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Rapid Report

Preparation of Recombinant Peptides with Site- and Degree-Specific Lysine ^{13}C-Methylation†

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ABSTRACT: Lysine methylation is an important post-translational modification that affects protein function; for example, the transcriptional activity of the p53 tumor suppressor protein. To facilitate structural characterization of complexes involving proteins and methylated targets by nuclear magnetic resonance spectroscopy, we devised a simple method for preparing recombinant ^{15}N/^{13}C-enriched peptides with a ^{13}C-methyl-labeled methylated lysine analogue. The method, which relies on the synthesis of ^{13}C-enriched alkylating agents, was applied to the production of 15-residue p53 peptides variously methylated at lysine analogue 370. The peptides were used to probe the methylation state-dependent interactions of mono, di, and trimethylated p53 with three different proteins.

Many proteins undergo post-translational modifications (PTMs) altering their physical and chemical properties, and consequently, their functions. There are several types of PTMs; one is lysine methylation, which is further classified as mono, di, or trimethylation. Our limited understanding of lysine methylation comes mainly from studies of histones and to a lesser extent of nonhistone proteins such as p53 (*1*). Although partial identification of methylases and demethylases have been made, it remains poorly understood how methylated sites are recognized by other proteins. This is due in part to the small number of three-dimensional (3D) structures available to elucidate these interactions at the molecular level. It has also been a challenge to generate high quality site- and state-specific methylations in these proteins or representative peptides useful for nuclear magnetic resonance (NMR) spectroscopy-based structural studies. Solid-state synthesis is a popular choice for producing peptides but is very costly when isotopic enrichment, which greatly facilitates structure−function studies by NMR methods, is involved. Furthermore, although methylation can be achieved via reductive alkylation of lysines (*2*) or by methyltransferases, the former lacks selectivity and gives dimethylated products only, and *in vitro* use of the latter on a large scale remains very challenging.

Here, we present a simple and efficient procedure for preparing pure lysine site-specific mono, di, and trimethylated peptides with selective ^{13}C-enrichment. We explored the techniques of recombinant peptide expression in bacteria and chemical installation of methyllysine analogues via reductive cysteine alkylation (*3*−*6*), and developed new synthetic pathways for the preparation of ^{13}C-enriched chemicals needed for the alkylation of cysteines. The new procedure was applied to the production of mono, di, and trimethylated p53 peptides (p53p, residues 363−377) with ^{13}C-enrichment of the methyl group(s) at the lysine of interest as well as non and ^{15}N/^{13}C-fully labeled p53p with or without lysine methylation. The peptides were used to probe p53 interactions with a known partner, 53BP1 (p53 binding protein 1), and possible targets: C20orf104 (Chromatin 20 orf104) and JMJD2A (Jumonji domain containing protein 2A) (*7*−*13*). C20orf104 has a tudor domain (C20orf104-tudor, residues 84−147) in tandem with an malignant brain tumor (MBT) domain, which are putative methyllysine binding modules. JMJD2A has hybrid tudor domains (JMJD2A-tudor, residues 897−1011). Previous *in vitro* studies have shown that the tudor domains of C20orf104 and JMJD2A bind histone H4 di and trimethylated at K20, respectively (*10*). The tudor domains of 53BP1 (53BP1-tudor, residues 1484−603) bind mono and dimethylated H4K20 (*10*−*12*). Since 53BP1 can also bind methylated p53 through its tudor domains, we reasoned that C20orf104-tudor and JMJD2A-tudor may likewise share this same target.

The p53 tumor suppressor protein is mutated in ∼50% of all human cancers (*14*). It has a key function as a transcriptional activator and is regulated by methylation at lysines K370, K372, and K382, which are monomethylated by Smyd2, Set9, and Set8 methyltransferases, respectively (*15*−*17*). The enzyme that dimethylates K370 has not been discovered, but the demethylase LSD1 (lysine-specific demethylase 1) of K370me2 (me = methyl; 2 = di) is known (*16*). To our knowledge, other forms of methylated p53 and coupled enzymes have not been observed *in vivo*. Growing data reveal that depending on the location and degree of methylation, p53 is either activated or inhibited for its transcriptional activities. Conceivably, lysine methylation could regulate the functions of p53 by affecting p53 stability and/or interactions with DNA and/or with other proteins. For example, K372me1 enhances

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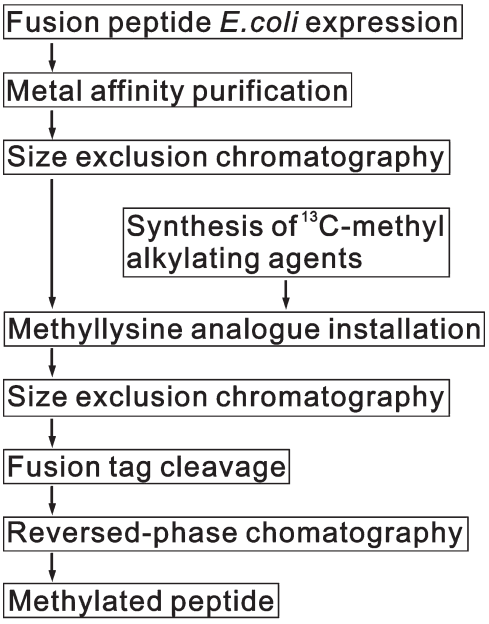

FIGURE 1: Production of recombinant p53 peptides and installation of MLAs. pGBm/p53p and pGBm/p53pK370C peptides were overexpressed in *E. coli* as a histidine tag-GBm fusion protein, subjected to reductive alkylation to incorporate methyllysine analogues, cleaved from the fusion, and purified by various chromatographic methods.

the stability of p53 by blocking K370 ubiquitination (*15*); K370me1 and K382me1 reduce p53 binding to DNA and inhibit transcription (*8*); and K370me2, by mediating p53 binding to the coactivator 53BP1, enhances transcription (*7*).

To produce nonmethylated and methylated peptides for structural studies, we first overexpressed in *E. coli* recombinant p53p, wild type and K370C mutant, fused to the C-terminus of a carrier protein GBm (B1 domain of streptococcal protein G with a single methionine before the peptide sequence), designated as pGBm/p53p and pGBm/p53pK370C, respectively. Depending on whether LB or isotope-enriched M9 media were used during cell culture, ensuing proteins were either nonuniformly or uniformly $^{15}N/^{13}C$-labeled. With a hexahistidine tag present at the N-terminus of GBm, the proteins were purified by metal chelation chromatography initially and then size exclusion chromatography (Figure S1, Supporting Information). Figure 1 is a flowchart of the steps leading to the production of methylated peptides. Experimental details are provided in Supporting Information.

Incorporation of methyllysine analogues (MLAs) at position 370 of the pGBm/p53pK370C mutant was accomplished following reductive alkylations as shown in Figure S2 (Supporting Information) (*5*). Mono, di, or triMLAs (designated as $K_C$me1, $K_C$me2, and $K_C$me3) are functionally similar to their methylated lysine counterparts (Kme1, Kme2, and Kme3) and can be obtained depending on whether a mono, di, or trimethylated ethylamine is used in the alkylation reaction. These alkylating agents are commercially available but only in nonisotopic forms. To increase their utility for structural studies by NMR spectroscopy, we developed the procedures for synthesizing ^{13}C-enriched methylated ethylamine reagents, as shown in Figure 2, which were then used for reactions

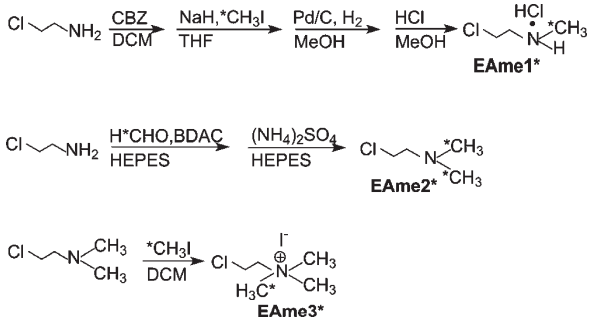

FIGURE 2: Syntheses of ^{13}C-enriched mono, di, and trimethylated 2-chloroethylamine. These reductive alkylations use ^{13}C-iodomethane and ^{13}C-formaldehyde as sources of ^{13}C-methyl. * indicates ^{13}C, and me stands for methyl. EAme1* and EAme2* have all their methyllysine analogue methyls ^{13}C-labeled. EAme3* has 3 methyls, but only 1 is ^{13}C-labeled.

similar to those in Figure S2 (Supporting Information). In such syntheses, we took advantage of commercially available and inexpensive ^{13}C-labeled iodomethane and formaldehyde as sources of methyl groups.

The reaction of 2-chloroethylamine (EA) with ^{13}C-formaldehyde, followed by reduction with borane dimethylamine complex (BDAC) proceeded nearly 100% giving dimethylated 2-chloroethylamine (EAme2*, where * designates ^{13}C-methyl) as the sole product. Alkylation of EA with ^{13}C-iodomethane could yield mono, di, and trimethylated products but was controlled to stop at monomethylation by protecting the reactive amino group of EA with benzyl chloroformate (CBZ). Palladium catalyzed hydrogenation removed the CBZ group, exclusively yielding a monomethylated 2-chloroethylammonium chloride (EAme1*), with a 30−40% yield. A one-step methylation of nonlabeled dimethylated 2-chloroethylamine using ^{13}C-labeled iodomethane produced trimethylated 2-chloroethylammonium iodide (EAme3*) with 1 ^{13}C-labeled methyl. The yield was excellent at 80−90%. EAme3* gave a weaker NMR methyl signal but otherwise behaved similarly as an EAme3 with 3 ^{13}C-labeled methyls.

The crude MLAs of pGBm/p53pK370C were next passed through a size exclusion column and then cleaved from the histidine tag-GBm fragment by either cyanogen bromide or TEV protease. Unmodified wild type pGBm/p53p and mutant pGBm/K370C were cleaved similarly. Final purification was attained by reversed-phase HPLC (Figure S3, Supporting Information). High qualities and correct sequences of the nonmethylated and variously methylated p53p (p53pK370C, p53pK_C370me1*, p53pK_C370me2*, p53pK_C370me3*) were confirmed by mass spectrometry (Figure S4, Supporting Information) and NMR spectroscopy (Figures S5 and S6, Supporting Information). Typical yields for the purified methylated peptides were ~3 mg/L cell culture, about as much as that for the nonmethylated peptides.

Using purified protein samples of nonlabeled 53BP1-tudor, C20orf104-tudor, and JMJD2A-tudor produced by overexpression from *E. coli*, we performed NMR titration experiments on p53pK_C370me1*, p53pK_C370me2*, and p53pK_C70me3*. Details of the titration experiments and sample preparations are contained in the Supporting Information. Representative $^1H-^{13}C$ heteronuclear single

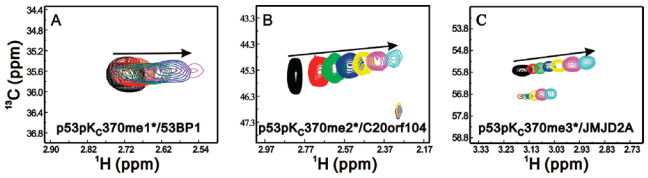

FIGURE 3: ^{1}H−^{13}C HSQC titration spectra of ^{13}C-methylated p53p peptides with nonlabeled 53BP1-tudor, C20orf104-tudor, and JMJD2A-tudor acquired at pH 7.5. Black peaks represent free p53pK$_C$370me1* (A), p53pK$_C$370me2* (B), and p53K$_C$370me3* (C), and cyan peaks, after the addition of excess 53BP1-tudor, C20orf104-tudor, and JMJD2A-tudor, respectively. Intermediate stages of the titrations are shown in other colors.

quantum coherence (HSQC) spectra of these methylated peptides, free and with stepwise addition of the partner proteins, were overlaid in Figure 3. The shifts in the NMR signals are indicative of specific protein−peptide interactions as changing the methylation states and sites of the peptides led to weaker or no interactions. For example, 53BP1-tudor interacts strongest with p53pK$_C$370me2* and more weakly with p53pK$_C$370me1*; it does not bind p53pK$_C$370me3*. C20orf104-tudor interacts with p53pK$_C$370me2* but not with p53pK$_C$370me3*. JMJD2A-tudor has the highest affinity for p53pK$_C$370me3* among all methylation states. None of the 3 proteins binds nonmethylated p53p. When reverse titrations using ^{15}N-labeled proteins and nonlabeled methylated peptides were performed, identical chemical shift perturbations were observed whether the peptides were prepared by solid-state synthesis or by the methods reported here (Figure S7, Supporting Information). The interactions among methylated p53p and the 3 proteins could be characterized further by solving the 3D structures of their complexes. Having p53p samples fully ^{15}N/^{13}C-labeled as well as ^{13}C-methyl-labeled at the methyllysine analogue can provide valuable intra- and intermolecular nuclear Overhauser effects (NOEs) that facilitate structure determination by NMR spectroscopy. The ^{13}C-labeled methyl group(s) will also be useful to probe the dynamics of interaction using NMR spectroscopy. Figure S8 shows the full ^{1}H−^{13}C HSQC spectrum of a ^{15}N/^{13}C-labeled p53pK$_C$370me2* peptide.

In conclusion, we have developed an efficient method for the preparation of high quality methylated peptides of various methylation sites and states, with selective ^{13}C-enrichment. The method can be applied to study the interactions and functions of proteins linked to lysine methylation.

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures and Figures S1 to S8. This material is available free of charge via the Internet at http://pubs.acs.org.

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