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Journal Title: Nucleic Acids Research
Volume: Volume 39, Number 15
Publisher: Oxford University Press | 2011-04-22, Pages 6380-6389
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1093/nar/gkr256
Permanent URL: http://pid.emory.edu/ark:/25593/cdt6h

Final published version: http://nar.oxfordjournals.org/content/39/15/6380

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Accessed October 3, 2019 4:03 PM EDT
Structural basis of SETD6-mediated regulation of the NF-κB network via methyl-lysine signaling

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Received January 25, 2011; Revised March 30, 2011; Accepted April 1, 2011

ABSTRACT

SET domain containing 6 (SETD6) monomethylates the RelA subunit of nuclear factor kappa B (NF-κB). The ankyrin repeats of G9a-like protein (GLP) recognizes RelA monomethylated at Lys310. Adjacent to Lys310 is Ser311, a known phosphorylation site of RelA. Ser311 phosphorylation inhibits Lys310 methylation by SETD6 as well as binding of Lys310me1 by GLP. The structure of SETD6 in complex with RelA peptide containing the methylation site, in the presence of S-adenosyl-L-methionine, reveals a V-like protein structure and suggests a model for NF-κB binding to SETD6. In addition, structural modeling of the GLP ankyrin repeats bound to Lys310me1 peptide provides insight into the molecular basis for inhibition of Lys310me1 binding by Ser311 phosphorylation. Together, these findings provide a structural explanation for a key cellular signaling pathway centered on RelA Lys310 methylation, which is generated by SETD6 and recognized by GLP, and incorporate a methylation–phosphorylation switch of adjacent lysine and serine residues. Finally, SETD6 is structurally similar to the Rubisco large subunit methyltransferase. Given the restriction of Rubisco to plant species, this particular appearance of the protein lysine methyltransferase has been evolutionarily well conserved.

INTRODUCTION

Mammalian nuclear factor κB (NF-κB) is a critical mediator of inducible transcription in the control of key physiological and pathological states, from immunity and inflammation to cancer [reviewed in (1)]. The NF-κB family of transcription factors consists of five members: p65 (RelA), p50 (NF-κB1), p52 (NF-κB2), c-Rel and RelB (2). The area of greatest homology among the NF-κB members occurs in the conserved N-terminal Rel homology region, which is composed of a DNA binding domain and a dimerization domain. Through the dimerization domain, different NF-κB members form a variety of homo- and hetero-dimers, with the p65/p50 combination being the most abundant. In addition, p65, but not p50, possesses a C-terminal transactivation domain (TAD) that is required for promoting transcription (see Supplementary Figure S1a). In unstimulated cells, the majority of NF-κB, including the p65/p50 heterodimer species, is sequestered in the cytosol by the ankyrin-repeat-containing IκB proteins. NF-κB activation by stimulants-like cytokines triggers a signaling cascade that results in degradation of IκB and releasing NF-κB to translocate into the nucleus and function as a transcription factor at target genes (3).

Recently, we described a previously uncharacterized mechanism in which, under basal conditions, the protein lysine methyltransferase (PKMT) SETD6 monomethylates chromatin-associated RelA at lysine 310, a residue located within the linker region between the dimerization and activation domains of RelA (4). Another PKMT, G9a-like protein (GLP), via its ankyrin-repeat domain, binds RelA methylated at Lys310 (Lys310me1) and acts to locally condense chromatin at several NF-κB-dependent target genes. The repressed chromatin state is terminated upon stimulating cells with TNFα, due to phosphorylation of RelA at serine 311 (4). Here we determine the SETD6–RelA peptide complex structure, which provides insight into the molecular basis for RelA peptide recognition by SETD6 as well as an understanding of the effects of modifications at nearby residues on SETD6-mediated Lys310 methylation. In addition, we
investigate the molecular basis of RelA Lys310me1 peptide recognition by the GLP ankyrin repeats. Finally, we generate a model that connects histone peptide-bound GLP with a DNA-bound NF-κB, using the existing structural information. Our study suggests that the methyl-phospho switch between two adjacent residues of RelA, Lys310 and Ser311, regulates localized chromatin state to influence NF-κB-target gene expression.

MATERIALS AND METHODS

Protein expression and purification

There are two splice variants of human SETD6 (Figure 1a): the longer 473 residue variant (isoform a) and the shorter 449 residue variant (isoform b), which lacks an in-frame segment (residues 40–63) and was used here for crystallography (residue numbering is based on the longer variant). SETD6 isoform b (residues 17–449) was sub-cloned into a His6-SUMO vector (generating plasmid pXC862) and confirmed by sequencing. All of the proteins were overexpressed in *Escherichia coli* strain BL21 (DE3) RIL-Codon plus strain (Stratagene). Cells expressing His6-SUMO-tagged SETD6 were induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 h at 16°C. Cells were collected, pelleted and then resuspended in 50 mM sodium phosphate, pH 7.4, 300 mM NaCl, 5% glycerol. The cells were lysed by two passes through a French pressure cell press and then centrifuged at 23,000g for 1 h. The soluble His6-SUMO fusion protein was first purified using the HisTrap HP column (GE Healthcare). The fusion protein was cleaved by Ulp-1 protease in overnight dialysis at 4°C. Only two extraneous N-terminal amino acids (HisAsn) were left as a result of a restriction site. The cleaved SUMO tag was removed by ion exchange purification (HiTrap Q HP, GE Healthcare). The SETD6 protein was further purified by gel-filtration chromatography (Superdex 200, GE Healthcare). All protein purification was performed at 4°C. For crystallization, the purified protein was concentrated to ~16 mg ml⁻¹ in the presence of 100 μM S-adenosyl-L-methionine (AdoMet).

The human RelA dimerization domain plus the linker region (amino acids 191–325; see Supplementary Figure S1a) was expressed as a His6-SUMO tagged construct in *E. coli* BL21(DE3)-Gold cells (Stratagene) with RIL-Codon plus plasmid (pXC875). For the RelA:p50 heterodimer, His6-SUMO-tagged human RelA (residues 1–325 including DNA binding domain, dimerization domain and linker region; pXC914) and non-tagged human p50 dimerization domain (residues 243–366; pXC902) in pET21b (Novagen) were coexpressed in *E. coli* BL21(DE3). Similar purification strategies were used for both complexes. The soluble fraction was isolated using a nickel-charged HiTrap chelating HP column (GE Healthcare). The fused SUMO tag was removed by Ulp-1 protease in overnight dialysis at 4°C. The product protein was further purified by cation-exchange and gel-filtration chromatography. Size exclusion chromatography was employed to evaluate dimer formation.

Crystallography

Crystallization of the SETD6–AdoMet–RelA peptide complex was carried out by the hanging-drop vapor-diffusion method at 16°C after mixing the protein with peptide in a ratio of ~1:1.2, and then mixing with an equal amount of well solution (1.5 μl). Both native and selenium-substituted SETD6–AdoMet, in the presence of RelA peptide, were crystallized using well solutions containing 15% (w/v) polyethylene glycol (PEG) 3350, 0.1 M di-ammonium hydrogen citrate, pH 4.6. The crystals belonged to space group P1 with two SETD6–AdoMet–peptide complexes per asymmetric unit. For data collection, the crystals were equilibrated in a cryoprotectant buffer containing reservoir buffer plus 20% (v/v) ethylene glycol. The native and selenium SAD data sets were collected at SER-CAT beamline 24ID of Advanced Photon Source (APS) at Argonne National Laboratory. All the data sets were processed using the program HKL2000. The structure was determined and refined utilizing components of the SGXPRO (5) and PHENIX (6) program packages. The program COOT (7) was used for building peptide and manual model manipulation between rounds of refinement with PHENIX. Structural figures were generated by the program MacPyMol (DeLano Scientific). The RelA peptide was synthesized.
Mass spectrometry-based peptide methylation assay

Human RelA peptide (residues 302–316) was used as substrate for SETD6. A reaction mixture contained 50 mM glycine pH 10.2 (or di-ammonium hydrogen citrate for pH 4.6, Bis–Tris–HCl for pH 6.4, Tris–HCl for pH 7.4, 8.2 and 8.6, and glycine/NaOH for pH 9.0–11.0), 5 mM dithiothreitol, 200–500 μM AdoMet, 10 μM SETD6 and 30 μM peptide. The assays were carried out at room temperature (∼25°C) for 1 h with a total volume of 20 μl. The reaction was terminated by addition of trifluoroacetic acid (TFA) to 0.1% (v/v). The resulting peptides were measured by MALDI-TOF on a Bruker Ultraflex II TOF/TOF instrument (Biochemistry Department, Emory University School of Medicine).

Peptide methylation analysis by the LC-MS/MS approach

Peptides methylated by SETD6 were analyzed by reverse-phase liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) as reported (8). The peptide mixtures were loaded onto a C18 column, eluted and monitored in a MS survey scan followed by data-dependent MS/MS scans on a LTQ-Orbitrap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The acquired MS/MS spectra were searched against a database containing the synthetic peptides. Modified methylation sites were determined by dynamic assignment of mass addition (14 Da) to lysine residues during the search. Finally, all modified peptide assignments were manually examined.

In vitro methylation of RelA by SETD6

Methylation assays were carried out in a 20 μl reaction (45 μM substrate, 5.5 μM [methyl-3H]AdoMet, with and without 32 μM recombinant SETD6) in 20 mM Tris, pH 8.5 and 5 mM DTT for 12 h at 25°C. Samples were analyzed by 17% SDS–PAGE gel and fluorography after 36 h of exposure.

Cell lines and transfections

Human embryonic kidney 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS, Gibco), 100 U ml−1 penicillin and 1-glutamine. Cells were transfected with TransIT 293 transfection reagent (Mirus) according to the manufacturer’s protocols.

Plasmids and mutagenesis

For overexpression in mammalian cells, the plasmids used were: pcDNA-RelA, pcCAG-Flag-SETD6 WT, pcCAG-Flag-SETD6N293A. The pGEX-derived plasmids used were pGEX–GLPANK WT, pGEX–GLPANK W843A, pGEX–GLPANK E851A and pGEX–GLPANK W881A. Mutants were constructed using the QuickChange site-directed mutagenesis kit (Stratagene).
Figure 2. Interactions of SETD6 and RelA K310 peptide. (a) SETD6 activity as a function of pH, (b) the surface representation of SETD6, colored with yellow (SET), orange (i-SET) and green (C-SET). For clarity, the C-SET domain has been sliced away. In addition, the surface nitrogen atoms are colored blue and surface oxygen atoms are red, (c) electrostatic interactions, hydrogen bonds and van der Waals interactions define SETD6 (in green) and RelA peptide (in light blue) interactions. The linear conformation of Lys310 of RelA is in cyan, and the bent conformation in light blue, (d) the bent conformation of the target lysine side chain forms a hydrogen bond with the side chain hydroxyl oxygen of Y297 and the main-chain carbonyl oxygen of S224, (e) a model of phosphated-Ser311 of RelA (pSer311) with the phosphate group potentially clashing with P228 of SETD6 and (f) In vitro methylation assays by SETD6 on unmodified RelA peptides (left panels) or phosphorylated at Ser311 (pS311) (right panels) followed by mass spectrometry of the reaction products (0 h, before assay; 3 h, after assay).
event would not occur and the methyl transfer between the donor methyl group (S'\text{CH}_3) and the acceptor amino group (NH_3\text{+}) would be inhibited.

Interestingly, the side chain of the target lysine 310 of RelA adopts two conformations: one is linear and the other is bent (Figure 2c). The linear conformation of lysine 310 points its ε-amino group to the transferable methyl group of AdoMet (Figure 2c) so that the methyl donor and acceptor are aligned in a nearly linear geometry (N...C distance of 3.4 Å and N...C-S angle of 161°) for SN2 nucleophilic transfer of the methyl group during catalysis. The terminal ε-amino group of the bent conformation sits in the carboxyl end of helix αG (Figure 2c and Supplementary Figure S2c). Thus the positive charge of the terminal ε-amino group of the bent conformation under the low pH condition is effectively balanced by the partial negative dipole charge at the carboxyl end of the helix and stabilized by hydrogen-bonding interactions with S224 (main chain carbonyl oxygen) and Y297 (side chain hydroxyl oxygen) of SETD6 (Figure 2d; one-letter code is used for SETD6 residues).

Specificity for the RelA peptide is determined primarily through recognition of side chains of RelA (Phe309, Ser311 and Ile312) before and after the target Lys310 (Figure 2c; three-letter code is used for RelA residues). The network of interactions includes the following: (i) the phenyl ring of Phe309 of RelA packs against M296 and Y297 of SETD6, (ii) The target nitrogen atom of RelA Lys310 forms a water-mediated hydrogen bond with the main chain carboxyl oxygen of L250 (the linear conformation) or with Y297 and S224 (the bent conformation); (iii) Ser311 of RelA is involved in a polar interaction with the main chain carboxyl oxygen of Q226 and a van der Waals contact with P228 of SETD6. Adding a phosphate group to the side chain hydroxyl oxygen of Ser311 of RelA would result in repulsion from SETD6. Adding a phosphate group to the side chain hydroxyl oxygen of Ser311 of RelA would result in repulsion from SETD6. As shown in Figure 2f, phosphorylation of Ser311 (14) causes a complete loss of Lys310 methylation in the context of peptide substrate; and (iv) the side chain of Ile312 of RelA fits into a surface pocket formed by F225, L260, N283 and T284 of SETD6.

Four aromatic residues (Y223, F225, Y285, Y297) and one polar residue (N283) of SETD6 form the largely hydrophobic active site and wrap around the aliphatic chain of the target lysine. Interestingly, only one of these residues (Y285) is conserved in Set7/9 (Figure 3a), another human protein lysine mono-methyltransferase. The hydroxyl group of Y285 of SETD6 is hydrogen bonded to the backbone carboxyl oxygen of L250 and is snug between the methyl group and adenine ring of AdoMet (Figure 3b). The Y285A mutation in SETD6 abolished its enzymatic activity (4), whereas the corresponding Y283F mutation in DIM-5 (a Neurospora histone H3 lysine 9 tri-methyltransferase) lost its AdoMet binding.

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**Figure 3.** Comparison of active sites of SETD6 and related SET domain proteins. (a) Superimposition of active sites of SETD6 (colored) and Set7/9-ER (estrogen receptor) complex (PDB 3CBM). (b) The hydroxyl group of Y285 of SETD6 is in contact with AdoMet. (c) Superimposition of active sites of SETD6 (colored) and Dim-5-H3 complex (PDB 1PEG) and (d) Superimpositions of active sites of SETD6 (colored) and LSMT (PDB 2H2J).
and thus activity (12). In contrast, all four aromatic residues are conserved and superimposable between SETD6 and DIM-5 (Figure 3c) as well as G9a methyltransferase [a mammalian histone H3 lysine 9 mono- and di-methyltransferase (15,16)], while N283 of SETD6 is in the place of F281 of DIM-5 (Figure 3c) or the corresponding Phe (F1205) of G9a. There are two differences between SETD6 and Rubisco LSMT (an enzyme that generates a tri-methyl-lysine) in the active site, Y223 and N283 of SETD6 replacing R222 and I285 of LSMT, respectively (Figure 3d). Despite the high level of conservation among the active site residues of the two
tri-methyltransferases (DIM-5 and LSMT) and SETD6. SETD6 is a mono-methyltransferase (see Discussion in Supplementary Data).

Structural and sequence similarities between SETD6 and LSMT

The structure of SETD6 shares high similarity with Rubisco LSMT (13) (Figure 4a), and the structure-based sequence alignment between the two enzymes reveals sequence conservation throughout the entire region (Figure 4b). SETD6 represents a sub-family of SET domain-containing protein lysine methyltransferases (PKMTs) with an insertion of about 100–200 amino acids in the middle of the SET domain (Supplementary Figure S2d). Two sequences share 18% identity (81/449) and 31% similarity (140/449). Only three regions have suffered insertions of more than five residues in SETD6: residues 232–239 (part of a disordered loop connecting i-SET to SET domain), residues 333–345 (helix αJ) and residues 440–444 (helix αP). There is one 9-residue deletion around SETD6 residue 265 (making a shorter loop between two strands of SET domain).

The SET domain was originally identified in three Drosophila proteins involved in epigenetic processes: the suppressor of position-effect variegation 3-9, Su(var)3-9; an enhancer of the eye color mutant zeste, En(zeste); and the homeotic gene regulator Trithorax (17). Based on the sequence similarity of the SET domain to that of plant methyltransferases including the Rubisco LSMT, the mammalian homologues of Drosophila Su(var)3-9 and of Schizosaccharomyces pombe Clr4 were the first histone lysine methyltransferases identified, and they specifically methylate lysine 9 of histone H3 (18). Here we show that human SETD6 shares a striking structural similarity to the Rubisco LSMT (13) throughout the entire protein. The unexpected resemblance of these two PKMTs, given the restriction of Rubisco to plant species, suggests that this particular appearance of the PKMT has been evolutionarily successful.

Model of the SETD6–RelA complex

Based on the fact that the RelA linker region—containing the methylation site Lys310—has a simple secondary structure (either a single helix or a flexible loop; Supplementary Figure S1b and c), we performed a rigid body docking of the RelA/p50 heterodimer into the V-cleft of SETD6, resulting in a very good overall fit (Figure 5a). In our docking, the RelA linker helical region localizes to the bottom of the cleft and the helical axis is nearly perpendicular to the longest dimension of SETD6, with the dimerization and the activation domains on either side of the V-cleft of SETD6, resulting in a very good overall fit (Figure 5a). In our docking, the RelA linker helical region localizes to the bottom of the cleft and the helical axis is nearly perpendicular to the longest dimension of SETD6, with the dimerization and the activation domains on either side of the V-cleft of SETD6, resulting in a very good overall fit (Figure 5a).

Recognition of RelA K310me1 by GLP ankyrin repeats

Methylation of RelA by SETD6 represses NF-κB signaling via the recognition of Lys310me1 by the ankyrin repeats of GLP (G9a-like protein) with a dissociation constant (K_D) of ~5 μM (4). G9a and GLP are euchromatin-associated methyltransferases that repress transcription by mono- and di-methylating histone H3 lysine 9 (H3K9me1/2) via their C-terminal SET domains (19). Previously, we showed that the centrally located ankyrin repeat domains of G9a and GLP bind to histone

Figure 5. Hypothetical complex model of SETD6/NF-κB. (a) The RelA subunit resembles a dumbbell with the dimerization and the activation domains (TAD) connected by a linker region. The SETD6-driven methylation site, Lys310, is part of the linker region that also harbors nuclear localization signal (see Supplementary Figure S1a). The truncated NF-κB heterodimer structure [PDB 1FNI; p50 (gray)/RelA (cyan)] (24) is docked onto the V-cleft of SETD6, which grips the linker region. (b) In vitro methylation of RelA, either as a RelA homodimer (lanes 2 and 3) or a RelA/p50 heterodimer (lanes 4 and 5), by SETD6. The top panel shows the Coomassie stain and the fluorography is presented at the bottom. (yellow) and 387–394 (green), located in the inner surface of the V-cleft and the rim of the C-terminal domain. We hypothesize that on association with the RelA/p50 heterodimer or RelA homodimer (Supplementary Figure S1d), the unstructured SETD6 loops adopt stable conformations that include contacts with the linker regions of RelA/p50.
H3 peptides containing H3K9me2/1, and phosphorylation of H3S10 in the context of H3K9me2 completely eliminates peptide binding (10). Analogous to H3S10 phosphorylation (see sequence alignment in Figure 6a), phosphorylation of Ser311 of RelA blocks the binding of Lys310me1 by GLP in vitro and in cells (4), probably due to steric hindrance from the Ser-interacting glutamate (E874 of human GLP) (Figure 6a). Mutations of the GLP residues involved in forming the methyl-lysine cage (W843A, E851A and W881A) abrogate the binding of GLP to the Lys310-monomethylated RelA peptide (Figure 6b).

**DISCUSSION**

Three classes of ankyrin repeat domain-containing proteins are involved in controlling NF-κB signaling.
The NF-κB precursor protein p105 possesses a N-terminal p50 amino acid sequence and its own inhibitory ankyrin repeat within its carboxy-terminal region (20). Once processed, the NF-κB dimer RelA/p50 exists in the cytoplasm of resting cells by its association with an IkB inhibitor protein (21). IkB uses its entire ankyrin repeat domain for interacting with the RelA linker region, which includes Lys310 [reviewed in (22)]. Active NF-κB accumulates in the nucleus where it preferentially binds a specific DNA sequence in the promoter regions of target genes to activate transcription. In addition, Levy et al. (4) suggested that SETD6-mediated Lys310 methylation and its recognition by the ankyrin repeat domain of GLP renders NF-κB inert due to downstream silencing events mediated by GLP-associated histone H3 lysine 9 methylation. Encouraged by the known structures of (i) the RelA/p50 heterodimer bound to DNA (23), (ii) the RelA/p50 heterodimer bound to IkB (24) and (iii) the GLP ankyrin repeat domain (10) and SET domain of GLP bound with H3 peptide (25), we modeled a quaternary complex involving DNA, NF-κB (RelA/p50), and GLP (ankyron repeats and SET domain) (Figure 6c). Our model supports the notion that the SETD6–RelA–GLP–H3K9me2/1 network constitutes a lysine methylation signaling cascade, initiated by SETD6-mediated RelA methylation at Lys310, followed by recruitment of a histone-modifying enzyme (GLP), which subsequently generates a repressive mark (H3K9me2/1).

Finally, it is interesting to note that a different class of nuclear, ankyrin-repeat-containing IkB proteins (26,27) bind homodimers of p50 (28) that lack tranactivation domains, and the nuclear IkB•NF-κB complexes can bind DNA as repressors of transcription. In this regard, we speculate that the ankyrin repeat-containing GLP might function as an interaction competitor of nuclear IkB for the RelA subunit, and the resulting H3K9 methylation may reinforce silencing. It is possible that the repressive GLP and G9a heterodimer (29) allows one of these methyltransferases to interact with RelA and the other to interact with histone H3 (Figure 6d).

**ACCESSION NUMBERS**

Protein Data Bank: The coordinates and structure factors of the SETD6–RelA peptide–AdoMet complex have been deposited with accession numbers 3QXY (with the target lysine in alternative bent and linear conformations) and 3RC0 (with the target lysine in bent conformation).

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

**ACKNOWLEDGEMENTS**

Y.C. performed SETD6 enzyme purifications for both wild-type and selenomethionyl proteins, mass spectrometry-based methylation assays, crystallization and X-ray data collection; D.L. performed the cell culture and the peptide pull-down experiments; J.R.H. participated in collecting X-ray data, determined structures and performed structural refinements; J.P. performed peptide methylation analysis by the LC–MS/MS approach; D.L. and O.G. provided initial expression constructs and the knowledge of specificities of SETD6 and RelA peptides. X.Z. developed and optimized the mass spectrometry-based assay; X.C. and O.G. organized and designed the scope of the study, and all were involved in analyzing data and helped in writing and revising the manuscript. The authors thank Ruth Tennen for reading and editing the manuscript.

**FUNDING**

The Department of Biochemistry at the Emory University School of Medicine supported the use of the SER-CAT synchrotron beamline at the Advanced Photon Source of Argonne National Laboratory, local X-ray facility and MALDI-TOF mass spectrometry. US National Institutes of Health (Grants GM068680 to X.C. and DA025800 to O.G.); (NIH/NCCR R21RR025822 to J.P.); European Molecular Biology Organization, Human Frontier Science Program, and the Machiah Foundation Fellowship (to D.L., partial); X.C. is a Georgia Research Alliance Eminent Scholar; O.G. is an Ellison Senior Scholar. Funding for open access charge: National Institutes of Health.

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