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De novo DNA methyltransferase DNMT3A: Regulation of oligomeric state and mechanism of action in response to pH changes

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ABSTRACT

Background: The oligomeric state of the human DNMT3A is functionally important and cancer cells are known to undergo changes in pH (intracellular).
Methods: Light scattering, gel filtration, and fluorescence anisotropy. Also, methylation and processivity assays.
Conclusions: Physiologically relevant changes in pH result in changes in DNMT3A oligomer composition which have dramatic consequences on DNMT3A function.
General significance: The pH changes which occur within cancer cells alter the oligomeric state and function of DNMT3A which could contribute to changes in genomic DNA methylation observed in vivo.
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1. Introduction

Covalent modification of DNA by methylation of cytosine residues is heritable and reversible and regulates a diverse range of biological processes. In mammals, DNA methylation is essential to cellular programing, memory formation, imprinting, silencing transposable elements, and gene regulation [1,2]. Malignant tumors undergo dramatic changes in their methylation patterns that correlate with altered transcriptional profiles [3]. DNA methylation is initially (de novo) established by DNMT3A and the closely related DNMT3B in a spatial and temporally regulated manner [4–8]. The DNMTs transfer a methyl group from the cofactor *S*-adenosyl-methionine (AdoMet) to cytosines primarily located in a CpG dinucleotide pair.

For many proteins, physiological conditions regulate function by altering substrate affinity, interactions with binding partners, and oligomeric state [9–11]. Hypoxia, extracellular acidification, and transient changes in intracellular pH occur frequently in tumors, and impact tumor growth and response to therapeutics [12,13]. Cancer cells usually have a decrease in extracellular pH from 7.8 to 6.8 [12]. Gene expression is greatly altered by intracellular changes in pH, due in part from changes in mRNA stability [14,15] and DNA binding proteins changing their protein–protein and protein-DNA complex affinities, including NF-κB, AP-1 [15] and Sp1 [16]. Sp1 binds CpG sites, protecting individual regions from methylation and is activated by increasing acidity [16]. pH changes are also known to alter the oligomeric state of many proteins, including a p53 mutant [17], where tetramerization is disrupted and thus DNA binding. It is proposed that the combination of the p53 mutation and altered pH in cells leads to adrenocortical carcinoma. DNA methylation patterns change during cancer progression but the mechanism that drives altered patterning is mostly unknown. Thus, small changes in pH that alter protein–protein and protein–nucleic acid interactions may contribute to the changes in methylation patterning observed in malignant cells. In addition, during development intercellular pH changes have been observed ranging from about 0.1 to 1.6 pH units in many diverse organisms [18].

Recently, the oligomeric state of DNMT3A was shown to be important in regulating the catalytic properties of the enzyme [19].[1] DNMT3A interacts with DNMT3L, a non-catalytically active homologue [20–22] to form a DNMT3L:DNMT3A:DNMT3A:DNMT3L heterotetramer complex and the interface along which DNMT3L binds (the tetramer interface) also supports DNMT3A homotetramerization in the absence of DNMT3L [19]. At the opposite side of DNMT3A's catalytic domain in the recognition domain, DNMT3A also oligomerizes (dimer interface) [23].

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DNMT3A can even further oligomerize. Disruption of either interface by mutations has modest effects on the catalytic activity of the enzyme but significant consequences to the ability of the enzyme to carry out multiple turnovers on the same strand of DNA. Disruption of homo-oligomerization in DNMT3A also has been shown to disrupt binding to nucleosomes and is necessary for heterochromatic localization [24]. Recurrent DNMT3A mutations are also found in AML and myelodysplastic syndrome MDS patients with poor survival outlook [25,26]. The majority of the mutations, mainly at the dimer interface and accounting for ~15% of adult AML cases, were found to disrupt oligomerization in vitro and suggest the disruption of oligomerization has a significant role in the function of DNMT3A in vivo.[1] Therefore, disruption of the oligomeric state of DNMT3A may provide a mechanism for the elimination of clustered methylation observed in cancer patients [19].

In this study, we evaluated the functional significance of pH on DNMT3A. Acidification by one pH unit, from 7.8 to 6.8, completely disrupts the dimer interface of DNMT3A as observed with light scattering, gel filtration, and fluorescence anisotropy. Disruption of the dimer interface by pH decreases catalytic activity as a result of changes in oligomerization and DNA binding. Homotetramers carry out multiple cycles of methylation on the same piece of DNA (processive catalysis), while dimers have faster product release and a non-processive mechanism [19]. Our results show environmental conditions regulate the oligomeric state, and thus the catalytic properties, of DNMT3A. pH titration and mutagenesis studies suggest the observed effect may be due to the protonation of His-873 and/or His-821 at the dimer interface. The activities of mutants previously shown to disrupt the dimer interface, including AML mutation R882H, are recovered with acidification, resulting in similar activity between R882H and the wild type enzyme at pH 6.8. Our data suggests the DNMT3A dimer interface is dynamic and small pH changes that occur through development, localized physiological processes, or cancer progression could change methylation patterns created by of DNMT3A.

2. Material and methods

2.1. Cloning and protein purification

The catalytic domain of DNMT3A and full length DNMT3L were purified as stated in Holz-Schietinger and Reich [27]. Plasmids used for protein expression and site-directed mutagenesis, include codon-optimized pET28a-hDNMT3A_CD (Δ1-611) [28], and pTYB1-3L for hDNMT3L [22]. DNMT3A was purified from BioRex and nickel affinity columns, DNMT3L was purified from nickel affinity and chitin columns, all purified to >95%. The catalytic domain of DNMT3A has similar kinetic parameters as the full-length enzyme, including k_{cat}, K_m^{DNA}, K_m^{AdoMet}, processivity, and DNMT3L activation [22,27,28], and was used for the DNMT3A/DNMT3L co-crystal structure [23]. Both the homo- and DNMT3L hetero-oligomerization interfaces are located on the catalytic domain.

2.2. DNA sequences

Substrates include duplex poly-dIdC (~1000 bp) (Sigma-Aldrich), plasmid $pCpG^L$ (non-CpG substrate [29]), linear human promoters (*RASSF1A*, *p15*, *Hoxd4* and *Oct-4*) in plasmid $pCpG^L$, single site substrates GCbox2 unmethylated (5′-GGGAATTCAAGGGGCGGGGCAATGTTAGGG-3′) and fluorescent DNA (GCbox30) with fluorescein (6-FAM) on the 5′ end of the top strand, and (5′/6-FAM/TGGATATCTAGGGGCGCTATGATA TCT-3′) GCbox2 purchased from Integrated DNA Technologies (HPLC purified). See Supplemental methods for human promoter sequences and creation.

2.3. Size-exclusion chromatography coupled to multi-angle light scattering

Experiments were done as described in Holz-Schietinger et al. [19]: here we used a light scattering buffer at either pH 7.8 or 6.8 (50 mM KH_2PO_4/K_2HPO_4, with 200 mM NaCl, 1 mM DTT, 1 mM EDTA and 0.2% azide).

2.4. Methylation assays

DNMT3A methylation assays measured the amount of tritiated methyl groups transferred from cofactor AdoMet to the DNA by the enzyme. Reactions were carried out at 37 °C in reaction buffer (50 mM KH_2PO_4/K_2HPO_4, 1 mM EDTA, 1 mM DTT, 0.2 mg/mL BSA and 20 mM NaCl) at pH indicated. Enzyme was at 150 nM total, which is 27 nM active enzyme (previously determined in Purdy et al. 2010) [28]. DNA was the multiple site substrate poly-dIdC at saturation (40 μM base pairs (bp)) unless stated otherwise, all other substrates were also used at saturation (40 μM bp). AdoMet was used at saturation (5 μM). Processivity assays and DNMT3L activation assays were carried out as described in Holz-Schietinger and Reich [27]. Briefly, processivity assays involve three separate reactions; positive control: 20 μM bp substrate, experiment: substrate followed at 20 min by chase DNA ($pCpG^L$) at 20-fold excess over substrate concentration, and negative control: chase and substrate at the start of reaction. Mathematical modeling was done as described in Holz-Schietinger and Reich [27], and detailed in Supplemental methods. A 1:1 ratio with a one hour pre-incubation (with AdoMet) was used for all DNMT3L assays. All reactions were quenched by addition of 500 μM AdoMet and 50 μg/ml proteinase K. Samples were spotted onto Whatman DE81 filters then washed, dried, and counted as described previously.

For K_m determination, data was fit to the Michaelis–Menten equation, k_{cat}, and processivity data was fit to either linear regression or a fit to a nonlinear regression using Prism v5 (GraphPad). Error bars are standard error from three reactions. Bar charts of kinetic values compared mutants to wild type using one-way ANOVA to determine p-value using Prism.

2.5. Fluorescence anisotropy

Enzyme was titrated with 5′ 6-FAM labeled GCbox30 duplex DNA at 20 nM in reaction buffer with saturating AdoMet (1 μM). Data fit to single exponential, pH 6.8 fit to two curves, before 230 nM and after due to the two phasic behavior.

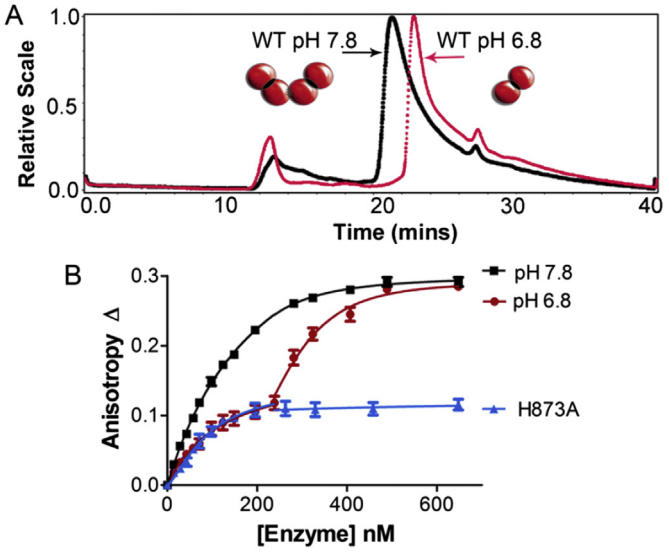

Fig. 1. Acidification disrupts tetramerization. A. Light scattering data of DNMT3A at pH 7.8 and pH 6.8. Size-exclusion chromatography of light scattering traces of the wild type catalytic domain at pH 7.8 (black trace) and at pH 6.8 (red trace). Molecular weights were determined from the amount of scattered light, in relation to the protein concentration determined by A_{280}. B. Anisotropy change observed with wild type DNMT3A at pH 7.8 (black) and pH 6.8 (red), and a mutant that disrupts the dimer interface, H873A (blue). DNA is a single site duplex labeled with 6-FAM at 20 nM and enzyme-varied.