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Brief Communication

Analysis of the Substrate Specificity of the Dim-5 Histone Lysine Methyltransferase Using Peptide Arrays

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SUMMARY

Histone methylation is an epigenetic mark essential for gene regulation and development. We introduce peptide SPOT synthesis to study sequence specificity of the Dim-5 histone-3 lysine-9 methyltransferase. Dim-5 recognizes R8-G12 of the H3 tail with T11 and G12 being the most important specificity determinants. Exchange of H3 tail residue S10 and T11 by E strongly reduced methylation by Dim-5, suggesting that phosphorylation of S10 or T11 may regulate the activity of Dim-5. In the Dim-5/peptide structure, E227 interacts with H3R8 and D209 with H3-S10. Mutations of E227 or D209 caused predictable changes in the substrate preference, illustrating that peptide recognition of histone methyltransferases can be altered by protein design. Comparative analyses of peptide arrays with wild-type and mutant enzymes, therefore, are well suited to investigate the target specificity of protein methyltransferases and study epigenetic crosstalk.

INTRODUCTION

Epigenetic regulation by covalent modification of histone proteins and methylation of DNA controls gene activity during development and disease processes (Egger et al., 2004; Feinberg and Tycko, 2004; Jones and Baylin, 2002; Li, 2002). Histones are posttranslationally modified by various enzymatic reactions including methylation, primarily at their flexible N termini (Berger, 2007; Margueron et al., 2005). Methylation of lysine residues occurs in histone H3 at residues K4, K9, K27, and K36; in histone H4 at K20; and in histone H1b at K26. All these modifications have different biological functions (Berger, 2007; Martin and Zhang, 2005); for example, H3K9 methylation leads to condensation of the chromatin and inhibition of gene expression, H3K27 methylation is correlated to gene silencing, while H3K4 methylation marks active chromatin (Li et al., 2007).

Most histone lysine methyltransferases (HKMTs) contain a SET (Su(var)3-9, *E*nhancer-of-zeste, *T*rithorax) domain which comprises approximately 130 amino acids and harbors the active center of the enzymes (Cheng et al., 2005). Different HKMTs not only vary in their substrate specificity but also in product pattern because lysine can be mono-, di-, or trimethylated (Zhang et al., 2003). Here, we investigate the target specificity of the H3K9 Dim-5 HKMT from *Neurospora crassa*. The enzyme generates trimethylated H3K9 (H3K9me3) in a processive reaction (Zhang et al., 2003) that controls DNA methylation in *N. crassa* (Selker et al., 2002; Tamaru and Selker, 2001; Tamaru et al., 2003). The three-dimensional structure of the protein alone and in complex with a peptide has been solved and allowed to identify the directly interacting moieties in atomic detail (Zhang et al., 2002, 2003).

While the sequence specificity of substrate and ligand interaction of proteases (Overall, 2002), kinases (Kreegipuu et al., 1998), and antibodies (Mariuzza et al., 1987) has been analyzed comprehensively, such in-depth studies have not yet been carried out for HKMTs because a large number of different peptides have to be analyzed to derive a complete specificity profile for one enzyme. Peptide array SPOT synthesis on cellulose membranes had been introduced to prepare large libraries of different peptides at amounts sufficient for biochemical assays and at moderate costs (Frank, 2002; Hilpert et al., 2007; Reineke et al., 2001; Wenschuh et al., 2000). The method was used to analyze the specificity of kinases and proteases (Hohne and Hilpert, 2005; Tegge and Frank, 1998) and to study the binding specificity of protein/protein interaction (Bialek et al., 2003; Frank, 2002; Hilpert et al., 2000, 2007). Here, we demonstrate that peptide arrays are an ideal approach to analyze the substrate specificity of HKMTs.

RESULTS AND DISCUSSION

We synthesized 21-residue peptides on a functionalized cellulose membrane by the SPOT synthesis method (Frank, 2002; Reineke et al., 2001; Wenschuh et al., 2000). The methylation of the respective substrates was analyzed by following the enzymatic transfer of radioactively labeled methyl groups from the coenzyme S-adenosyl-L-methionine (AdoMet) to the immobilized peptides. As a pilot experiment, several spots with the sequence of the first 21 amino acids of the N-terminal tail of histone H3 and spots of the K9A peptide variant in which the target lysine had been replaced by alanine were prepared. After incubation with Dim-5, a clear methylation signal was detected at the H3 peptides, while no methylation occurred on H3 K9A peptides (Figure 1A). The methylation signal at H3 peptides was linearly increasing with incubation time and enzyme concentration

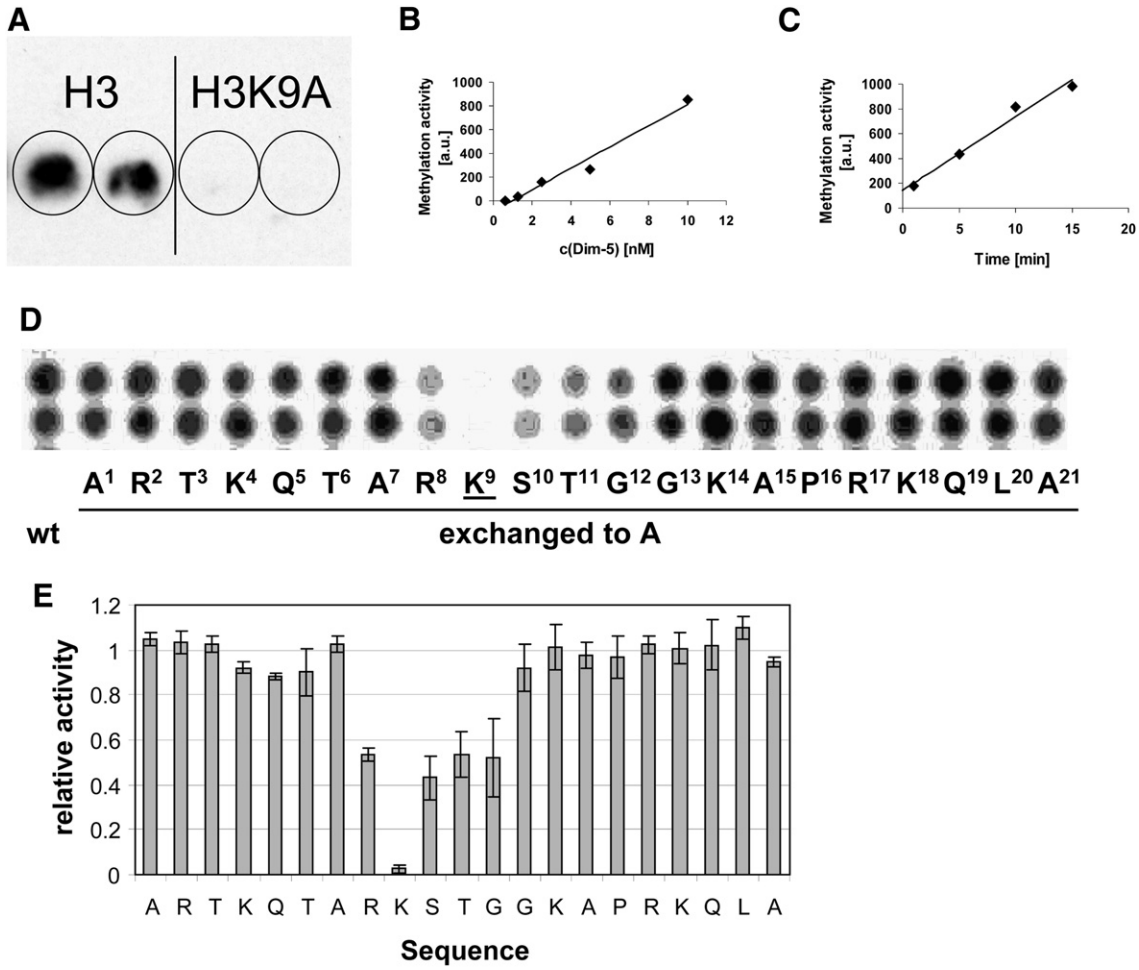


Figure 1. Application of the Peptide SPOT Synthesis to Study the Activity of Histone Methyltransferases

(A) Example of the methylation of peptides on solid support. After SPOT synthesis of the wild-type histone H3 N-terminal tail sequence (H3) and a K9A variant, the membrane was incubated with Dim-5 and radioactive AdoMet. Methylation was visualized by fluorography.

(B and C) The methylation rate of peptide spots increases linearly with enzyme concentration and time.

(D) Alanine scan of H3 tail methylation by Dim-5. In this assay, all 21 positions of the H3 tail were exchanged individually against alanine. The spot labeled with wt contains the wild-type H3 tail sequence.

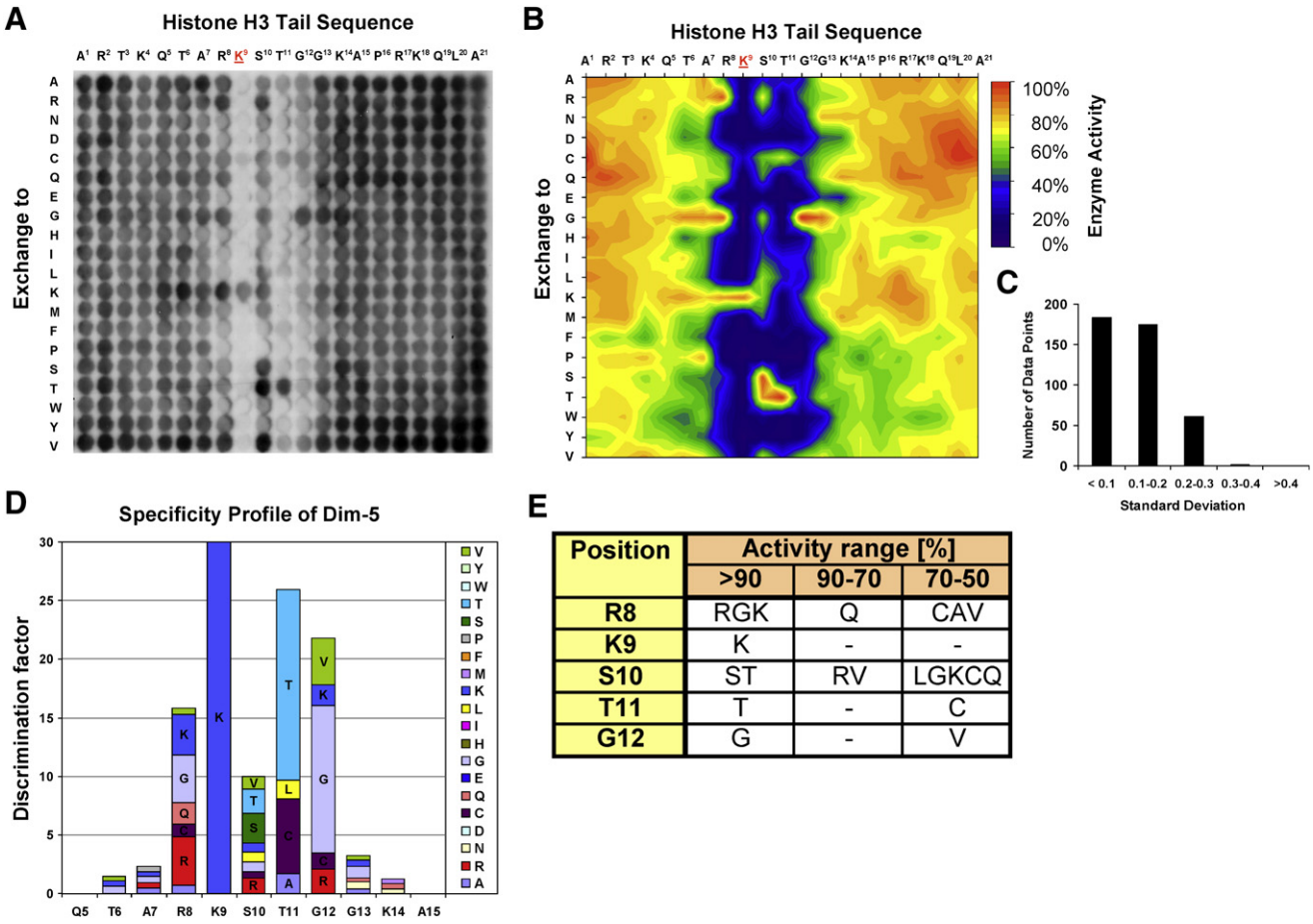
(E) Quantitative analysis of four independent alanine scans indicating the average activity and standard error for each target peptide.

(Figures 1B and 1C). To analyze if the activity of Dim-5 on immobilized substrates is comparable with data obtained in solution, we determined the K_m value for AdoMet by using H3 tail peptides as substrates either bound to the cellulose membrane or in solution. For both experiments, similar K_m values between 1.2 and 1.3 μM were obtained (data not shown).

Substrate Specificity of Dim-5

To study the influence of each residue on peptide recognition by Dim-5, an alanine scanning experiment was performed by synthesizing a small array of 21 peptides each carrying an exchange of a single residue against alanine (Figure 1D). The reduced methylation of peptides carrying substitutions at positions 8–12 demonstrated an important role of R8, K9 (the target of methylation), S10, T11, and G12 in the peptide recognition by Dim-5. The residual methylation activities were measured on four independent membranes, and standard deviations were determined (Figure 1E). The average standard error in all four experiments was ± 7%; standard errors of individual substrates were generally smaller than ± 15%, indicating that the assay is reliable and accurate. We conclude that the peptide methylation assay on solid support allows rapid methylation analysis of several target peptides. One inherent advantage of the method is that all peptide spots are methylated in competition, which ensures that equal amounts of active enzyme and cofactor are available for all substrates. Under the experimental conditions used here, the relative rates of methylation correspond to ratios of k_{cat}/K_d values for the respective peptide, the latter representing an established parameter for quantification of enzyme specificity (Fersht, 1998) (Supplemental Experimental Procedures, see the Supplemental Data available with this article online).

Next, we determined the influence of each possible amino acid exchange at each position of the peptide substrate on the activity of Dim-5. Therefore, H3 tail arrays comprising 420 individual peptides were used, in which each peptide contains an exchange of one amino acid of the wild-type H3 tail sequence



Position	Activity range [%]		
	>90	90-70	70-50
R8	RGK	Q	CAV
K9	K	-	-
S10	ST	RV	LGKCQ
T11	T	-	C
G12	G	-	V

Figure 2. Specificity of Peptide Methylation by Dim-5

(A) Example of one full H3 peptide tail array. The sequence of the H3 tail is given on the horizontal axis. Each residue was exchanged against all 20 natural amino acid residues (as indicated on the vertical axis) and the relative efficiency of methylation by Dim-5 analyzed.

(B) Compilation of the results of the peptide scan experiments with Dim-5. Data are averaged numbers from three independent experiments.

(C) Distribution of standard deviations for the data shown in (B) after normalizing full activity to 1.0.

(D) Bar diagram showing the discrimination factors of Dim-5 at the positions tested.

(E) Compilation of the specificity of Dim-5 for its target peptide.

against each of the 20 natural amino acids (Figure 2A). Three independent membrane arrays were synthesized and incubated with Dim-5 (Figure 2B). After normalization, the results showed excellent reproducibility as indicated by the distribution of standard deviations (Figure 2C). To quantify the contribution of each amino acid to the recognition of the substrate and display it graphically, a discrimination factor of Dim-5 for each position was calculated and displayed in Figure 2D. Strikingly, and in excellent agreement with the structure of Dim-5 in complex with an H3 residue 1–15 peptide, where residues 7–12 are ordered (Zhang et al., 2003) (Figure 3A), the data highlights the importance of substrate residues R8 to G12 for the activity of Dim-5. These results are consistent with a more limited data set previously obtained testing Dim-5 on modified recombinant histones (E. Selker, personal communication).

Among histone H3 residues R8–G12, recognition of T11 and G12 was most pronounced. The spectrum of residues accepted at position 11 (T, C, A, and L) suggests that hydrophobic contacts are important, which is in agreement with the Dim-5 peptide structure because the residues within 4 Å of the side chain of T11 are F206, I250, and L265. The Dim-5 atoms in contact with G12 are approximately 5 Å away. The preference for G12 suggests that either glycine-specific backbone angles are preferred at this position as the H3 peptide meanders off the face of Dim-5 after G12 and/or the presence of a Cβ atom would sterically interfere with complex formation. R8 could only be substituted by K and G. The preference for K and R suggests recognition of positively charged residues at this position, which is in agreement with a contact between R8 and E227 seen in the Dim-5 peptide structure (Figure 3A). Surprisingly, G is favored as well, probably because it gives conformational flexibility to the peptide chain. The specificity for recognition of S10 is lower as compared to R8, T11, and G12, and peptides containing various amino acids (including T, V, and R) at this position were methylated. However, serine and threonine are the preferred residues, which can be explained by the hydrogen bond between S10 and D209 observed in the Dim-5 peptide structure (Figure 3A).

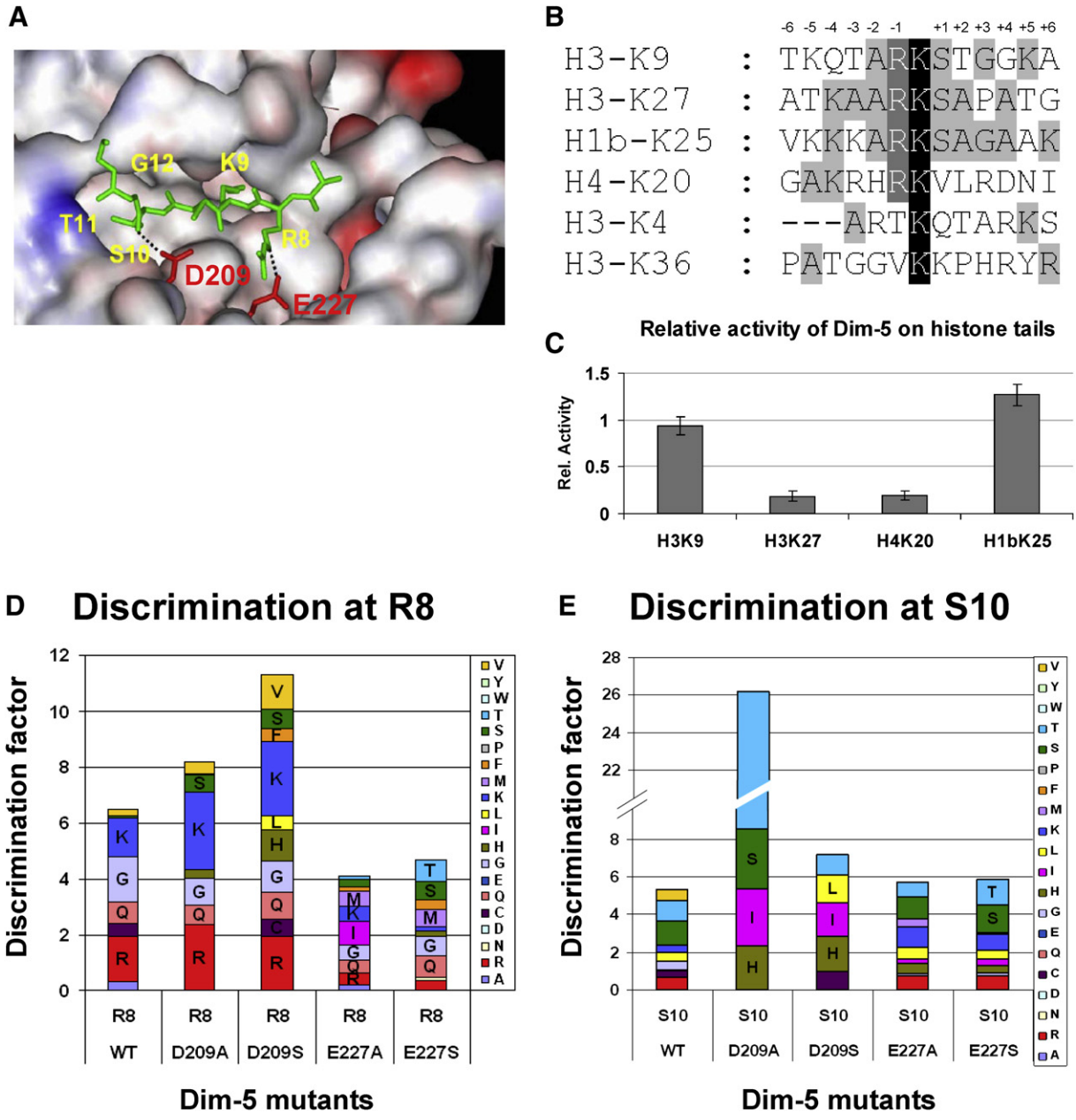


Figure 3. Analysis of Dim-5 Peptide Contacts

(A) Structure of the Dim-5 binding cleft (Zhang et al., 2002). H3 peptide residues from A7-G12 are indicated in green; Dim-5 residues contacting the peptide (D209 and E227) are shown in red. For clarity of visualization, the side chains of D209 and E227 were not included in the calculation of the surface of Dim-5.

(B) Alignment of some lysine residues in the different histone tails.

(C) Relative rates of modification of H3K9, H3K27, H4K20, and H1bK25 peptides by Dim-5. Error bars denote the standard deviation of the mean.

(D) Substrate discrimination of Dim-5 wild-type and mutants on H3 tail substrates with all 20 natural amino acid substitutions at position R8.

(E) Substrate discrimination of Dim-5 wild-type and mutants on H3 tail substrates with all 20 natural amino acid substitutions at position S10.

The peptide arrays included exchanges of histone H3 residues S10 and T11 by E. Substitution of S10 and T11 by E strongly reduced enzymatic activity of Dim-5 (86% inhibition for S10E, 91% for T11E). Because S10 and T11 are known phosphorylation sites in eukaryotes (Margueron et al., 2005) and E resembles a phosphoserine in steric and electrostatic terms, this results suggest that the activity of Dim-5 can be regulated by phosphorylation of S10 (or T11), the same way as phosphorylation of H3S10 prevents H3K9 methylation by Suv39H1 and Clr4 (Nakayama et al., 2001; Rea et al., 2000). In contrast, a T6E exchange did not affect Dim-5 activity, in agreement with structural result and specificity profile.

A search of the entire proteome of *Neurospora crassa* using Scansite (Obenauer et al., 2003) revealed that histone H3 is the only *N. crassa* protein that contains a (R/G/K)-K-(S/T)-T-G sequence, which represents the ideal Dim-5 target sequence

(Figure 2E). In particular, the recognition of T11 and G12 is important to distinguish H3K9 from H3K27 and H4K20 (Figures 3B and 3C). In agreement with the profile, Dim-5 can methylate the tail of human histone H1b in vitro (Figure 3C), but this protein is not present in *N. crassa*.

Influence of Dim-5 Residues on H3 Peptide Recognition

To investigate the role of the H3R8-E227 and H3S10-D209 contacts for peptide recognition by Dim-5, mutant enzymes were created with substitutions of D209 or E227 to A and S, respectively. In solution experiments with the wild-type H3 sequence, the D209A and S mutants showed a 100-fold and the E227A and S mutants a 10-fold reduced activity as compared to wild-type Dim-5 (data not shown). All four mutants were analyzed for methylation of peptides carrying all possible substitutions at position 8 and 10 of the N-terminal histone tail (Figures 3D and 3E). The E227A and S variants had lost the preference for R or K at position 8 of the H3 tail confirming charge complementarity as specificity determining factor at this position. The broad spectrum of amino acids accepted at this position by E227A and S could be explained because besides E227, no other residue in wild-type Dim-5 closely contacts the side chain at this position. As expected, the D209A and S influenced the specificity profile at position 8 only to a minor degree.

The D209A and S variants showed clear changes of their interaction with substrate position 10 because they both displayed a preference for hydrophobic amino acids at this position. While the wild-type Dim-5 equally accepts S and T, the D290A variant showed a pronounced preference for T over S (5.6-fold), followed by I, and about 18-fold above the average of all amino acids other than T at position 10. The D209S variant also preferred hydrophobic residues at position 10 (L, I, and T). These results can be interpreted in the light of the Dim-5 peptide structure because after mutational exchange of D209 to A or S, an extra space is generated close to the substrate position 10. In addition, the side chain of S209 (in D209S) as well as the main chain carbonyl of A207 may serve as hydrogen bond partners for the side chain of substrate position 10. These potential contacts might explain the specificity profile of D209A and S at substrate position 10, because S, T, and H may form hydrogen bonds, and T, I, L, and H may occupy the extra space generated due to the loss of a larger protein side chain. As expected, the E227A and S exchange did not influence the recognition at position 10 substantially as indicated by the finding that both variants still preferred S and T, and all in all, the relative preferences for most amino acid residues were similar to wild-type Dim-5.

Interestingly, the D209A variant showed a new specificity for T at position 10, which was preferred about 18-fold over all other residues at this position. This level of specificity is even higher than the preference of wild-type Dim-5 for T11 and G12 (Figure 2D), illustrating that peptide recognition by Dim-5 is malleable by protein design, and one can create synthetic enzyme-substrate pairs by using existing HKMTs as a starting point.

SIGNIFICANCE

Methylation of histone tails is an important epigenetic signal (Martin and Zhang, 2005). The first HKMT was identified in 2000 (Rea et al., 2000), and today about 30 different enzymes are known in different species (Kouzarides, 2007). However, many of the enzymatic properties of these enzymes are still uncharacterized. We established the peptide SPOT array technology as a method to investigate the specificity of protein methyltransferases. It allows at moderate costs to perform a rapid analysis of methylation of a large number of different peptides (>1000 per array) to determine the specificity signature for each particular enzyme. The substrate preferences determined by this way are of importance because they allow us to predict new potential methylation targets (as we did for G9a) (Rathert et al., unpublished data), since the intrinsic preferences of the HKMT most likely reflect some functional constraints and adaptation of the enzyme to its substrates. In addition, the specificity profiles provide a detailed knowledge about the substrate's chemical properties recognized by the enzyme and, thereby, may help to design compounds for the inhibition of these enzymes. Furthermore, the histone tails contain residues that are subject to one or sometimes several different modifications at different positions. Crosstalk occurs between different histone modification marks, such that methylation of H3K9 precludes H3K4 methylation or phosphorylation of H3S10 (Margueron et al., 2005) and methylation of H3R2 prevents H3K4 methylation (Guccione et al., 2007; Kirmizis et al., 2007). Such epigenetic crosstalk can occur at the level of the modification reading domains or the modifying enzymes. For example, H3R8, which is an important specificity determinant of Dim-5, can be methylated by Prmt5 (Pal et al., 2004) in vivo. The peptide spot array method is well suited to investigate the sensitivity of HKMTs toward mutations on the histone tails—that can be easily expanded to include pre-existing posttranslational modifications. This allows to decode the effect of the complex posttranslational modification pattern of histone tails on HKMT activity and is, therefore, of great value for an understanding of the cellular role of these enzymes.

EXPERIMENTAL PROCEDURES

Enzyme Purification

Dim-5 wild-type and variants were expressed and purified as described previously (Zhang et al., 2002). The *E. coli* strain XL1 blue was used as cloning host for Dim-5 variants. The plasmid pXC379 (Zhang et al., 2002) encoding Dim-5 was mutated at different positions to yield Dim-5 D209A/S and E227A/S variants by site-directed mutagenesis methods (Jeltsch and Lanio, 2002). All mutants were sequenced to verify the presence of the intended mutation and the absence of additional mutations.

Peptide Methylation in Solution

A synthetic peptide corresponding to the first 19 amino acids of histone H3 tail plus a methionine (Bt-MARTKQTARKSTGGKAPRKQ), which contains a biotin at its N terminus, was purchased from IRIS Biotech (Marktredwitz, Germany) in HPLC-purified form and was dissolved in water. Purity of the peptide was greater than 95%, as confirmed by HPLC and MALDI-TOF mass-spectrometric analysis. Methylation reactions on micro plates were carried out as described (Gowher et al., 2005).

Synthesis of Peptide Spot Arrays

Peptide arrays were synthesized as described by the SPOT synthesis method (Frank, 2002; Wenschuh et al., 2000). Successful synthesis of each peptide was confirmed by bromphenol blue staining of the membranes (Figure S1).

The peptide spots used in our assay had diameters of 2 mm and contained approximately 9 nmol of peptide (Autospot Reference Handbook, Intavis AG).

Methylation of Peptide Spot Arrays

For methylation, the membranes containing 420 peptide spots were washed for 20 min in methylation buffer containing 50 mM glycine (pH 9.8), 2 mM dithiothreitol (DTT), 25 μg/ml BSA, 10% glycerol, and subsequently incubated with 20 nM enzyme in methylation buffer at ambient temperature in the presence of 0.35 μM labeled [methyl-^{3}H]-AdoMet (2.93×10^{15} Bq/mol) (NEN Life Sciences). After 45 min, the membranes were washed four times with 50 mM NH_4HCO_3, dried between whatman papers (Whatman GmbH, Dassel, Germany), and washed once with Amplify NAMP100V solution (GE Healthcare, Munich, Germany). The membranes were incubated on Hyperfilm high performance autoradiography films (GE Healthcare, Munich, Germany) in the dark. After 3–7 days, the films were developed by using AGFA Curix 60 developing machine (Agfa Deutschland Vertriebsgesellschaft mbH & Co. KG, Cologne, Germany).

Data Analysis

All experiments were carried out at least in triplicate. Data are reported as mean values and standard errors. To compare the accuracy of recognition of each residue in the substrate quantitatively, the relative contribution of each amino acid i at position x for peptide recognition was calculated by a discrimination factor D:

$$D = \frac{\overline{v_{j\neq i}}}{v_i} - 1$$

where v_i is the rate of modification of peptide carrying amino acid i and $\overline{v_{j\neq i}}$ is the average rate of methylation of all 19 peptides carrying a different amino acid $j \neq i$ at position x (including the wild-type sequence). For example in Figure 2D, the discrimination factor of 10 for a Thr at position 11 indicates that the peptide with Thr at that position is methylated ten times faster than the average of all peptides carrying any of the other amino acids at this site. Since the detection limit of the experiments was at about 3% of the full activity, the discrimination factor for K9, which could not be replaced by any other residue, was 30.

Supplemental Data

Supplemental Data include one figure that shows an example of Bromophenol blue stained peptide membrane and Supplemental Experimental Procedures that give a detailed description of data analysis and are available with this article online at http://www.chembiol.com/cgi/content/full/15/1/5/DC1/.

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