Lysine Trimethylation of Retinoic Acid Receptor-α

A NOVEL MEANS TO REGULATE RECEPTOR FUNCTION*

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Retinoic acid receptors (RARs) belong to the nuclear receptor superfamily. The mechanism of ligand-dependent activation of RARs is well known. The effect of protein phosphorylation on the activity of RARs has also been demonstrated. However, it is unclear whether other types of modifications exist and if so whether they can affect the activity of RARs. In a mass spectrometric analysis of mouse RARα expressed in insect cells, we identified a trimethylation site on Lys347 in the ligand binding domain. The modification site was verified in mammalian cells, and site-directed mutagenesis studies revealed the functionality of Lys347 methylation in vivo. Constitutive negative mutants, mimicking hypomethylated RARα, were prepared by replacing methylated Lys347 with either alanine or glutamine. A constitutive positive mutant partially mimicking the hypermethylated RARα was generated by replacing the methylated lysine residue with phenylalanine, a bulky hydrophobic amino acid, to introduce a site-specific hydrophobicity similar to that contributed by lysine methylation. Studies of these mutants revealed that trimethylation of Lys347 of RARα facilitated its interactions with cofactors p300/CREB-binding protein-associated factor and receptor-interacting protein 140 as well as its heterodimeric partner retinoid X receptor, suggesting that site-specific hydrophobicity at Lys347 enhanced molecular interaction of RARα with its modulators. This study uncovers the first example of lysine trimethylation on a mammalian non-histone protein that has an important biological consequence. Our finding also provides the evidence for lysine methylation for the family of nuclear receptors for the first time. Molecular & Cellular Proteomics 6:677–688, 2007.

Nuclear receptors comprise a superfamily of transcription factors. The interaction of nuclear receptors with co-activators such as steroid receptor co-activators (SRC-1, -2, and -3), p300/CREB1-binding protein (CBP), and p300/CBP-associated factor (PCAF) or co-repressors including histone deacetylases, nuclear receptor co-repressor (NCoR), receptor-interacting protein 140 (RIP140), and silencing mediator of retinoid and thyroid hormone receptor (SMRT) mediates the recruitment of enzymes that modify chromatin templates or facilitates receptor interaction with basal transcription machineries (1, 2). This event ultimately leads to the action of nuclear receptors to activate genes in the presence of ligands or to repress target genes in the absence of ligands.

Retinoic acid receptors (RARs) are activated by retinoic acid (RA) and regulate many genes involved in growth, development, differentiation, and apoptosis (3). RA signaling is complicated by the participation of two families of RA receptors, the RARs and RXRs (4, 5). Each family has three isotypes (α, β, and γ) that are encoded by different genes. Further complexity is generated by alternative promoter usage and splicing (5). The RAR family can be activated by both all-trans-RA and 9-cis-RA, whereas the RXR family is activated by 9-cis-RA (4). RAR and RXR form heterodimers that bind to specific DNA sequences known as RA response elements (RAREs) and regulate transcription in a ligand-dependent manner. Activation of RARs by RA has been studied extensively (5, 6), and the effects of protein phosphorylation on receptor activity has also been reported (7, 8). To determine whether RAR can be modified by other types of modifications, we conducted mass spectrometric analysis of RARα expressed in insect cells. A trimethylated lysine residue at Lys347 was identified in its ligand binding domain (LBD). The modification site was also confirmed in mammalian cells. The biological relevance of this modification was then addressed by site-directed mutagenesis studies, which revealed its functional role in modulating receptor interaction with various coregulators and, consequently, its biological activity in RA activation of target genes. This study uncovers the first example of lysine trimethylation of a mammalian non-histone protein that has an important biological implication.
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**EXPERIMENTAL PROCEDURES**

**Plasmids**—Full-length RAR, His-tagged RARLBD, Gal4V16-RIP140, Gal4VP16-NCoR, Gal4VP6-RXRLBD, and Gal4-RARLBD plasmids were constructed as described previously (9, 10). Gal4VP16-PCAF was subcloned from CMV-PCAF by ligating the PCR-amplified cDNA into Gal4VP16 vector at XbaI and EcoRI sites.

**Expression and Purification of Mouse RARα**—For expression of murine RARαs in the baculovirus system, the cDNA of full-length mouse RARα tagged with a FLAG epitope was inserted into pVL1393 (Invitrogen) at the EcoRI and XbaI sites. Sf21 insect cells (1×10⁶) were infected with recombinant baculovirus vector. After 72 h, the cells from a 500-ml culture were harvested by centrifugation at 6000 rpm for 10 min at 4 °C, and the pellet was kept frozen at −80 °C. The cell extract was prepared, and the protein was subjected to affinity purification over anti-FLAG M2-agarose following the protocol for FLAG M purification kit (Sigma) provided by the manufacturer. The purified protein was resolved by 8% SDS-PAGE.

**Mass Spectrometric Analysis of RARα**—The mass spectrometric analysis of RARα protein was conducted as described previously (11, 12). FLAG-tagged RARα purified from insect cells was resolved by SDS-PAGE. The gel slice was subjected to overnight in-gel tryptic digestion. The tryptic digest was analyzed by LC-ESI-MS/MS as described previously (11, 12). The LC system was on line with Applied Biosystems, Inc. (Foster City, CA) QSTAR Pulsar quadrupole TOF mass spectrometer, which was equipped with a Protean nanoelectrospray source. The information-dependent acquisition (IDA) was used to acquire MS/MS, IDA mode was set to measure continuous cycles of full scan TOF MS from 400 to 1200 m/z plus three product ion scans from 50 to 4000 m/z. The data from IDA experiments were searched using the MASCOT (Matrix Science) MS/MS data search. The mass tolerance of both precursor ions and the MS/MS fragment ions was set at ±0.1 Da, carboxymethylmethionine was specified as a static modification, and methylated lysine and oxidized methionine were specified as variable modifications. All MS/MS spectra were analyzed manually to verify sequence with the help of Bioanalyst software (Applied Biosystems, Inc.).

**Site-directed Mutagenesis**—Site-directed mutagenesis on the methylated lysine residue of full-length RAR, His-RARLBD, and Gal4-RARLBD was performed using the QuickChange XL site-directed mutagenesis kit (Stratagene) following the manufacturer’s protocol. Replacement of lysine residues with alanine, glutamine, and phenylalanine were made by using mutagenic primers. The Lys→Ala/Gln and Lys→Phe point mutations represented the constitutive negative (CN) and the constitutive positive (CP) mutants, respectively. The mutagenic primers used to generate the mutant (bold characters) constructs are: K347A: sense, 5′-CTG GAG CAG CCA GAC GGG GTG GAC ATG CTG CAA-3′; antisense, 5′-TTG CAG CAT GTC CAC CGC GTC TGG CTG CTC CAG-3′; K347Q: sense, 5′-CTG GAG CAG CCA GAC GGG GTG GAC ATG CTG CAA-3′; antisense, 5′-TTG CAG CAT GTC CAC CGC GTC TGG CTG CTC CAG-3′; K347F: sense, 5′-CTG GAG CAG CCA GAC TTC GTC GAC ATG CTG CAA-3′; antisense, 5′-TTG CAG CAT GTC CAC GGA GTC TGG CTG CTC CAG-3′. The positive clones were verified by DNA sequencing.

**Cell Culture, Transfection, and Reporter Assay**—COS-1 cells were maintained in Dulbecco’s modiﬁed Eagle’s medium supplemented with 10% fetal bovine serum. Transient transfection in cells was performed using Lipofectamine™ 2000 (Invitrogen). In the reporter assay, 0.1 μg of Gal4BD-RARs and control plasmid (Gal4BD empty vector), Gal4-tk-luciferase (0.5 μg) reporter, and CMV-lacZ as an internal control (0.05 μg) were used in each well of 24-well plates. At 24 h post-transfection, the medium was replaced with a fresh medium containing dextran-charcoal-treated fetal bovine serum and treated with either all-trans-RA (aRA; 2 μM) in the presence or absence of 20 μM methylamine inhibitor (MITI) adenosine-2,3-dialdehyde (Sigma) for 12 h. To monitor the methylation effect on RAR full length activity, a luciferase reporter assay containing RARE (DR5 element) derived from RARβ2 gene was conducted. To test the methylation effect on protein-protein interaction of RAR with its coregulators (RIP140 and PCAF) and its heterodimeric partner (RXR), a mammalian two-hybrid test was conducted in COS-1 cells on a Gal4 reporter using Gal4BD-fused RARLBD and Gal4VP16 constructs of PACTF, RIP140, RXR, and NCoR. 36 h post-transfection total cell extracts were prepared by freeze-thaw cycles and tested for luciferase and lacZ activities. The -fold relative luciferase activity was calculated by normalizing relative luciferase unit activity of the experimental groups to the relative luciferase unit activity of the empty vector control group.

**Metabolic Labeling, RT-PCR, Immunoprecipitation (IP), and Western Blot**—Metabolic labeling of ectopically expressed RAR was conducted in COS-1 cells using radiolabeled S-adenosylmethionine (l[l]H]AdoMet) (25 μCi/ml). Briefly the confluent cells were washed with methionine-free Dulbecco’s modiﬁed Eagle’s medium (Invitrogen) twice, and [l]H]AdoMet (Sigma) was added directly to the medium and incubated for 6 h in the presence or absence of MTI (20 μM). The cells were washed twice with PBS and harvested. The cell lysates were prepared in co-IP buffer by freeze-thaw cycles for the immunoprecipitation experiment. To detect the lysine methylation status of RARα in mammalian cells, IP was conducted in RARα (Gal4-RARα-LBD and RARα full length)-overexpressing COS-1 cells using either anti-Gal4 antibody (Santa Cruz Biotechnology) or anti-RARα antibody (Affinity Bioreagents) followed by detection with anti-methylated lysine antibody (Stressgen Bioreagents) in the Western blot. For RT-PCR analyses, total RNA was isolated from COS-1 cells using a TRizol® kit (Invitrogen), and the RT reaction was conducted using Superscript™ (Invitrogen) reverse transcriptase enzyme following the manufacturer’s protocol. The expression pattern of chimeric Gal4-RAR wild type and its mutants were monitored by RT-PCR using the specific primers for the chimeric cDNA. The sense primer was designed at the Gal4BD domain, and the antisense primer was designed at the LBD, producing a 500-bp PCR product. Western blot to detect His-RAR and RXR was conducted using anti-His (Upstate) and anti-RXRα (Affinity Bioreagents) antibodies.

**His Pulldown Assay**—His fusion proteins were partially purified from bacteria by affinity chromatography using nickel-nitrotriacetic acid-agarose (Qiagen) metal affinity beads. Preliminary binding studies were done for the wild type and mutant His-RARs to determine the amount of bound sample that would yield approximately an equal amount of protein on a Coomassie-stained SDS-PAGE gel. After binding, the beads were washed twice with 20 volumes of PBS and once with a binding buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 100 mM glycerol). 35S-Labeled PCAF or RIP140 (2 μl) prepared with the TNT kit (Promega) was then added to the samples in 300 μl of binding buffer. The samples were incubated at 4 °C for 90 min followed by three washes with a 20-bead volume of binding buffer. The beads were collected by centrifugation and suspended in the binding buffer (20 μl) and 4× SDS sample buffer (20 μl). Samples were divided into two parts, and an equal amount was resolved on two separate SDS-PAGE (10%) gels. One gel was stained with Coomassie Blue, and the second gel was fixed, dried, and exposed to a Phosphorimager screen (GE Healthcare) overnight to detect the bound PCAF and RIP140 proteins.

**Ligand Binding Assay**—The in vitro ligand binding assay was performed using His-RARLBD along with its mutant proteins purified from bacteria. In a saturation binding assay, 10 nM His-RARLBD was incubated at room temperature for 40 min with variable concentrations of radiolabeled atRA ([l]H]atRA; 37 Ci/mmol) (American Radio-labeled Chemicals, Inc.) in 300 μl of binding buffer (50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, pH 8.0). In the competition binding assay, the equimolecular concentration (10
of receptor and [3H]RA was incubated for 40 min at room temperature in the presence of excess cold atRA. Then His-tagged receptor was affinity-captured on nickel-nitrilotriacetic acid-agarose affinity beads (Qiagen) for 2 h at 4 °C, and the unbound ligand was washed twice with the binding buffer. The ligand-bound receptor on beads was dispersed in scintillation mixtures, and the radioactivity was measured in a liquid scintillation counter (Beckman).

RESULTS

Expression and Purification of RARα in S21 Cells—Many studies including our own have validated the expression of recombinant proteins in insect cells as an efficient way to generate large quantities of low abundance proteins for the study of protein post-translational modification (PTM) (13–15). Although the stoichiometry could vary, the sites of modification identified in proteins expressed in insect cells usually occur in proteins expressed in mammalian cells (13–15). To identify the PTM sites on RARα, mouse FLAG-tagged RARα was expressed in S21 cells and purified over anti-FLAG M2-agarose affinity beads, allowing the enrichment of the purified protein as shown on an 8% SDS-PAGE gel (Fig. 1).

Mass Spectrometric Analysis of RAR—The protein preparation was subjected to in-gel trypsin digestion. The tryptic peptides were then analyzed by LC-ESI-MS/MS to identify the protein and the potential PTM sites. IDA was used to acquire MS/MS data. The data from IDA experiments were searched on line using MASCOT. The result revealed coverage of 38% of the protein sequence (Table I). Furthermore the MS/MS data predicted a trimethylated tryptic peptide (spanning aa 340–360) within the LBD (aa 230–388, bold characters) of the receptor. The low coverage of the protein sequence was attributed to the lack of tryptic cleavage sites (Lys/Arg) in the amino-terminal (aa 1–100, A/B or AF-1) domain of the receptor. However, the LBD sequences were mostly covered, including most of the lysine residues (as shown in blocks), except Lys347.

Identification of Trimethylation Site on Lys347—To identify the methylation site in the total ion chromatogram (TIC) of the tryptic digests of RARα, 14-, 28-, and 42-amu positive mass shifts due to covalent modification of lysine by mono-, di- and trimethylation, respectively, were considered. To distinguish trimethylation from acetylation, which appeared to display the same integral mass (42 Da), the marker ions for those modifications were analyzed as described previously (12, 16). The TIC of the LC-ESI-MS revealed a trimethylated tryptic peptide spanning aa residues 340–360 located in the LBD domain of RAR (17, 18). The MS/MS data of the methylated peptide were analyzed manually to confirm the position of the modified site. The modified peptide displayed a quadruply charged ([M + 4H]4+ mono) precursor ion at m/z 628.093 (theoretical molecular mass = 2509.305 Da; calculated, 2508.372 Da) and a triply...
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**Top**, the modified peptide (aa 340–360): precursor m/z 628.1 (z = 4) and 837.4 (z = 3). **Bottom**, the unmodified peptide: precursor m/z 823.4 (z = 3). The y8 ion at m/z 912.54 and a6 ions at m/z 683.38 for both the modified and the unmodified peptides were identical. The a8* ion at m/z 951.54 showed a 42-unit positive mass shift, suggesting the modification of Lys347 by acetylation or trimethylation. The marker ion at m/z 126 specific for acetylated lysine was absent, whereas a marker ion for di-/trimethylated lysine at m/z 84 and a doubly charged y14* (y14 – NH₃) ion at m/z 803.9 due to loss of trimethylamine [y14* – 59]⁺ appeared only in the spectrum of the modified peptide, suggesting the modification by trimethylation rather than acetylation.

Fig. 2. Identification of Lys347 trimethylation on mouse RARα by LC-ESI-MS/MS analysis. Top, the modified peptide (aa 340–360): precursor m/z 628.1 (z = 4) and 837.4 (z = 3). **Bottom**, the unmodified peptide: precursor m/z 823.4 (z = 3). The y8 ion at m/z 912.54 and a6 ions at m/z 683.38 for both the modified and the unmodified peptides were identical. The a8* ion at m/z 951.54 showed a 42-unit positive mass shift, suggesting the modification of Lys347 by acetylation or trimethylation. The marker ion at m/z 126 specific for acetylated lysine was absent, whereas a marker ion for di-/trimethylated lysine at m/z 84 and a doubly charged y14* (y14 – NH₃) ion at m/z 803.9 due to loss of trimethylamine [y14* – 59]⁺ appeared only in the spectrum of the modified peptide, suggesting the modification by trimethylation rather than acetylation.
charged \([\text{M} + 3\text{H}]^{3+}\) precursor ion at \(m/z\) 837.116 (theoretical molecular mass = 2509.305 Da; calculated, 2508.348 Da) at 51.09 min, and the precursor ion of the unmodified version appeared as a triply charged ion at \(m/z\) 823.439 (theoretical molecular mass = 2467.258 Da; calculated, 2467.317 Da) at 57.78 min in the TIC. The precursor ion mass of the modified peptide showed a 42-unit positive mass shift as compared with the unmodified peptide. This suggested that the peptide could be modified by either acetylation or trimethylation. The CID of the precursor ion (\(m/z\) 628.093) of the peptide yielded a quality MS/MS spectrum, which provided almost a total coverage of the amino acid sequence of the peptide contributed by both y ions and b ions (Fig. 2). In the MS/MS spectrum of the modified peptide, the singly charged y8 ion at \(m/z\) 912.5 (Fig. 2, top) was identical to that of the unmodified peptide (Fig. 2, bottom). Furthermore the a6 ions at \(m/z\) 683.3 were also identical for both peptides. The a8* (a8 – \(\text{NH}_3\)) ion at \(m/z\) 951.5 of the modified peptide displayed a 42-unit mass shift, suggesting modification at Lys347. The challenge was to confirm whether the modification was contributed by acetylation or trimethylation. The immonium ions for acetylated lysine or trimethylated lysine have been proposed as marker ions to differentiate these two possibilities (16, 19). Typically immonium ions at \(m/z\) 126, \(m/z\) 98, and \(m/z\) 84, corresponding to the acetylated, monomethylated, and di- or trimethylated lysine, respectively, have been used effectively to differentiate methylation from acetylation. Furthermore the neutral loss of trimethylamine (\(\text{MH}^+ – 59\)) is considered as a unique feature for trimethylated lysine. First, we determined whether the marker ion at \(m/z\) 126, specific for acetylated lysine, was present or not in the MS/MS spectrum. The MS/MS spectrum showed no signal at \(m/z\) 126, suggesting that the modification is likely by trimethylation rather than acetylation. Second, the marker ion at \(m/z\) 84, which was supposed to originate from a di- or trimethylated lysine (19), was only prevalent in the MS/MS spectrum of the modified peptide (Fig. 2, top) but not in the unmodified version (Fig. 2, bottom). Therefore, this modification was likely to be due to trimethylation rather than acetylation. This was further substantiated by a doubly charged y14* (y14 – \(\text{NH}_3\)) ion that appeared at \(m/z\) 803.9 (y14* – 59) due to the loss of trimethylamine (59 Da) from the site of modification. Together the data confirmed the modification of Lys347 by trimethylation.

The Effects of Methylase Inhibitor on RAR\(\alpha\) Activity—We then monitored the effects of methylation on RAR\(\alpha\) activation by RA in COS-1 cells under a hypomethylated condition induced by adenosine-2,3-dialdehyde, a global MTI. First we tested the transactivation of Gal4-RLARBD by RA using a Gal4BD-fused reporter system. It appeared that, in the presence of MTI, transactivation of Gal4-RLARBD by RA was dramatically reduced (Fig. 3A), supporting the role of methylation in RAR function. Similarly we evaluated the effects of methylation in the context of RAR full-length protein using a reporter system containing a RARE derived from the RAR\(\beta2\) gene promoter. The result also confirmed a similar attenuation of RAR activation by RA in the presence of MTI (Fig. 3B), suggesting a role for methylation in the activation of RAR\(\alpha\).

The Effects of Lys347 Trimethylation on RAR\(\alpha\) Activity—To unambiguously verify the role of Lys347 trimethylation in the manifestation of RAR\(\alpha\) activity, we conducted site-directed mutagenic analyses in the context of both RARLBD and RAR full-length proteins (Fig. 4A). First we generated a CN (mimicking unmethylated/hypomethylated) mutant by replacing Lys347 with Ala. It appeared that RA activation of the mutant Gal4-RLARBD (K347A mutant) was dramatically reduced as compared with the wild type Gal4-RLARBD. As expected, in the presence of MTI, the wild type activity was reduced, but the mutant RAR was not affected. Similar results were obtained using the full-length RAR wild type and mutant protein determined on the DR5 (RARE) reporter (data not shown). These data suggested that Lys347 trimethylation positively modulates RA activation of RAR. To confirm that the effect was specific to lysine methylation, rather than to the alanine mutation, we also generated a mutant by replacing Lys347 with a glutamine, which was capable of introducing a charge similar to that of lysine. This mutant (K347Q) indeed behaved
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The Effects of Trimethylation on Heterodimerization with RXR—RARs are known to form heterodimers with RXRs to regulate RA-dependent gene expression. Therefore, we also tested whether methylation on RARα could affect the heterodimerization process. The mammalian two-hybrid test demonstrated that 9-cis-RA-dependent RAR/RXR heterodimer formation of the CN (K347A) mutant was interrupted, but it was significantly rescued by the CP (K347F) mutant (Fig. 6). A direct protein-protein interaction test was also conducted in an in vitro IP experiment using His-RAR and GST-RXR purified from bacteria. The heterodimeric complex was precipitated with anti-RXRβ antibody, and the interacting partners, His-RAR and GST-RXR, were detected on the Western blots (Fig. 6B, bottom). This result clearly demonstrated that RAR wild type and the CP mutant, but not the CN mutant, could efficiently form heterodimer with RXR. The data further support the notion that the site-specific hydrophobicity induced by lysine methylation on RARα enhances its molecular interaction.

The Effect of Trimethylation on Ligand Binding—The above protein-protein interaction data demonstrated that hydrophobic mimicry hypermethylation could enhance the potential of RARs for ligand-dependent recruitment of co-activators and the formation of heterodimer with RXR. This prompted us to test whether methylation affects the ligand binding property of the receptor. An in vitro ligand binding assay was conducted using the wild type His-RARLBD and its CP and CN mutants (Fig. 7A). The saturation binding using the radioactive

like the Ala (K347A) mutant, supporting the specific effect triggered by Lys347 trimethylation (Fig. 4B). One of the primary effects of lysine/arginine methylation is to increase site-specific hydrophobicity of the protein (20). Similarly methylation could also enhance the bulkiness of the amino tail of lysine/arginine, which could probably promote steric hindrance either to block protein-protein interaction or to enhance the van der Waals/London forces for molecular interaction. To test this hypothesis, we made a CP mutant by replacing Lys347 with phenylalanine, a bulky amino acid, to impart hydrophobicity. As expected, this CP mutant (K347F) behaved like the lys347 methlatable RARα wild type in COS-1 cells (Fig. 4B).

As compared with the constitutive negative mutants (K347A and K347Q), this CP mutant (K347F) was robustly activated by RA. This supported the notion that Lys347 trimethylation could introduce site-specific hydrophobicity to affect the biological activity of RARα, specifically RA activation of the receptor.

The Effects of RARα Trimethylation on Interaction with Co-factors—To gain insights into the molecular interaction affected by methylation, we used a mammalian two-hybrid system (Fig. 5A) to test the interaction of RAR with PCAF. In this system, RA-dependent interaction of PCAF with Ala (K347A) and Gln (K347Q) mutants was dramatically reduced. However, RA-dependent interaction of the CP mutant with PCAF was not affected (Fig. 5A). This suggested that the hydrophobic mutant, mimicking, at least partially, the hypermethylated RAR, could enhance the ligand dependent co-activator recruitment. In vitro direct protein-protein interaction was conducted to examine how the phenylalanine-induced hydrophobicity could affect protein-protein interaction (Fig. 5A, bottom panel). In a pulldown experiment, the CP (Lys → Phe) mutant preferentially interacted with PCAF as compared with the Lys → Ala mutant. This supports the notion that Lys347 is crucial for interaction with PCAF, and site-specific hydrophobicity by methylation on this residue increases the potential of RAR activation.

We then examined the ligand-dependent interaction with RIP140, a ligand-dependent co-repressor. The data also showed that the CP mutation could enhance the ligand-dependent interaction with RIP140, as compared with the Ala mutant, in both mammalian two-hybrid assay and direct protein-protein interaction test (Fig. 5B). This indicates that methylation on Lys347 probably modulates protein-protein interaction of RAR by promoting hydrophobicity-induced van der Waals/London forces for molecular interactions.

The Effects of Trimethylation on Ligand Binding—The above protein-protein interaction data demonstrated that hydrophobicity mimicking hypermethylation could enhance the potential of RARs for ligand-dependent recruitment of co-activators and the formation of heterodimer with RXR. This prompted us to test whether methylation affects the ligand binding property of the receptor. An in vitro ligand binding assay was conducted using the wild type His-RARLBD and its CP and CN mutants (Fig. 7A). The saturation binding using the radioactive...
RA ligand ([3H]atRA) demonstrated no significant difference in the ligand binding affinity among the RAR mutants (Fig. 7A, top). Also no difference was found in a competition assay using an excess amount of cold RA (Fig. 7A, bottom). This suggests that hydrophobicity induced by trimethylation does not seem to have an effect on the RAR ligand binding affinity.

According to the reported structure of RARLBD, Lys347 is located within helix 9 on the surface of the LBD (17, 18). Presumably Lys347 does not impact the interior of the ligand-binding pocket because it is located on the surface area, but its methylation could increase the surface hydrophobicity of helix 9, which in turn could enhance the overall surface hydrophobicity of the receptor in the proximity of the cofactor binding area, thereby promoting interaction of the receptor with coregulators without affecting ligand binding.

To provide further evidence for the effect of this residue on protein-protein interaction, we conducted an in vivo protein-protein interaction test for wild type RAR and its mutants with respect to their interaction with a co-repressor, NCoR, which is known to interact with RAR in the absence of RA (Fig. 7A, bottom). This suggests that hydrophobicity induced by trimethylation does not seem to have an effect on the RAR ligand binding affinity. According to the reported structure of RARLBD, Lys347 is located within helix 9 on the surface of the LBD (17, 18). Presumably Lys347 does not impact the interior of the ligand-binding pocket because it is located on the surface area, but its methylation could increase the surface hydrophobicity of helix 9, which in turn could enhance the overall surface hydrophobicity of the receptor in the proximity of the cofactor binding area, thereby promoting interaction of the receptor with coregulators without affecting ligand binding.

To provide further evidence for the effect of this residue on protein-protein interaction, we conducted an in vivo protein-protein interaction test for wild type RAR and its mutants with respect to their interaction with a co-activator PCAF. The constitutive negative mutants (K347A and K347Q) failed to interact with co-activator PCAF in a mammalian two-hybrid test (subpanel, top). The CP mutant (K347F) demonstrated preferential interaction with PCAF. The CP mutant exhibited similar interaction with PCAF in a direct protein-protein interaction test by pulldown experiment (bottom). This suggests that hydrophobicity induced by trimethylation does not seem to have an effect on the RAR ligand binding affinity.

To provide further evidence for the effect of this residue on protein-protein interaction, we conducted an in vivo protein-protein interaction test for wild type RAR and its mutants with respect to their interaction with a co-repressor, NCoR, which is known to interact with RAR in the absence of RA (Fig. 7B). If methylation promotes protein-protein interaction without affecting ligand binding, it might also significantly affect its interaction with NCoR, which requires no RA. As expected, both the CP and CN mutants behaved similarly to the wild type receptor in terms of RA-independent interaction with NCoR and the sensitivity of this interaction to RA. However, the interaction seemed to be stronger for the CP and wild type receptors (Fig. 7B). This supports that methylation of RAR at Lys347 enhances primarily the hydrophobicity-induced van der Waals/London forces, promoting the overall potential of the receptor in protein-protein interaction but not the ligand binding activity per se.
Lysine Methylation of RARα/H9251 in Mammalian Cells and Cofactor Recruitment—The status of lysine methylation on RARα and its direct role in cofactor recruitment were verified in mammalian cells. We first examined whether RARα could be methylated in mammalian cells by performing metabolic labeling experiments using [3H]AdoMet as methyl donor as well as by Western blot detection of methylated RAR using a methylated lysine-specific antibody (Fig. 8A). The metabolic labeling (left panel, top) and Western blot (left panel, middle) analyses demonstrated that RARα-LBD is methylated in mammalian cells, and the level of methylation was reduced (left panel, lane 3) in the presence of MTI. In another experiment, Western blot analyses of immunoprecipitated full-length RAR using anti-RARα confirmed that RAR is methylated at the lysine residue (Fig. 8A, right panel). As expected, the level of lysine methylation was also attenuated in the presence of MTI (right panel, lane 2), attesting to the specificity of the lysine methylation site on RAR. Given that the titration capacity of all antibodies was equal based on the equal amount of IgG used for IP and Western blot, it was estimated that around 30% of total RAR was methylated in COS-1 cells (Fig. 8, A and B). Similarly considering the similar ionization efficiency of the precursor ions of the modified and unmodified peptides with the same charge state, it was calculated from the TOF mass signal intensity that around 45% of the total RAR was methylated at Lys347 expressed in insect cells (data not shown).

We further confirmed the specificity of Lys347 methylation by IP experiment using the Lys347 mutant RAR. For this purpose, we used the Lys→Phe mutant because this mutant, if not mimicking lysine methylation, at least can be predicted to be structurally less disrupted. It appeared that mutation of Lys347 reduced the level of RAR-LBD methylation albeit the expression level of both the wild type and the mutant RARs were comparable, confirming the specificity of Lys347 methylation on RARα in mammalian cells (Fig. 8B, left panel). Interestingly blockage of lysine methylation for Lys347 mutant RAR-LBD by MTI was not complete, suggesting the existence of other potential lysine methylation sites that have yet to be identified.

To test the direct role for lysine methylation of RARα in regulating its interaction with cofactors, we tested the interaction of the receptor with PCAF under a hypomethylated condition induced by MTI (Fig. 8B, right panel). It appeared that, under the hypomethylated condition induced by MTI, interaction of Gal4-RAR-LBD with PCAF was significantly reduced, supporting a direct positive role for lysine methylation in enhancing RAR interaction with its cofactors. In parallel, we also tested the Lys→Phe (K347F) mutant that appeared to interact with PCAF even better than the wild type. This supported that the Lys→Phe mutant could mimic, at least partially, hypermethylated RAR in mammalian cells.

To provide further evidence, the Lys→Phe (K347F) mutant was tested for transactivation in mammalian cells with and
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**DISCUSSION**

Epigenetic modification of protein by a variety of PTMs, such as phosphorylation, acetylation, methylation, and glycosylation, is known to be involved in the regulation of protein functions and plays an important role in multiple cellular processes including DNA repair, protein stability, nuclear translocation, protein-protein interactions, cellular proliferation, differentiation, and apoptosis (21–23). The major challenge is to identify PTMs occurring on proteins in vivo and to demonstrate and verify their biological significance. It has been difficult, if not impossible, to purify mammalian proteins to homogeneity for biochemical analyses (24). In this work, we identified the sites of lysine methylation in the LBD of RARα by examining proteins expressed and purified from Sf21 cells. Many studies focusing on PTMs of nuclear hormone receptors and coregulators showed variation mostly in the stoichiometry in mammalian cells but not the sites of PTM on proteins expressed in insect cells (13–15). Therefore, we predicted that the modified residues identified on RAR proteins from insect cells could be found in mammalian cells. The modification of RAR by lysine methylation in mammalian cells was verified by site-specific mutagenesis and detected by a methylated lysine-specific antibody.

Lysine methylation of core histone protein is widely known. However, very little is known about lysine methylation of non-histone proteins. Recently it has been shown that transcription factors p53 and TAF10 could be modified by lysine methylation, representing unprecedented examples of lysine methylation for non-histone proteins (25, 26). The reports showed that both p53 and TAF10 would be modified by methylated RARα.

**Fig. 8. Lysine methylation of RARα in mammalian cells.** A, metabolic labeling of Gal4-RARLBD in COS-1 cells using [3H]AdoMet suggested that RARα is methylated in mammalian cells as depicted by IP using anti-Gal4 antibody followed by autoradiography (left panel, top). The level of methylation was shown to be reduced under hypomethylated condition induced by MTI (20 μM) (left panel, top, lane 3). IP experiment using anti-RARα antibody and Western blot detection with anti-methylated lysine antibody revealed that RARα was indeed methylated at the lysine residue (left panel, middle, lane 2). The lysine methylation was also reduced in the presence of MTI (20 μM, 12 h), suggesting the specificity of lysine methylation on RARLBD (left panel, middle, lane 3). The methylated lysine-specific antibody detected two methylated species from IP samples for Gal4-RARLBD in the Western blot. According to the molecular mass, the lower band was considered to be specific and the high molecular unknown band indicated by "?” was considered to be nonspecific methylated species. The IP experiment in COS-1 cells using anti-RARα antibody and Western blot detection with methylated lysine-specific antibody suggested that full-length RARα was methylated in mammalian cells (right panel). B, the lysine methylation status of hydrophobic without MTI (Fig. 8C). The data also showed that RA activation of the wild type receptor was significantly reduced, whereas the Lys → Phe mutant receptor was barely affected. Together these data support a specific positive role for lysine methylation in regulating RAR activity and its molecular interaction, which can be mimicked by the site-specific hydrophobicity on Lys347.

Lys → Phe (K347F) mutant of Gal4-RARLBD was tested in COS-1 cells using methylated lysine-specific antibody (left panel). Mutation on Lys347 attenuated the level of lysine methylation of RARLBD (lanes 1 and 3). This suggested the specificity of the Lys347 methylation site on RARLBD. The direct role of lysine methylation of RARα on its interaction with in vitro translated (TNT) PCAF was tested by pulldown experiment using affinity-purified Gal4-RARLBD from COS-1 cells in the presence or absence of MTI (right panel). The data showed that hypomethylated RAR indeed interacted less with PCAF (lane 1 and 3). C, the effect of MTI on the Lys → Phe (K347F) mutant on RA activation was tested on a Gal4 reporter in COS-1 cells. The wild type RA activation was significantly attenuated in the presence of MTI (20 μM, 12 h), but the constitutive positive (K347F) mutant was not affected. RLU, relative luciferase unit; IB, immunoblot; MeK, methylated lysine; MeRAR, methylated RAR; SAM, S-adenosylmethionine.

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knowledge, our finding of lysine trimethylation of RARα is the first example of trimethylation of mammalian non-histone proteins as well as for the family of nuclear receptors. A recent study described lysine trimethylation of a yeast ribosomal protein, L12ab (27). Therefore, it is likely that more proteins, in addition to the widely demonstrated modification on the histone proteins, can be modified by lysine trimethylation. It would be important to investigate how this modification regulates the functions of the modified proteins.

Several enzymes that methylate specific lysine residues within the histone tails, the histone-lysine methyltransferases, have been isolated and characterized (28). Among these known members, SET9 (also known as SET7) is a protein-lysine methyltransferase (PKMT) with a specific histone methyltransferase activity for H3 Lys4, and it can mono- and dimethylate these substrates. SET7/9 methylates the tumor suppressor p53, recognizes a conserved (K/R-S/T/A) motif preceding the lysine substrate and has a propensity to bind aspartates and asparagines on the C-terminal side of the lysine target (29). According to a sequence-based approach to identify novel substrates for this PKMT, TAF7 was found to be methylated at Lys6 by this enzyme in vitro. The modified Lys347 (DLEOPDKVDM) on RARα does not belong to the conserved sequence for SET9/7. Therefore, RARα is likely to be methylated by other PKMTs that could trimethylate lysine residues. Proteins containing a SET domain constitute a family and are classified into at least four groups on the basis of structural or sequence similarities. Among these members, the SUV39 protein, a mammalian homologue of *Drosophila* position-effect variegation modifier Su(var)3-9, has H3 Lys9 methyltransferase activity. SET1 and SET2 show a H3 Lys4 and a H3 Lys36 methyltransferase activity, respectively. PR-SET7 targets H4 Lys20, whereas G9a functions as a “dual” methyltransferase, catalyzing reactions for histone H3 Lys9 and H3 Lys27 (25). However no non-histone substrates have been reported for these enzymes. Although the methylated residue of RARα was identified on proteins expressed in insect cells in this study, the residue has been confirmed in the mammalian system. However, it remains to be determined which methylase enzyme could trimethylate RARs in the mammalian cells. It is known that the consensus target sequences of lysine-specific methylase, from yeast to mammalian cells, are very similar, and they exert very similar catalytic activities for lysine methylation (28). Therefore, the insect system can be appropriate for systematic identification of protein modifications in vivo. Whether all potentially modifiable residues in mammalian cells could be identified from proteins expressed in insect cells needs further investigation.

Many histone methyltransferase are known to modulate the activity of transcription factors (30, 31). However, it remains to be investigated whether this involves, directly, lysine methylation. In this study, we report lysine trimethylation as a novel means to modulate the biological activity of RARα. This modification made RARα a facile accessible platform for molecular interaction with a number of associated proteins such as its heterodimeric partner RXR and recruitment of cofactors PCAF and RIP140. To understand how Lys347 trimethylation can play a role in such interactions, the molecular structure analysis of methylated RAR as reported elsewhere (32) was used to locate the motif where this particular methylation could occur. According to the reported structures, Lys4347 is located within helix 9 on the surface, which is directly involved in heterodimer formation with RXR. Lys347 is not located in the cofactor-contacting surface area of RARLBD on the basis of the co-crystal structures of RARLBD and a short co-activator peptide. Perhaps the overall receptor-coregulator complex could involve an even greater surface area of the receptor for interaction that may be modulated by helix 9 where this particular lysine residue is located. Furthermore it has been shown as the prototype for the folding of nuclear hormone receptors family that helices H1–H3 form one face of the LBD; helices H4, H5, H8, and H9 correspond to the central layer of the LBD; and helices H6, H7, and H10 constitute the second face (33). Because helix 9 takes part in folding of LBD, Lys347 methylation-induced hydrophobicity on helix 9 could influence the folding of the RARLBD, which could in turn promote overall cofactor interaction. This awaits further structural studies.

Apparently an important question is what triggers RARα to be methylated. How the PKMTs are regulated is also not known. As trimethylated RAR can be better activated by RA, it is possible that cellular RA concentration may play a role in lysine trimethylation of RAR to enhance the signal of RA. Recently we have shown that cellular RA concentration is better maintained by cholesterol input (34). Some forms of metabolic links may exist to modulate PTM. For example, oxidized and native low density lipoproteins have been reported to up-regulate the expression of protein-arginine methyltransferases, the enzymes responsible for protein arginine methylation (35). It may also be possible that PKMTs are regulated by some metabolic pathways. Another interesting question is whether any link exists between lysine trimethylation of RAR and the nutritional status, such as the amino acid metabolic pathways for the synthesis of methyl donor (S-adenosylmethionine).

In this study, we further uncovered that site-specific hydrophobicity imparted by lysine methylation altered the behavior, property, and activity of RARα. A mutant RARα mimicking, at least partially, constitutive hypermethylation was generated by substitution with a bulky phenylalanine residue. This appeared to be specific to the methylation on lysine residues because substitution with a glutamine, which has a similar charge, or with a hydrophobic short chain amino acid, alanine, failed to recover the effects of methylation in vivo. Based upon ligand binding data, there was no difference in the ligand binding affinity of the wild type and the mutant RARs. Therefore, it is unlikely that the Lys → Ala and Lys → Gln mutants were structurally defective. Thus, the overall site-directed mu-
tagenesis studies validated the specificity of Lys347 methylation. This further suggests that methylation on this particular lysine residue, rather than the lysine itself, is one of the most critical factors that modulates the biological activity of RARα.

It has been reported that trimethylated lysine or dimethylated lysine of histone is specifically recognized by plant homeodomain (PHD) of several chromatin remodeling factors including nucleosome remodeling factor (NURF) and inhibitor of growth protein 2 (ING2) (36–38). The binding site of PHDs consists of hydrophobic aromatic amino acids, including tryptophan and tyrosine, essential for binding to trimethylated lysine by van der Waals and cation–π interactions. This indicated that site-specific hydrophobicity is an important factor for such interaction. It is also known that PHDs specifically recognize trimethylated lysine at Lys4 of histone H3 but not the trimethylated lysine at Lys9 and Lys27 of histone H3. This suggested that the recognition of trimethylated lysine by PHDs is very specific. Our data showed that lysine trimethylation enhanced the interaction of RAR with its effector molecules. The specific domain in the effector molecules that recognizes trimethylated lysine of these non-histone proteins remains to be determined. Our data also suggested that site-specific hydrophobicity imparted by lysine trimethylation could be generated by replacing the methylated lysine with a bulky aromatic hydrophobic amino acid. Presumably the spatial orientation of the phenyl ring favors the van der Waals interaction similar to that of a trimethyl group. However, it remains to be verified if our strategy of replacing methylatable lysine with phenylalanine to increase hydrophobicity can be applied in general.

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