The Lysine 831 of Vascular Endothelial Growth Factor Receptor 1 Is a Novel Target of Methylation by SMYD3

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Abstract

We previously identified SMYD3 as a histone methyltransferase and showed that its expression was elevated in colorectal, hepatocellular, and breast carcinomas. In the investigation of methyltransferase activity of SMYD3, we have found that vascular endothelial growth factor receptor 1 (VEGFR1) was also methylated by SMYD3. We further identified the methylated residue at VEGFR1 lysine 831, which is located in the kinase domain and is conserved among VEGFR1 orthologues. We also found that the lysine is followed by serine, which is conserved among some of the methylation targets of histone methyltransferases. Furthermore, methylation of VEGFR1 enhanced its kinase activity in cells. These data should be helpful for the profound understanding of the biological role of SMYD3 and regulatory mechanisms of VEGFR1. Additionally our finding may facilitate the development of strategies that may inhibit the progression of cancer cells. [Cancer Res 2007;67(22):10759–65]

Introduction

Modifications of histone tails play a crucial role for the regulation of transcription, telomere maintenance, DNA replication, and chromosome segregation (1). The modifications include acetylation, phosphorylation, methylation, and/or ubiquitination, and the covalent modifications regulate not only the chromatin structure but also the interaction with chromatin binding proteins (1–3). Conformation of chromatin is one of the key regulators of transcription; untranscribed genes are compacted in heterochromatin, whereas transcribed genes are in euchromatin, wherein transcriptional complexes are accessible to the target DNA (4). In addition, modification of histone residue facilitates the interaction with its binding protein(s) and affects subsequent modifications on other histone tails (2–5). For instance, phosphorylation of H3 serine 10 (H3S10) suppresses methylation of H3 lysine 9 (H3K9) (5). Conversely, methylation of H3K9 antagonizes phosphorylation of H3S10 (5). Phosphorylation of H3S10 promotes acetylation of H3K14 by GCN5 (5). These data indicate the complex nature of histone modification regulated by the interplay between different modifications. Indeed, a growing number of these proteins have been shown to promote or inhibit tumorigenesis through their histone methyltransferase activity (6–8). Although methylation of histone tails has been intensively studied, that of nonhistone protein remains unclear. Recent studies reported that SET7/9, a histone H3K4 methyltransferase, catalyzes TAF10 and p53 as substrates (9, 10).

Vascular endothelial growth factor receptor 1 (VEGFR1) is a receptor tyrosine kinase (RTK) that plays a role in physiologic and pathologic angiogenesis in the context of receptor dimerization and an interaction with its ligands (11, 12). VEGFR1 shares structural similarity with the FMS/KIT/PDGFR family, containing an extracellular domain, seven immunoglobulin-like sequences, and a cytoplasmic tyrosine kinase domain with a long kinase insert. VEGFR1 is expressed as two forms: a full-length tyrosine kinase receptor and a soluble form that carries only the extracellular domain. Through the homodimerization or heterodimerization with other RTKs, such as VEGFR2 and VEGFR3, the full-length form of VEGFR1 mediates signaling positively on the binding with its ligands. However, the soluble form of VEGFR1 acts as an inhibitor through ligand trapping and suppresses angiogenesis (13). The regulatory mechanism of these opposite functions remains elusive. Although vascular endothelial growth factor-A (VEGFA), one of its ligands, associates with both VEGFR1 and VEGFR2; the affinity of VEGFA to VEGFR1 is at least one order of magnitude higher than that to VEGFR2 (14). On the other hand, the endogenous tyrosine kinase activity of VEGFR1 is extremely low compared with that of VEGFR2. Upon binding with VEGFA, these receptors dramatically increase their autophosphorylation levels and induce growth of endothelial cells, recruitment of endothelial cell progenitors, adhesion of natural killer cells to endothelial cells, and monocyte migration (11–13). Although several VEGFR1 tyrosine phosphorylation sites and their potential interacting partners have been described in different overexpression models (13), the downstream signaling events remain to be delineated because of low biological activity of this receptor.

In our earlier studies, we reported that SMYD3 has dimethyltransferase and trimethyltransferase activity on H3K4, and that elevated SMYD3 expression plays a crucial role in the proliferation of colorectal carcinoma, hepatocellular carcinoma, and breast cancer cells (7, 15); overexpression of SMYD3 conferred growth-promoting effect on NIH3T3 cells, and its knockdown induced growth inhibition and apoptosis of the cancer cells. In search for other substrates of SMYD3 methyltransferase, we have found that SMYD3 methylates lysine 831 of VEGFR1 *in vitro*, and that the VEGFR1 methylation enhances its kinase activity. These findings will help a better understanding of the regulatory mechanisms of VEGFR1 and human carcinogenesis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell lines, reagents, and Western blot analysis. Human embryonic kidney cell lines (HEK293 and HEK293T) and a human colon cancer cell line (SW480) were obtained from the American Type Culture Collection. SNU423 hepatocellular carcinoma cells and MCF7 breast cancer cells were kindly provided from Korea cell-line bank and Cancer Institute of the Japanese Foundation for Cancer Research, respectively. Extraction of protein, immunoprecipitation, and Western blot analysis were done as previously described elsewhere (7). Immunoblot analysis was carried out with anti-SMYD3 (Abcam) with anti–Flt-1 antibody (Santa Cruz Biotechnology), anti-HA (Sigma), anti-Flag (Sigma), anti–glutathione *S*-transferase (GST; BD PharMingen), anti–β-actin (Sigma), or anti–phosphorylated tyrosine (PY20; Sigma) antibodies. Anti-VEGFR1 antisera were raised against the C-terminal domain of human VEGFR1 (residues 1123–1338) in rabbits. Methylated lysine 831–specific antibody (anti-K831me2) was purified from the sera of rabbits immunized with RERLKLGK^{m2}SLGRGA, synthetic peptides containing dimethylated lysine 831. Horseradish peroxidase–conjugated second antibody was used for the ECL Detection System (GE Healthcare). Recombinant histone H3 and VEGFR1 were purchased from Upstate Biotechnology and Calbiochem, respectively.

Preparation of plasmids. Plasmids expressing SMYD3 were prepared as described elsewhere (7). We also prepared plasmids expressing HA-tagged or 3xFlag-tagged VEGFR1 by cloning various reverse transcription–PCR products containing either wild-type or deleted forms of VEGFR1 into an appropriate site of pCMV-HA (Clontech) and p3xFlag-CMV14 (Sigma). We prepared plasmids expressing GST-fused VEGFR1 using pGEX6P-3 vector (GE Healthcare). These plasmids expressed VEGFR1#1 (codons 800–1,000), VEGFR1#2 (codons 1,000–1,200), VEGFR1#3 (codons 1,200–1,338), N1 (codons 800–841), N2 (codons 842–878), N3 (codons 879–920), N4 (codons 842–900), N5 (codons 879–900), N6 (codons 842–900), N7 (codons 800–878), or N8 (codons 842–1,000). Primers used for the preparation of plasmids are shown in Supplementary Table S1. Mutant VEGFR1 plasmids containing substitution of amino acid sequence were generated using Quickchange II XL site-directed mutagenesis kit according to the supplier's protocol (Stratagene). We transfected cultured cells with the mammalian plasmids using FuGENE 6 reagent according to the supplier's protocol (Roche). Recombinant GST-fused VEGFR1 protein was purified from *Escherichia coli* BL21 bacterial cells using glutathione Sepharose 4B (GE Healthcare).

***In vitro* methyltransferase assay.** We transfected 293T cells with plasmids expressing Flag-tagged wild-type SMYD3 (p3xFLAG-CMV-SMYD3) and purified the tagged SMYD3 protein by immunoprecipitation using anti-Flag antibody. *In vitro* methyltransferase assay was done with a slight modification as described elsewhere (7). Briefly, immunoprecipitated or recombinant SMYD3 protein was mixed with 1 μg of recombinant histone H3 or VEGFR-1 protein in the presence of 2 μCi of [methyl-^{3}H]–labeled *S*-adenosyl-L-methionine (GE Healthcare) as methyl donor with or without *S*-(5′-adenosyl)-L-homocysteine hydrolase (Sigma) in methyltransferase buffer [50 mmol/L Tris-HCl (pH 8.5), 100 mmol/L NaCl, 10 mmol/L DTT] and was incubated at 30°C for 1 h. Labeled protein was measured by liquid scintillation counter or detected after SDS-PAGE by fluorography.

***In vivo* methylation assay.** *In vivo* methylation of VEGFR1 was analyzed according to the method described by Liu and Dreyfuss (16) with slight modifications. Briefly, HEK293 cells expressing SMYD3 (HEK293-SMYD3) and control cells (HEK293-Mock) were transfected with plasmids expressing HA-tagged VEGFR1. The cells were treated with 100 μg/mL of cycloheximide at 37°C for 30 min and then further maintained in a medium containing 10 μCi/mL of L-[*methyl*-^{3}H] methionine for 3 h. VEGFR1 was precipitated with anti-HA antibody from the cell extract, separated by SDS-PAGE, and subsequently analyzed by BAS imaging system (BAS-TR2040, FUJI) or by immunoblot analysis.

***In vitro* kinase assay.** *In vitro* kinase assay was done with gastrin precursor (tyrosine 87) biotinylated peptides as substrates using a VEGFR1 kinase assay kit that recognizes phosphorylated tyrosine of the substrates (Cell Signaling). The peptides were incubated with cytoplasmic region of VEGFR1 that was treated with/without SMYD3, and the phosphorylation was recognized with anti–phosphorylated tyrosine antibody and measured by DELFIA assay kit according to the supplier's recommendations (Cell Signaling). Methylation of VEGFR1 by SMYD3 was confirmed using [^{3}H]BAS system (data not shown).

Autophosphorylation of VEGFR1 in cells. Phosphorylation of VEGFR1 was measured using HEK293-SMYD3 cells and HEK293-Mock cells. To stimulate VEGFR1, these cells were starved in serum-free medium for 24 h and subsequently treated with placental growth factor (PLGF) or VEGFA$_{165}$ (R&D Systems) at a concentration of 50 ng/mL for 10 min. VEGFR1 was precipitated with anti-VEGFR1 antisera. The immunoprecipitants were analyzed by immunoblot analyses with anti–Flt-1 antibody (Santa Cruz) or anti–phosphorylated tyrosine antibody (Sigma).

Statistical analysis. All analyses were done using Statview software (SAS Institute).

Results

SMYD3 methylates VEGFR1 *in vitro*. Because recent studies have suggested that VEGFRs are involved in human carcinogenesis, we investigated VEGFR1 and VEGFR2 as candidates of methylation targets of SMYD3 by means of *in vitro* methyltransferase assay. We prepared recombinant proteins of three cytoplasmic regions of VEGFR1 for the substrates, VEGFR1#1 (codons 800–1,000), VEGFR1#2 (codons 1,000–1,200), and VEGFR1#3 (codons 1,200–1,338), and incubated them with SMYD3 or control protein immunoprecipitated from HEK293-SMYD3 or HEK293-Mock cells, respectively. As a result, VEGFR1#1 was significantly methylated by SMYD3 compared with control protein (Fig. 1*A*). However, no methylation was observed in VEGFR1#2 or VEGFR1#3, suggesting that VEGFR1#1 contained the methylated residue(s). Approximately 3-fold higher methylation of VEGFR1#1 was detected compared with recombinant histone H3 *in vitro* (data not shown). Although we also tested methylation of VEGFR2 by SMYD3, we did not detect any methylated bands (data not shown). Because *S*-adenosylhomocysteine hydrolase catalyzes metabolites of *S*-adenosylmethionine, we additionally carried out *in vitro* methyltransferase assay with or without *S*-adenosylhomocysteine hydrolase using recombinant SMYD3 protein. Again, we observed methylation of VEGFR1#1, but not of VEGFR1#2 or VEGFR1#3 (Fig. 1*B*). In addition, *S*-adenosylhomocysteine hydrolase slightly increased the methylation, which was consistent with our other experiments (data not shown).

To determine the methylated residue(s), we prepared additional VEGFR1 proteins containing different fragments of the VEGFR1#1 construct (Fig. 1*C*). *In vitro* methyltransferase assay showed that VEGFR1-N1 containing codons 800 to 841, and VEGFR1-N7 containing codons 800 to 878 were methylated by SMYD3. However, no methylated bands were observed in VEGFR1-N2, VEGFR1-N3, VEGFR1-N4, VEGFR1-N5, VEGFR1-N6, or VEGFR1-N8; none of which contained codons 800 to 841 (Fig. 1*C*). These data suggested that a region between 800 and 841 was most likely to contain methylated residue(s).

VEGFR1 lysine 831 is methylated by SMYD3. Because most of the SET-containing methyltransferases, including SMYD3, modify lysines, we focused on lysines within this region. We found that lysines 819, 828, 831, and 840 are conserved among VEGFR1 orthologues in other species (data not shown). Although an earlier report showed that K/R-S/T/A-K is a consensus methylation motif of histone methyltransferase SET7/9 (17), other target lysines of histone methyltransferases, such as p53 K370 and histone H3K4 and H3K27, were not flanked by K/R-S/T/A-K but S/T-K-X or X-K-S (Fig. 2*A*). Therefore, among the lysines, we further focused on lysines 819 and 831, which matched these motifs. We did *in vitro*

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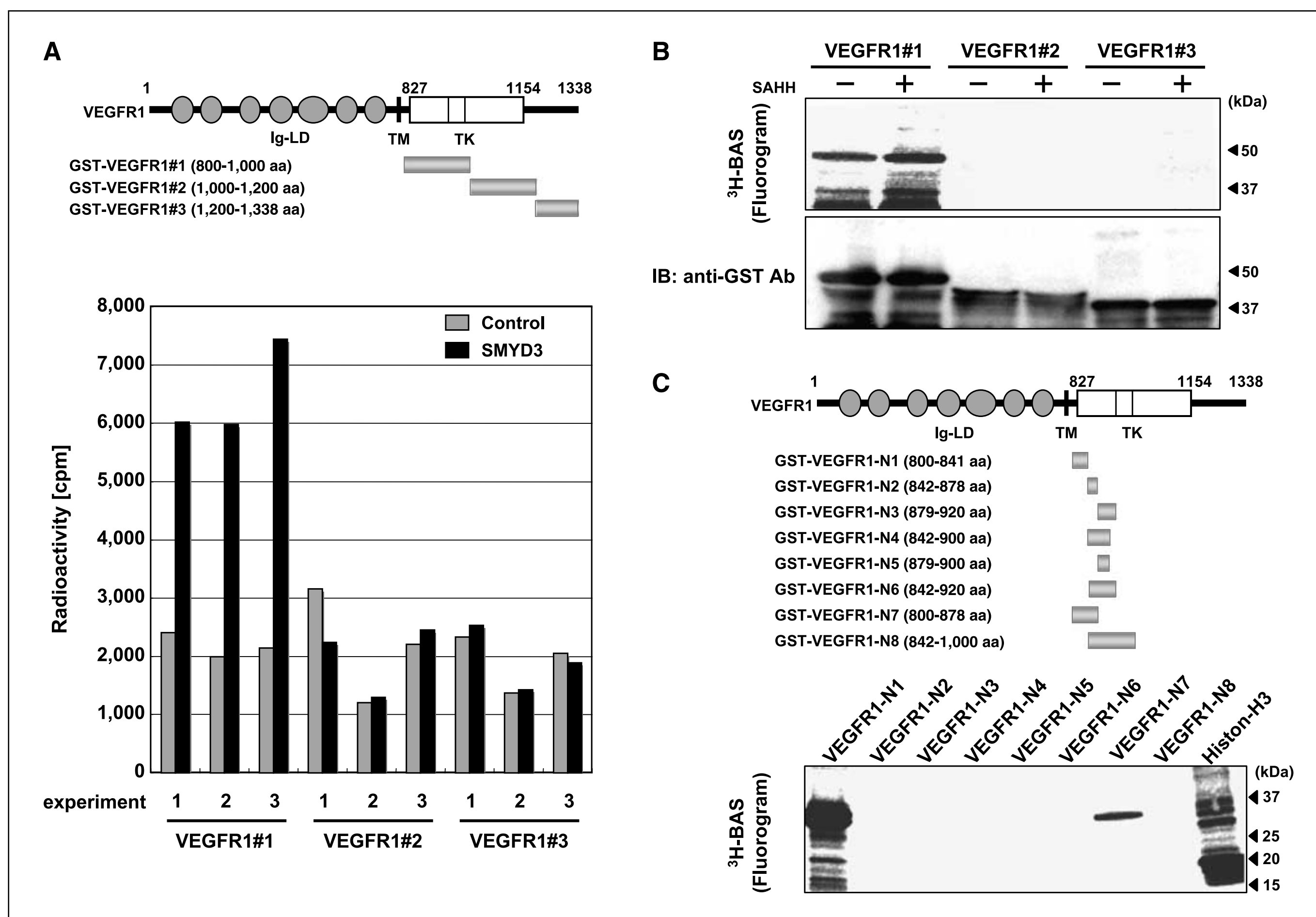

Figure 1. Methylation of the cytoplasmic region of VEGFR1 *in vitro*. *A, in vitro* methyltransferase assay of SMYD3 using recombinant protein containing different cytoplasmic regions of VEGFR1 as a substrate. *Ig-LD,* immunoglobulin-like domains; *TM,* transmembrane domain; *TK,* tyrosine kinase domain. Recombinant VEGFR1#1, VEGFR1#2, and VEGFR1#3 proteins were incubated with [^{3}H]-labeled *S*-adenosylmethionine (*SAM*), a methyl donor, in the presence of immunoprecipitated Flag-tagged SMYD3 (*closed box*). Immunoprecipitants from cells transfected with mock plasmid were used for control (*shadowed box*). Independent experiments were carried out thrice with different immunoprecipitants. *B,* detection of methylated VEGFR1 by fluorogram. Recombinant SMYD3 was incubated with recombinant VEGFR1 in the presence or absence of *S*-(5′-adenosyl)-L-homocysteine hydrolase (*SAHH*). The substrates were quantified by immunoblot analysis with anti-GST antibody (*bottom*). *C,* identification of the methylated region in VEGFR1#1.

methyltransferase assay using wild-type and mutant VEGFR1-N1 protein containing a substitution of the lysines to alanine (Fig. 2*A*). Although the assay showed methylated band in wild-type and K819A mutant, K831A or other K831 mutants did not show any methylated bands (Fig. 2*A*; Supplementary Fig. S1*A*). To confirm the methylation of lysine 831, we prepared methylation-specific antibody against the lysine (anti-K831me2). *In vitro* methyltransferase assay confirmed the methylated lysine 831 of recombinant VEGFR1 by fluorogram, as well as Western blot analysis with the anti-K831me2 antibody (Fig. 2*B*).

Methylation of VEGFR1 in cells. We expressed HA-tagged VEGFR1 in HEK293-SMYD3 cells and control cells (HEK293-Mock) and carried out *in vivo* methylation assay. As expected, we detected augmented methylation of VEGFR1 in HEK293-SMYD3 cells compared with HEK293-Mock cells (Fig. 2*C*). Consistently, immunoblot analysis with anti-K831me2 antibody confirmed elevated methylation of lysine 831 compared with control cells (Fig. 2*D*). Furthermore, small interfering RNA that knocked down SMYD3 reduced dimethylation of VEGFR1 K831 (Supplementary Fig. S1*B*). These data suggested that lysine 831 of VEGFR1 is methylated by SMYD3 in cells.

Interaction between SMYD3 and VEGFR1. Because VEGFR1 was methylated by SMYD3, we investigated whether SMYD3 interacts with VEGFR1. We expressed Flag-tagged cytoplasmic regions of VEGFR1 together with HA-tagged SMYD3 in HEK293 cells. Immunoprecipitation with anti-HA antibody and subsequent immunoblot analysis with anti-Flag antibody disclosed that SMYD3 associates with VEGFR1#1 (codons 800–1,000) but not VEGFR1#2 or VEGFR1#3 (Fig. 3*A*). Consistently, immunoprecipitation with anti-Flag antibody and subsequent immunoblot analysis with anti-HA antibody corroborated the interaction between SMYD3 and VEGFR1#1 (Fig. 3*A*). To examine the interaction of endogenous SMYD3 with VEGFR1, we additionally did immunoprecipitation with anti-SMYD3 antibody using extracts from SNU423, SW480, or MCF7 cells that expressed both SMYD3 and VEGFR1 (data not shown). As a result, we found that endogenous SMYD3 coimmunoprecipitated with VEGFR1 by immunoprecipitation with anti-SMYD3 antibody (Fig. 3*B*), suggesting the interaction between SMYD3 and VEGFR1. We also searched a responsible region of SMYD3 for the binding using plasmids expressing wild-type and three deleted forms of SMYD3: SMYD3-N20 (codons 20–428), SMYD3-Δ2 (codons 100–428), and SMYD3-Δ3 (codons 250–428). Although wild-type SMYD3, SMYD3-N20, and

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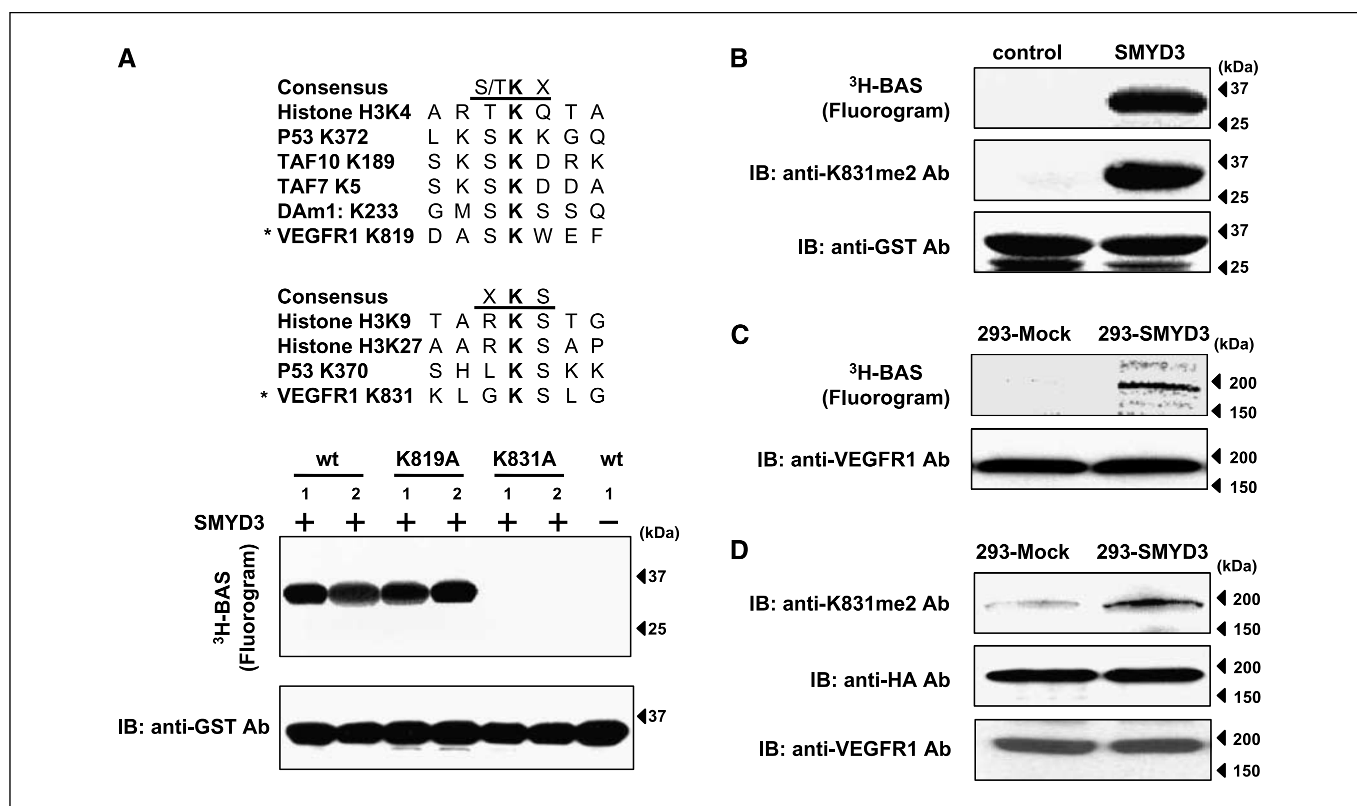

Figure 2. Methylation of VEGFR1 lysine 831. *A,* alignment of flanking sequences of methylated lysines by histone methyltransferases. *, candidate lysines in VEGFR1. Methylation of wild-type and mutant forms (K819A and K831A) of VEGFR1-N1 *in vitro* (*top*). Immunoblot analysis with anti-GST antibody (*bottom*). *B,* immunoblot analysis of methylated VEGFR1 with anti-K831me2–specific antibody (*middle*). Methylation was confirmed in a different experiment by fluorogram (*top*). *C,* methylation of VEGFR1 in HEK293-SMYD3 cells expressing SMYD3 and HA-tagged VEGFR1. The cells were incubated with L-[methyl-^{3}H] methionine in the presence of protein synthesis inhibitor. VEGFR1 was immunoprecipitated with anti-HA antibody and examined by fluorogram (*top*). *D,* methylation of VEGFR1 in the cells was analyzed by immunoblot analysis with anti-K831me2–specific antibody.

SMYD3-Δ2 interacted with VEGFR1, SMYD3-Δ3, lacking the SET domain, did not (Fig. 3*C*). Additional plasmids expressing the SET domain (100–250) showed an association with the cytoplasmic domain of VEGFR1 (Supplementary Fig. S2). These data suggested that the SET domain is responsible for the interaction.

Enhanced kinase activity of VEGFR1 through its methylation by SMYD3. Because lysine 831 localizes within the kinase domain, we explored the effect of VEGFR1 methylation on its kinase activity. As a result, *in vitro* kinase assay showed that methylated VEGFR1 had significantly higher levels of kinase activity compared with unmethylated VEGFR1 (Fig. 4*A*). No phosphorylation was observed by SMYD3 alone without VEGFR1. To examine whether SMYD3 might enhance VEGFR1 autophosphorylation in cells, we treated HEK293-SMYD3 and HEK293-Mock cells with recombinant PLGF, a VEGFR1-specific ligand that stimulates its autophosphorylation. As expected, we found augmented VEGFR1 autophosphorylation in HEK293-SMYD3 cells compared with HEK293-Mock cells (Fig. 4*B*). Furthermore, VEGF-A, another VEGFR1 ligand, also induced higher level of VEGFR1 autophosphorylation in HEK293-SMYD3 cells compared with the control cells (Fig. 4*C*). These data evidenced that SMYD3 enhances ligand-dependent VEGFR1 autophosphorylation through its methylation.

Discussion

We have shown in this report that VEGFR1 is a novel nonhistone target of SMYD3 histone methyltransferase. Modification of histone tails plays crucial roles in transcription, DNA repair, telomere maintenance, DNA replication, and chromosome segregation through in part alteration of chromatin structure. Recent molecular studies have disclosed the importance of lysine modifications in histone tails, and its dynamic regulation by histone methyltransferases and demethylases (18). More than 17 histone methyltransferases have been identified thus far, and these methyltransferases have specificity for their substrate. H3K4 is monomethylated, dimethylated, or trimethylated by SET7/9, Set1, MLL1, MLL2, MLL3, MLL4, MLL5, ASH1, and SMYD3 (1, 18). Importantly, among histone methyltransferases, SET7/9 and SMYD2 have been shown to methylate nonhistone proteins (9, 10, 19). SET7/9 methylated lysine 372 in p53 *in vitro* and *in vivo*, which increased the stability of p53 (10). SET7/9 also showed methyltransferase activity on lysine 189 of TAF10 (9) and lysine 5 of TAF7 (human TATA box-binding protein–associated factors; ref. 17). In addition, methylation of p53 K370 has been shown by SMYD2, another member of the SMYD (SET and MYND domain) family (19). Our data have added VEGFR1 as a novel nonhistone target of an H3K4 histone methyltransferase. The subcellular localization of SMYD3 changes according to the cellular growing condition (7); SMYD3 is accumulated in the nucleus at S phase and G_2-M, but located mainly in the cytoplasm when the cells were arrested at G_0-G_1. Because VEGFR1 is localized at endoplasmic membrane, cytoplasmic SMYD3 should play a role in the methylation of VEGFR1. Therefore, SMYD3 may enhance cellular response to VEGFR1 ligands by increasing methylation of VEGFR1 when cells are at resting state.

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VEGFRs are RTKs comprising of an extracellular ligand-binding domain, a transmembrane domain, a kinase domain, and a carboxyl-terminal region. All RTKs, including VEGFR1, contain an evolutionary conserved kinase domain containing GXGXXG, ATP binding site, HRDLA, a motif essential for catalysis, and one or two tyrosine autophosphorylation sites (12, 13). In this study, we disclosed that SMYD3 induced methylation of lysine 831, resulting in an enhanced kinase activity of VEGFR-1. This result is quite rational because the lysine was located three amino acid N-terminal from "GXGXXXG" motif in the kinase domain. Methylation of lysine 831 may alter conformation of the kinase domain. Alternatively, methylation of VEGFR1 may suppress the inhibitory domain to increase the kinase activity because the C-terminal region of the cytoplasmic domain of VEGFR1 has an inhibitory role for the kinase activity (13). Recent studies on histone tails have evidenced that their modified residues are recognized by specific molecules; phosphorylated H3S10 recruits GCN5, PVAF, and p300, methylated H3K9 recruits heterochromatin protein 1, and methylated H3K4 recruits CHD1, SNF2L/ISWI, WDR5, BPTF/NURF301, and ING2. Therefore, methylated lysine 831 may recruit methylation-specific protein complex(es) that may affect the kinase activity of VEGFR1.

Modification of a histone tail is associated with modifications in other histone tails, indicating the interplay between covalent modifications (2–5). Phosphorylation of H3S10 facilitates GCN5-mediated acetylation of H3K14 but inhibits methylation of H3K9 by SUV39H1 (5). On the contrary, methylation of H3K9 suppresses phosphorylation of H3S10 (5). Similarly, methylation of H4K3 facilitates acetylation of H4K8 and H4K12 (5). The view of interplay between covalent histone modifications is tempting us to speculate that methylation of VEGFR1 may alter its autophosphorylation by its enhanced kinase activity and subsequent phosphorylation of VEGFR2 through the intermolecular phosphorylation. Here, we showed that autophosphorylation of VEGFR1 was enhanced by SMYD3 in a ligand-dependent fashion. Hence, SMYD3 may increase downstream signaling of VEGFR1 on binding with its ligands. VEGFR1 contains phosphorylated tyrosines, including tyrosines 1169, 1213, 1242, 1327, and 1333 (20), and it remains unresolved which tyrosine(s) phosphorylation is enhanced by the methylation. Although lysine 831 seems to be far from the candidate tyrosines, these residues may show a structurally close localization to lysine 831. Future studies on phosphorylation of the tyrosines by different ligands and effect of covalent modifications in VEGFR1 may lead to a better understanding of the precise role of VEGFR1 signaling.

Earlier studies reported that the recognition motif (K/R-S/T/A-K) is the consensus recognition site for SET7/9 methyltransferase (17). However, the flanking sequences of methylated lysines by other histone methyltransferases do not always agree with this motif. Comparison of the amino acid sequences of histone H3K9 and H3K27 and lysine 370 of p53 has found that X-K-S is another conserved motif of histone lysine methyltransferase. Importantly, the methylated lysine 831 in VEGFR1 is in good agreement with this notion. Although the kinase domain of VEGFR2 shared 70.1% amino acid similarity with that of VEGFR1, VEGFR2 was not methylated by SMYD3. This result is compatible with the conserved motifs, because the corresponding lysine to lysine 831 in VEGFR2 was not flanked by the consensus sequences. It is interesting that VEGFR2 containing high kinase activity compared with VEGFR1 was not methylated by SMYD3. Although SMYD3 may not affect the methylation of VEGFR2, it may affect phosphorylation of VEGFR2

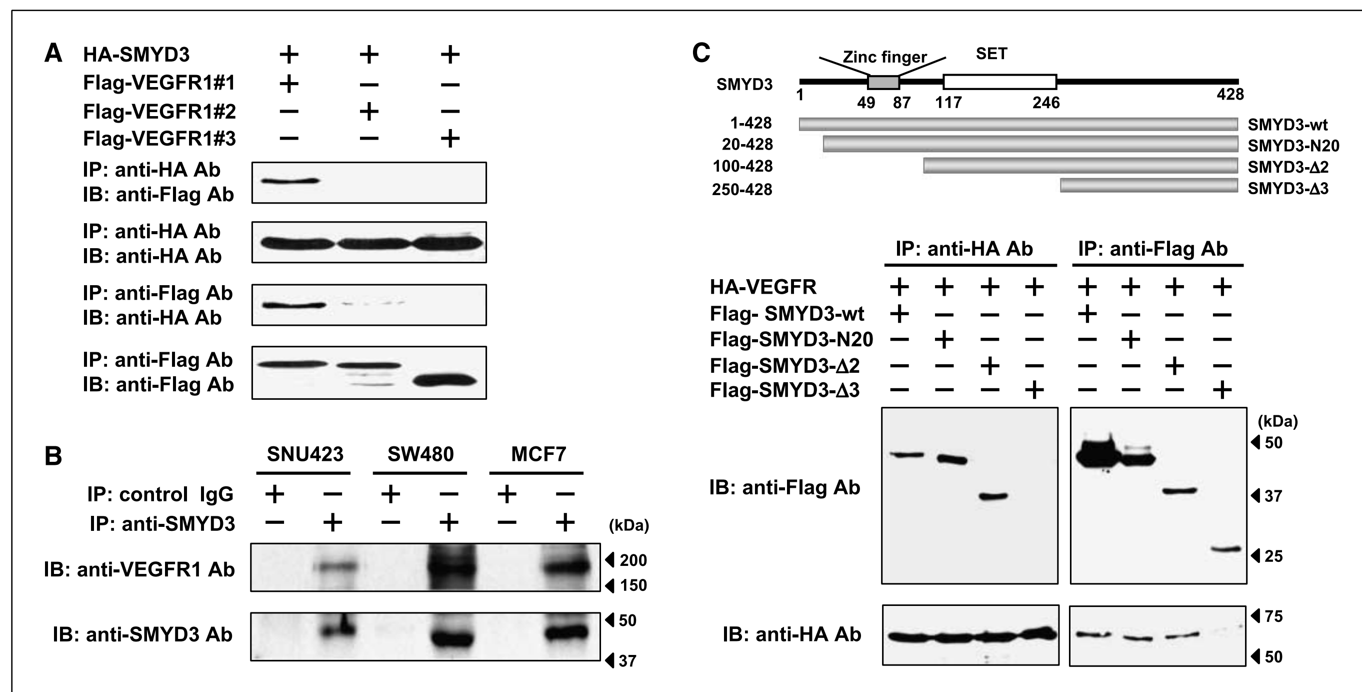

Figure 3. Interaction between SMYD3 and VEGFR1. *A,* interaction between SMYD3 and VEGFR1 was examined by coimmunoprecipitation assay using extracts from HEK293 cells expressing HA-tagged SMYD3 and different regions of Flag-tagged VEGFR1 (#1, #2, and #3). *B,* interaction between SMYD3 and VEGFR1 in SNU423, SW480, and MCF7 cells expressing both proteins. Equal amount of extract was used for immunoprecipitation with anti-SMYD3 antibody or control IgG. The precipitants were immunoblotted with anti-VEGFR1 antibody (*top*) or anti-SMYD3 antibody (*bottom*). *C,* the SET domain of SMYD3 is responsible for the association with VEGFR1. Wild-type or deleted forms of Flag-tagged SMYD3 (N-20, Δ2, or Δ3) were coexpressed with HA-tagged cytoplasmic region of VEGFR1 in HEK293 cells. Immunoprecipitation was done with either anti-HA or anti-Flag antibody, and the precipitants were analyzed by Western blot analysis with anti-Flag (*top*) and anti-HA (*bottom*) antibodies.

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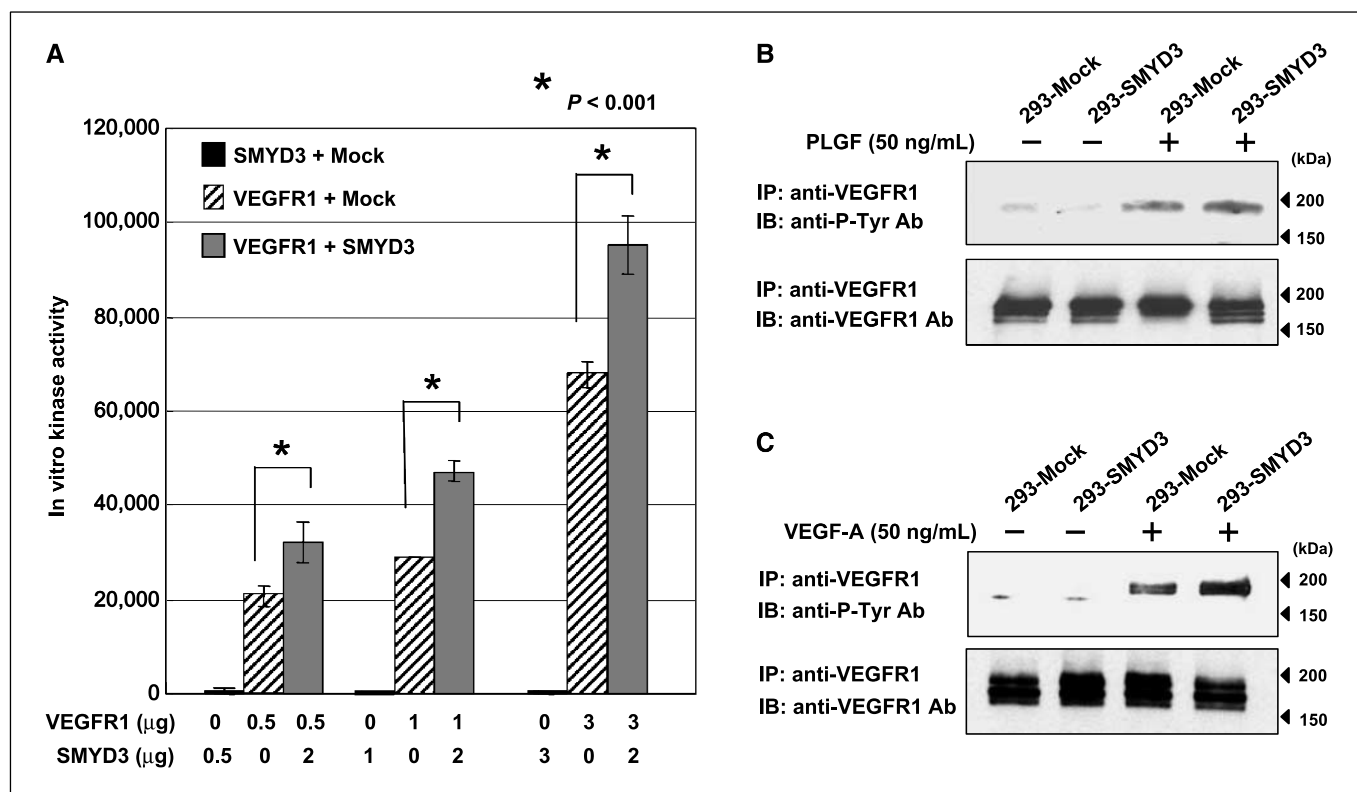

Figure 4. Enhanced kinase activity of VEGFR1 by its methylation. *A, in vitro* kinase activity of methylated or unmethylated VEGFR1. *In vitro* kinase assay was carried out using recombinant VEGFR1 treated with (*shadowed box*) or without (*hatched box*) SMYD3. Kinase activity of SMYD3 alone was also measured (*closed box*). *B* and *C,* phosphorylation of VEGFR1 in HEK293-SMYD3 and HEK293-Mock cells. VEGFR1 extracted from the cells stimulated with human PLGF (*B*) or VEGFA (*C*) was used for Western blot analysis with anti–phosphorylated tyrosine (*top*) or anti-VEGFR1 antibody (*bottom*).

through the phosphorylation of VEGFR1, because these two molecules form a heterodimer (12, 13). It is well known that phosphorylation of one receptor affects phosphorylation of the other receptor by intramolecular phosphorylation (13). It is additionally of note that yeast Rkm1, a SET domain–containing enzyme, revealed methyltransferase activity to Rpl23a and Rpl23b (21). The comparison of the target sequences around the methylated lysines suggested that N/P-P-K might be a target for the methylation. Therefore, the flanking sequence of lysine may be different among targets of methyltransferases.

Although VEGFR1 had been believed to be expressed exclusively on vascular endothelial cells, recent studies have shown VEGFR1 expression in nonendothelial cells. VEGFR1 is expressed in a wide range of human tissues, including colon (22–25), breast (26, 27), pancreatic (28, 29), prostate (30), renal (31, 32), and ovarian (33, 34) cancer tissues and cancer cell lines (23, 24, 27, 30, 35, 36). Reportedly, VEGFR1 expression was faint in primary colon cancer specimen, but its expression was clearly detected in liver metastasis (23). Furthermore, it was reported that VEGFR1 is implicated in tumor growth and progression; exogenous expression of VEGFR1 enhanced migration and invasion of pancreatic cancer cells. Hence, enhanced expression of SMYD3 may render invasive and/or metastasizing property to cancer cells. In this context, therapeutic approaches targeting VEGFR1 methylation may benefit patients by inhibiting invasion and metastasis of cancer cells. Suppression of the methyltransferase activity of SMYD3 may help the inhibition of VEGFR1 mediated cancer progression.

In this study, we disclosed that SMYD3 methylates lysine 831 of VEGFR1, and that methylated VEGFR1 has augmented its kinase activity compared with unmethylated VEGFR1. Therefore, cancer cells expressing abundant SMYD3 protein may show enhanced signal transduction pathway mediated by VEGFR1. These data should shed light on the novel regulatory mechanism of VEGFR1 and the deregulated VEGFR1 signaling that is involved in human carcinogenesis.

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The Lysine 831 of Vascular Endothelial Growth Factor Receptor 1 Is a Novel Target of Methylation by SMYD3

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