. 6b). This hydrogen bond is absent for peptides

(Fig. 6, c–h). Superposition of all atoms in the conserved six-residue sequence in all peptides reveals an root mean square deviation of 0.207 Å (Fig. 7). The largest difference is that the carbonyl of the glutamate in position +2 is oriented in the opposite direction in the SETd1a and SETd1b structures compared with that of the MLL1–4 structures (Fig. 7). The observed differences within the conserved six-residue Win motif do not correlate with the 5–51-fold differences in affinity for peptide binding to WDR5, suggesting that the non-conserved residues that flank the Win motif may be responsible for differences in affinity. Indeed, the largest differences in the six structures are observed in the residues flanking the N and C termini of the Win motif.

Although there is some structural heterogeneity in the non-conserved residues flanking the N terminus of the Win motif, in four of the six structures, the N-terminal flanking residues (−4 to −7) adopt a U-shaped conformation in which the concave surface forms a pocket that interacts with the side chain carboxylate of the conserved +2 glutamate (Fig. 8). In each of these structures, the +2 carboxylate forms two hydrogen bonds with the side chain and main chain atoms of the residue in the −4 position (either histidine or threonine). We speculate that these additional interactions may stabilize the bound conformation of the 3₁-helix of the peptide when free in solution, which may enhance binding affinity. Similar N-terminal interactions may also be present in the other WDR5-Win motif complexes of MLL1 and SETd1b in solution, but these interactions could not be observed in the crystal structures in this investigation. The N-terminal flanking residues of the SETd1b peptide co-crystal structure were disordered and could not be modeled. In contrast, most of the N-terminal residues of the 19-residue MLL1 Win motif peptide were ordered (Fig. 6c), and the model reveals that they adopt a significantly extended conformation that is stabilized by interaction with another molecule in the asymmetric unit (not shown). This extended conformation is therefore likely a crystallization artifact.

In five of the six structures, at least two residues flanking the C-terminal end of the Win motif could be modeled unambiguously (Fig. 6). In the WDR5-MLL3 Win motif structure, all four C-terminal flanking residues could be modeled because of its high resolution (1.22 Å). However, the last two residues (+5 and +6) do not appear to make any contacts with WDR5. In contrast, in the WDR5-SETd1b structure, only one residue C-terminal to the conserved +2 glutamate could be unambiguously modeled (Fig. 6h).

A comparison of the C-terminal flanking residues among WDR5-Win motif peptide structures reveals that the +4 residue interacts with one of two shallow hydrophobic pockets on WDR5, which we arbitrarily designate as the A- and B-pockets (Fig. 9a). WDR5 residues Tyr-191, Pro-173, Phe-149, and Asp-172 line the A-pocket, which interacts with the +4 amino acid in the MLL1 and MLL4 peptides. The B-pocket is lined by residues Tyr-191, Pro-216, and Leu-234, which interact with the +4 amino acid side chain of the MLL2, MLL3, and SETd1a Win motif peptides. In addition, B-pocket peptides form an additional main chain hydrogen bond between the amide nitrogen of the +4 amino acid and the carbonyl oxygen of WDR5 residue Lys-259 (Fig. 9b). This hydrogen bond is absent for peptides
FIGURE 6. Human SET1 family Win motif peptides bind WDR5 using the same arginine-binding pocket. a–h, the WDR5 molecule is shown as a surface representation (gray) and the different Win motif peptides are shown in different colors. a, superposition of Win motif peptides shown with a schematic representation. b, cut-away view of the arginine-binding pocket of WDR5 is shown. All Win motif peptides insert the conserved arginine into the central tunnel in WDR5. The side chain of the conserved arginine is shown as a stick representation. c–f, simulated annealing Fo−Fc omit maps showing the different Win motif peptides bound to WDR5. MLL1 (c) and MLL3 (e) Win motif peptides are contoured at 3σ, and MLL2 (d), MLL4 (f), SETd1a (g), and SETd1b (h) Win peptides are contoured at 3σ. The peptides are shown as sticks and color-coded as follows: MLL1, green; MLL2, pink; MLL3, yellow; MLL4, orange; SETd1a, cyan; and SETd1b, dark blue.)
that bind in the A-pocket. All of the B-pocket Win motif peptides, albeit to varying degrees, have higher affinity for WDR5 compared with that of the MLL1 Win motif peptide. This may be due to the presence of this additional main chain hydrogen bond and van der Waals interactions with the +4 amino acid in the B-pocket.

Binding of the +4 amino acid in the A- or B-pocket is not correlated with the identity of the residue in the +4 position as tyrosine is seen in both A- and B-pockets of the MLL4 and SETd1a structures, respectively (Fig. 9a). Instead, A- or B-pocket binding appears to be correlated with the identity of the preceding residue in the +3 position. Common among A-pocket peptides is the presence of a valine in the +3 position of MLL1 and MLL4 structures. The valine side chain in the +3 position interacts with WDR5 residue Tyr-260 and the aliphatic portion of the Lys-259 side chain (Fig. 9b). In contrast, B-pocket peptides have either proline (MLL2 and MLL3) or glycine (SETd1a and SETd1b) in the +3 position. The +3 proline interacts more extensively with the Tyr-260 side chain and to a lesser extent with the Lys-259 side chain (Fig. 9b). These results suggest that it is the different interactions between WDR5 and the side chain or main chain atoms of the +3 amino acid of the Win motif that determines at least in part the location of the +4 amino acid.

Despite differences in the positions of the C-terminal amino acids of the different Win motif peptides, there is remarkably little difference in the positions of WDR5 residues in the different complexes. Main chain atom positions of WDR5 are highly similar among the different Win peptide-bound structures and superpose with root mean square deviations of 0.1–0.4 Å. The largest difference is a rotation around the Cβ–Cγ bond of Tyr-191, which rotates to accommodate interactions with the +4 amino acid of the different Win motif peptides (Fig. 9c). In addition, the side chain of WDR5 residue Lys-259 is oriented differently in the SETd1a and SETd1b Win motif peptide structures compared with that of the MLL1–4 structures (Fig. 9c).

The −2 and +4 Amino Acids of SET1 Family Win Motifs Contribute to High Affinity Binding to WDR5—The amino acid sequences of the MLL1 and MLL4 peptides differ in only two positions (−2 and +4) (Fig. 10, a and b, respectively); however, the MLL4 peptide binds WDR5 with 31-fold greater affinity (Table 1). Comparison of the three-dimensional structures reveals that the tyrosine hydroxyl atom of the +4 position in the MLL4 Win motif peptide forms a direct hydrogen bond with WDR5 residue Asp-172 in the A-pocket (Fig. 10b). A similar direct hydrogen bonding pattern is absent in the MLL1 Win

FIGURE 7. Conserved Win motif residues adopt structurally similar conformations. Shown is model superposition of the six conserved Win motif residues from different WDR5-Win motif peptide co-crystal structures. Conserved Win motif residues are indicated and numbered with respect to the conserved arginine (position 0). Refer to Table 1 for residue numbering. WDR5 molecule is shown as a surface representation (gray), and the different Win motif peptides are shown as sticks (color-coded as in Fig. 5).

FIGURE 8. Structural comparison of N-terminal flanking residues from different SET1 family Win motif peptides. a–f, N-terminal flanking residues (−3 and −4) of MLL2 (b), MLL3 (c), MLL4 (d), and SETd1a (e) Win motif peptides adopt a U-shaped conformation where the concave surface interacts with the +2 glutamate side chain. Red dashes show hydrogen bonds between the −4 and +2 residues. Refer to the text for a more detailed description.
Win motif peptides, that this contribution results from an increase in entropy associated with easier desolvation of the alanine in the −2 position compared with that of serine for insertion into a relatively hydrophobic pocket on WDR5.

DISCUSSION

We showed previously that a peptide that mimics the MLL1 Win motif disrupts the assembly of the MLL1 core complex in vitro and inhibits its H3K4 dimethylation activity (36). These results suggest that disrupting the interaction between MLL1 and WDR5 using Win motif peptides or related compounds may be an effective strategy for the inhibition of H3K4 methylation in malignant cells (65). It is expected that such a strategy would be far more specific compared with efforts to design S-adenosylmethionine-related mimics that may bind to a wider variety of methyltransferases. A challenge remains in the identification of Win motif inhibitors that target specifically MLL1 over other human SET1 family members. However, little is known about the relative stability of SET1 family complexes. To begin to address this question and to identify more potent Win motif inhibitors of the MLL1 core complex, we compare thermodynamic binding parameters, inhibition constants, and three-dimensional crystal structures of several Win motif peptides derived from the other members of the human SET1 family (MLL2, MLL3, MLL4, SETd1a, and SETd1b).

We demonstrate that Win motif peptides from the other human SET1 family members bind WDR5 with 5–51-fold greater affinity than that of a similar length MLL1 Win motif peptide. Our three-dimensional structures reveal that each peptide inserts the conserved arginine of the Win motif into the central tunnel of WDR5. Surprisingly, despite the large differences in affinity, the three-dimensional structure of WDR5 displays remarkably little variation when bound to the different peptides. Instead, most of the variation is observed in the non-conserved residues located C-terminal to the Win motif in SET1 family sequences. WDR5 appears to have evolved two separate hydrophobic pockets to accommodate different sequences in the +3 and +4 positions of the Win motif peptides. In each case, high affinity binding appears to be conferred by an additional direct hydrogen bond either through main chain or side chain interactions between WDR5 and the +4 amino acid of SET1 family sequences. These interactions likely account for the majority of the affinity differences among SET1 family peptides. However, our data also suggest that subtle variation within the conserved six-residue Win motif sequence also contributes to the observed differences in binding energy. We speculate that binding may be facilitated if the conformation of the α3-helix is stabilized in solution by a hydrogen bond between the side chain in the −2 and +1 positions of the Win motif. Indeed, the MLL2, MLL3, and SETd1b peptides possess such a hydrogen bond, and they bind WDR5 with the greatest affinity. For those peptides lacking this hydrogen bond, binding may be facilitated by residues that are easier to desolvate when binding to WDR5. This may explain why replacing the −2 serine in the MLL1 peptide with alanine results in 3-fold greater affinity to WDR5.

Although the strength of the interaction between WDR5 and different full-length SET1 family members is unknown, the dis-
Differences in the binding affinities of the peptides shown here suggest that the SET1 family complexes may have differing overall stabilities. If this hypothesis is correct, then it is predicted that the weakest complex would be disrupted at the lowest concentration of Win motif inhibitors before other SET1 family complexes are disrupted. Our studies suggest that MLL1 has the weakest affinity for WDR5 and therefore would likely be most sensitive to inhibition by peptides or other compounds that mimic the MLL1-WDR5 interaction interface. The methylations in the wild-type MLL3 Win motif contains a cysteine and serine in the same respective positions. Our three-dimensional structure of the wild-type MLL3 Win motif reveals that the Cys and Ser are within hydrogen bonding distance. This hydrogen bond is absent in the mutant MLL3 peptide, which may explain its reduced affinity for WDR5. It is also possible that reduced affinity for the mutant MLL3 peptide could be due to the absence of the four N-terminal flanking residues, which hydrogen bond with the conserved Glu. The presence of these features in the wild-type MLL3 peptide may improve overall affinity by reducing the number of different conformations of the peptide that WDR5 must sample in solution.

In summary, we have shown that the MLL1 Win motif is essential for the assembly of the MLL1 core complex in mammalian cells. We have also shown that Win motif peptides bind to WDR5 with a wide range of affinities, suggesting that
human SET1 family complexes may be differentially sensitive to Win motif inhibitors with the MLL1 core complex being the most sensitive. Our structure-function results provide the basis for the rational design of improved Win motif inhibitors. We expect that the MLL3 Win motif peptide will be a good template for future rational drug design efforts.

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