EWS is a substrate of type I protein arginine methyltransferase, PRMT8

JUN-DAL KIM[1], KOICHIRO KAKO[1,2], MISAKO KAKIUCHI[1], GWI GUN PARK[3] and AKIYOSHI FUKAMIZU[1,2]

[1]Graduate School of Life & Environmental Science; [2]Center for Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, 1-1-1 Tenno-dai, Tsukuba, Ibaraki 305-8572, Japan; [3]Department of Food & Bioengineering, Kyungwon University, San 65 Bokjung-dong, Sujung-gu, Seongnam, Kyunggi-do 461-701, Korea

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Abstract. EWS, a pro-oncoprotein which is encoded by the *Ewing sarcoma* (*EWS*) gene, contains arginine-glycine-glycine repeats (RGG box) in its COOH-terminus. We previously found that the RGG box of EWS is a target for dimethylation catalyzed by protein arginine methyltransferases (PRMTs). Although it has been observed that arginine residues in EWS are dimethylated *in vivo*, the endogenous enzyme(s) responsible for this reaction have not been identified to date. In the present study, we determined that EWS was physically associated with PRMT8, the novel eighth member of the PRMT family, through the COOH-terminal region of EWS including RGG3 with the NH_2-terminal region of PRMT8 encompassing the *S*-adenosyl-L-methionine binding domain, and that arginine residues in EWS were asymmetrically dimethylated by PRMT8 using amino acid analysis with thin-layer chromatography. These results suggested that EWS is a substrate for PRMT8, as efficient as for PRMT1.

Introduction

EWS protein is characterized as a gene responsible for causing Ewing sarcoma family tumor, a peripheral primitive neuroectodermal tumors (PNET) (1,2). The chromosomal translocations that fuse the *Ewing sarcoma* (*EWS*) gene to various genes encoding transcription factors such as ATF-1, Fli-1, WT1 and nuclear orphan receptors result in tumorigenesis (3). Whereas much is known about the oncogenic functions of EWS fusion proteins, the cellular roles of EWS have not yet been elucidated. The product of the EWS gene encodes a potential transcriptional activation domain in its NH_2-terminal domain (NTD), RNA recognition motif (RRM) and arginine-glycine-glycine repeats (RGG boxes) in its COOH-terminal domain (CTD).

Several reports have shown that the RGG box in the CTD of RNA binding proteins is frequently a target sequence of methylation catalyzed by protein arginine methyltransferases (PRMTs) (4-6). We have reported that EWS enhances a transcriptional coactivator CBP required for hepatocyte nuclear factor-4α (HNF-4α)-mediated transcription (7). In addition, we demonstrated that PRMT1 methylates EWS and down-regulates its transcriptional activity through nuclear exclusion of EWS by arginine methylation (8). Belyanskaya *et al* (9) reported that EWS is not only localized in the nucleus and cytosol but also on the surface of cells and membrane-associated EWS is post-translationally methylated at arginine residues. Recently, the methylation of arginine residues present in cellular proteins has come to light as an important post-translational modification involved in the regulation of transcriptional regulation, RNA processing, DNA repair and signal transduction (10). The formation of methylarginine is catalyzed by PRMTs with *S*-adenosyl-L-methionine (AdoMet) as a methyl donor. Although there are currently eight proteins in humans known to possess PRMT activity, eight of the nine PRMTs are being intensively investigated (11). Two major types of PRMTs are known in mammalian cells. The type I enzymes, PRMT1 (12), PRMT3 (13), PRMT4 (14) and PRMT6 (15), catalyze the formation of ω-N^G-monomethylarginine (MMA) and asymmetric ω-N^G, ω-N^G-dimethylarginine (ADMA), whereas the type II enzymes, PRMT5 (16) and PRMT7 (17), catalyze the formation of MMA and symmetricω-N^G, ω-N'^G-dimethylarginine (SDMA) (6). On the other hand, no activity has yet been found in PRMT2 (18).

PRMT8 has recently been identified as a brain specific PRMT (19,20), and is most likely a type I enzyme, because of its ~80% amino acid sequence identity with PRMT1, but it has a distinctive NH_2-terminal region that harbors a myristylation motif. This enzyme is post-translationally modified by the attachment of a myristate to the glycine residue after the initiator methionine, resulting in its association with the plasma membrane (19). Besides the myristylation, automethylation of PRMT8 was recently observed at the two arginine residues located in its NH_2-terminal tail (21). However, no endogenous substrate for PRMT8 has been identified to date.

Correspondence to: Professor Akiyoshi Fukamizu, Graduate School of Life and Environmental Science/TARA Center, University of Tsukuba, 1-1-1 Tenno-dai, Tsukuba, Ibaraki 305-8572, Japan
E-mail: akif@tara.tsukuba.ac.jp

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In this study, we first screened all the known human PRMTs for their ability to methylate EWS *in vitro*, and clarified that arginine residues of RGG3 in EWS are intensively methylated by PRMT8 *in vitro*.

Materials and methods

Plasmids. Flag-EWS, GST-EWS, GST-EWS (aa 1-333), GST-EWS (aa 246-504), GST-EWS (aa 328-656), HA-PRMT1 and GST-PRMT1 expression plasmids have been described previously (8). For the construction of GST-EWS (aa 543-656), we designed restriction sites (*Eco*RI/*Sal*I)-tagged primer-pairs and carried out PCR with GST-EWS (7) expression plasmid as a template. The amplified fragment was digested with *Eco*RI/*Sal*I and subcloned into pGEX5X-1 (GE Healthcare).

cDNAs of PRMT2 to 6 were obtained by RT-PCR as follows: the first strand cDNA was synthesized with HeLa cell total RNA as template and PCR was performed using restriction sites (*Bam*HI/*Eco*RI)-tagged primer-pairs designed with reference to previous studies (13-16,18). These PCR amplified fragments were digested with *Bam*HI/*Eco*RI and subcloned into pGEX5X-1 and HA-tagged pcDNA3 (Invitrogen). For the construction of GST-PRMT7 and HA-PRMT7, we used human PRMT7 (GenBank accession no. NM019023) sequence to design primers tagged *Eco*RI site for RT-PCR amplification with total RNA from HeLa as a template. These amplified fragments were digested with *Eco*RI and subcloned into pGEX4T-2 (GE Healthcare) or HA-tagged pcDNA3.

The cDNA of human PRMT8 was amplified by PCR with Human Fetal Brain Matchmaker cDNA Library (Clontech) as a template. PCR was performed with primers set (fused to *Eco*RI/*Sal*I sites) according to PRMT8 (GenBank accession no. NM019854) sequence. The PCR product was digested with *Eco*RI/*Sal*I and used to construct both GST-PRMT8 and HA-PRMT8 by subcloning in-frame into pGEX4T-2 or HA-tagged pcDNA3.

For the construction of GST-PRMT8 (aa 1-213) and GST-PRMT8 (aa 214-394), we synthesized another primer set with *Xho*I/*Eco*RI sites [5'-GCCGTCGACGCAAGCCCT CCAGGTTTCAG-3' for GST-PRMT8 (aa 1-213); 5'-CGGA ATTCATGCCGCCATGTTTCCAGAC-3' for GST-PRMT8 (aa 214-394)]. PCR amplification was performed with pGEX4T-2/PRMT8 as a template. The PCR amplified fragment was digested with *Xho*I/*Eco*RI and resubcloned into pGEX4T-2.

For the construction of GST-nucleolin (aa 345-710) and GST-fibllarin (aa 1-148), we used human nucleolin (GenBank accession number NM005381) and fibllarin (GenBank accession number NM001436) sequences to design primer sets with *Eco*RI/*Xho*I sites [5'-CCGGAATTCATGACTA GGAAATTTGTTTAT-3' and 5'-CCGCTCGAGCTATTCA AACTTCGTCTTCT-3' for GST-nucleolin (aa 345-710); 5'-CCGGAATTCGCCATGAAGCCAGAATTC-3' and 5'-CGCCTCGAGTCAGATTGCTGCTGCTAG-3' for GST-fibllarin (aa 1-148)] for RT-PCR amplification with total RNA from HeLa as templates. Both of the PCR fragments were digested with *Eco*RI/*Xho*I and subcloned into pGEX5X-1.

Antibodies. Anti-Flag monoclonal antibody (M2) was purchased from Sigma; anti-HA monoclonal antibody (3F10) was from Roche Molecular Biochemicals.

Cell culture and transfections. Human embryonic kidney 293T (HEK293T) and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Nissui) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL) in a humidified atmosphere containing 5% CO_2 at 37˚C. These cells were transfected using GeneJuice transfection reagent (Novagen).

GST pull-down assay. HEK293T cells were transfected with 4 μg of HA-tagged PRMTs or Flag-EWS plasmids. All GST fusion proteins were expressed in *Escherichia coli* BL21 (DE3) cells and purified on glutathione-sepharose 4B beads (GE Healthcare) as described previously (7). Protein levels were estimated by comparison to increasing amounts of bovine serum albumin using Coomassie Brilliant Blue (CBB, Serva) staining. For protein interaction, 5 μg of glutathione-beads bound GST-EWS or glutathione-beads alone were incubated overnight at 4˚C with the cell extracts prepared using buffer A [0.1% NP-40, 400 mM NaCl, 1 mM PMSF, 20 mM HEPES pH 7.9), 1 mM DTT and 1X protease inhibitor cocktail (Roche)]. After washing three times with buffer B (50 mM Tris-HCl pH 8.0, 400 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF and 0.2 mM 2-mercaptoethanol), bound proteins were fractionated by SDS-PAGE and analyzed by Western blotting.

Immunoprecipitation. Whole cell extracts from HEK293T were prepared using lysis buffer (50 mM Tris-HCl pH 7.5, 400 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1X protease inhibitor cocktail). Immunoprecipitation with anti-Flag (M2) antibodies and Western blot analysis of the immunoprecipitates were performed as described previously (7).

In vitro methylation assay. GST-PRMT1 to 8 (1 μg) was added to 2 μg of GST, GST-EWS, GST-EWS (aa 1-333), GST-EWS (aa 246-504), GST-EWS (aa 328-656) and GST-EWS (aa 543-656), and incubated with 2 μl of *S*-adenosyl-L-[methyl-^{3}H]methionine (specific activity of 15.0 Ci/mmol, GE Healthcare) for 1 h at 37˚C in a final volume of 50 μl of methylation buffer (1X phosphate buffered saline (pH 7.4) (OXOID), 1X protease inhibitor cocktail). After washing the beads with the same buffer, the reaction products were fractionated by SDS-PAGE. Gels stained with CBB staining solution (0.05% CBB, 50% methanol, 10% acetic acid) were incubated for 30 min with Amplify Fluorographic Reagent (GE Healthcare), dried and exposed to high performance autoradiography film (GE Healthcare) at -80˚C.

Thin-layer chromatography (TLC) analysis. *In vitro* methylation reaction products were hydrolyzed with 300 μl of 6 N HCl (Wako) for 24 h at 110˚C and the hydrolysed amino acids were dried *in vacuo*. Fifteen microliters of water were added to the residue, and the solution was loaded on silica gel plate (Polygram® SIL G/UV_{254}, Macherey-Nagel)

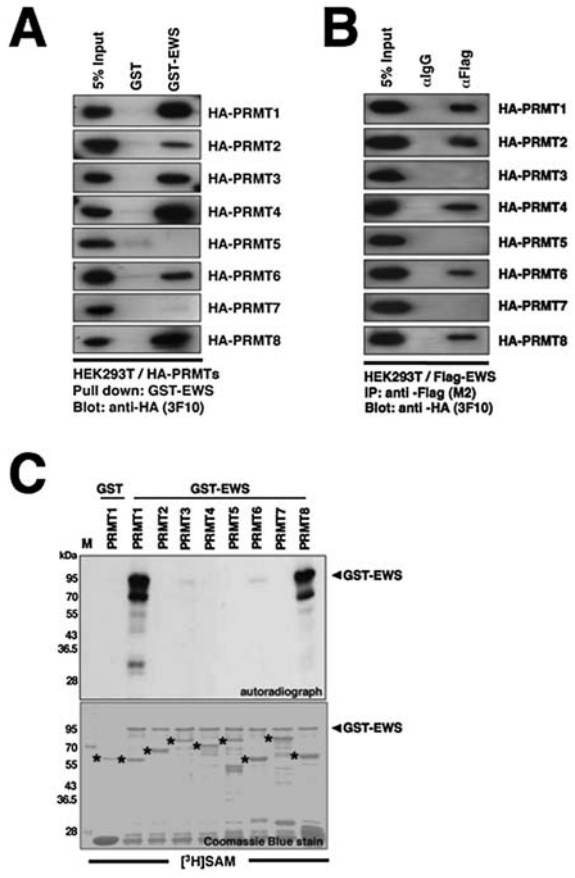

Figure 1. EWS was methylated by PRMT8 *in vitro*. (A) Pull-down assay with GST or GST-EWS and extracts from HEK293T cells overexpressing HA-tagged each PRMT expression plasmid. Western blot analysis was carried out using an anti-HA antibody (3F10). (B) Coimmunoprecipitation of HA-PRMTs with Flag-tagged EWS or normal mouse IgG. Whole cell extracts from HEK293T cells transfected with HA-PRMTs and Flag-tagged EWS expression plasmids were immunoprecipitated with anti-Flag antibody (M2) or normal mouse IgG and subjected to immunoblotting with anti-HA antibody. (C) *In vitro* methylation assay. Recombinant PRMTs fused to GST were incubated with recombinant GST-EWS or GST in the presence of *S*-adenosyl-L-[methyl-^{3}H]methionine as described in Materials and methods. Reactions were directly loaded onto a 10% gel for SDS-PAGE analysis. Gels were stained with CBB (bottom panel), treated with Amplify Fluorographic Reagent, dried and exposed with film for two days (upper panel). *Position of corresponding GST-PRMTs.

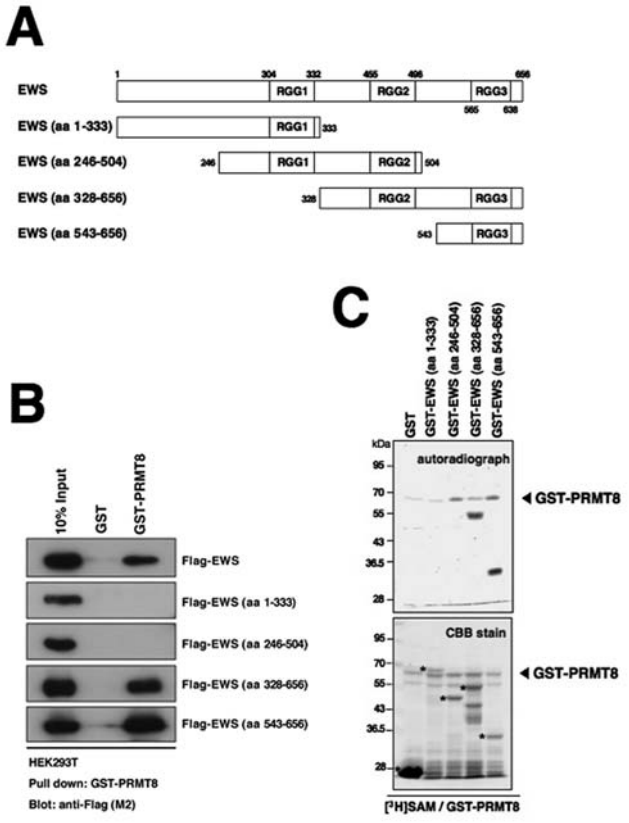

Figure 2. PRMT8 specifically methylates the COOH-terminal region of EWS. (A) Schematic representation of EWS and its deletion mutants. (B) Pull-down assay with GST or GST-PRMT8 and extracts from HEK293T cells overexpressing Flag-tagged EWS fragment expression plasmids. Western blot analysis was carried out using an anti-Flag antibody. (C) *In vitro* methylation assay using GST-PRMT8 and GST-EWS deletion mutants or GST under the conditions described in Fig. 1C. *Position of corresponding GST-EWS deletion mutants.

and separated with ammonium hydroxide:chloroform: methanol:water (2:0.5:4.5:1). Color was developed with a Ninhydrin spray (Wako). As standards for arginine derivatives, L-arginine (Wako), MMA (Dojindo), ADMA and SDMA (Calbiochem) were commercially purchased. Chromatographs were coated with 3 applications of En3hance Spray (PerkinElmer Life Sciences) and exposed to high performance autoradiography film (GE Healthcare) at -80˚C for 24 days.

Results

Physical association of EWS with type I PRMTs. Previously, we demonstrated that EWS physically associated with PRMT1 through its COOH-terminal region including RGG repeats, the major target for methylation by PRMTs (5,6,8). To further understand the essential role of methylated EWS, we first screened to see which enzymes associated with EWS *in vitro* and *in vivo*. HA-tagged constructs of PRMT1 to PRMT8 were transfected into HEK293T cells and each binding activity to GST-EWS was determined by GST-pull down assay. As shown in Fig. 1A, PRMT1, 2, 3, 4, 6 and 8, but neither PRMT5 nor 7, were bound to EWS *in vitro*. Furthermore, co-transfection experiments using Flag-EWS and each HA-tagged PRMT construct revealed that PRMT1, 2, 4, 6 and 8 interacted with EWS *in vivo* (Fig. 1B).

EWS is methylated by both PRMT1 and PRMT8. To examine which enzymes methylated recombinant human EWS, GST fusions of eight PRMTs were purified and their methyltransferase activities were determined by using the purified GST-EWS or GST as substrates. Proteins were incubated in the presence of *S*-adenosyl-L-[methyl-^{3}H]methionine, electrophoresed on an SDS-polyacrylamide gel, and exposed to X-ray film as described in Materials and methods. PRMT1 and 8 significantly methylated EWS (Fig. 1C), although faint methyltransferase activities were found in PRMT3 and 6. No activity was seen with PRMT2 and 4 in these conditions.

Arginine residues in the COOH-terminal region of EWS are major targets for PRMT 8. The significant catalytic activity of PRMT8 in relation to EWS is illustrated in Fig. 1C. To biochemically characterize the interaction between EWS and PRMT8 and the methylation of EWS, we constructed a series

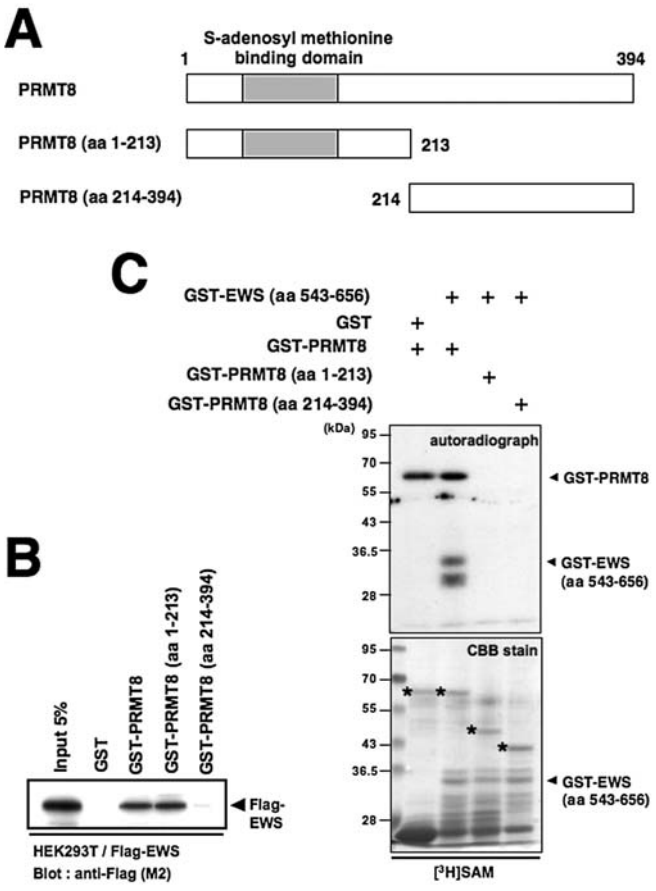

Figure 3. PRMT8 associates with EWS through its NH_2-terminal domain. (A) Schematic representation of PRMT8 and its fragments. (B) Pull-down assay with GST or GST-PRMT8 derivatives and extracts from HEK293T cells overexpressing Flag-tagged full-length EWS. Western blot analysis was carried out using an anti-Flag antibody. (C) *In vitro* methylation assay using GST-PRMT8 derivatives and GST-EWS (aa 543-656) or GST under the conditions described in Fig. 1C. *Position of corresponding GST-PRMT8 derivatives.

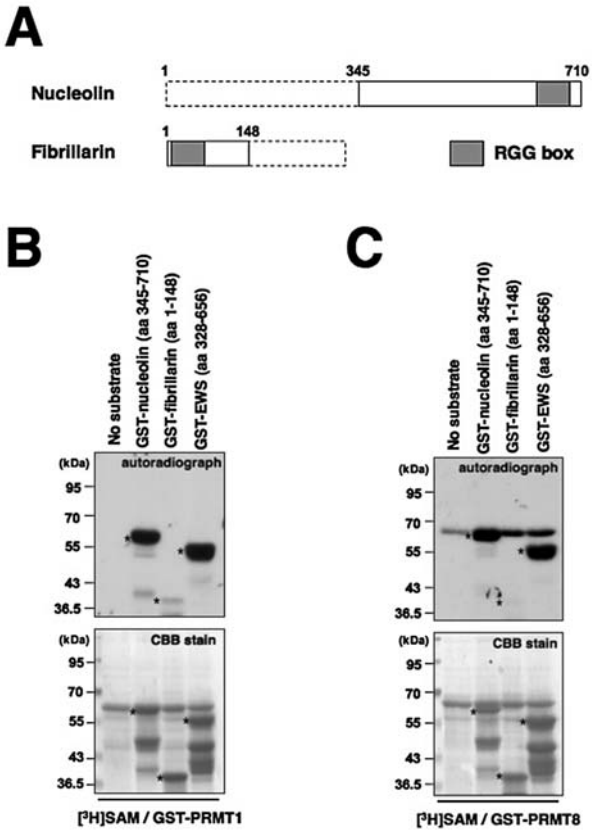

Figure 4. Relative *in vitro* methyltransferase activities of GST-PRMT1 and GST-PRMT8. (A) Schematic representation of the GAR fragments from nucleolin and fibrilarin as popular substrates for type I PRMTs. *In vitro* methylation assay using GST-PRMT1 (B) or GST-PRMT8 (C) and GST-nucleolin (aa 345-710), GST-fibllarin (aa 1-148), GST-EWS (aa 328-656) or GST under the conditions described in Fig. 1C. *Position of corresponding GST-substrates.

of Flag-tagged EWS deletion mutants (Fig. 2A) and carried out *in vitro* binding and methylation assay using GST-PRMT8 and cell extracts from HEK293T cells transfected with Flag-EWS fragments. Fig. 2B and C shows that PRMT8 specifically coupled with and significantly methylated EWS (aa 328-656) and EWS (aa 543-656).

Mapping of the EWS-interacting and catalytic domains of PRMT8. To map the PRMT8 region involved in the interaction with EWS using GST-pull down assay (Fig. 3), NH_2- or COOH-terminal fragments of the PRMT8 protein were fused to the GST (Fig. 3A) and tested for their ability to bind full-length EWS as described above. The NH_2-terminal fragment, GST-PRMT8 (aa 1-213) had approximately the same affinity as full-length GST-PRMT8. Empty vector coding only the GST-tag or the COOH-terminal fragment of PRMT8, GST-PRMT8 (aa 214-394), did not interact with EWS (Fig. 3B).

Moreover, we performed an *in vitro* methylation assay with the above set of GST-PRMT8 fragments to confirm the methyltransferase activities using GST-EWS (543-656) including RGG3 as a substrate (Fig. 3C). Fig. 3C also shows the equal loading and input controls of the tested proteins. Notably, whereas both PRMT8 (aa 1-213) and full-length PRMT8 interacted with EWS, PRMT8 (aa 1-213) failed to methylate not only EWS (aa 543-656) but also PRMT8 (aa 1-213) itself. This result indicated that the NH_2-terminal half of PRMT8 is necessary for association with the substrate, however, the whole structure of PRMT8 is indispensable for exerting catalytic activity as a methyltransferase.

PRMT8 has a similar enzymatic activity to PRMT1. As shown in Fig. 2C, the EWS is directly methylated by PRMT8 through the COOH-terminal region *in vitro*. We tested whether GST-PRMT1 and 8 substantially methylate arginine residues in this fragment of EWS with two GAR-fused GST constructs (one, an NH_2-terminal portion of human fibrillarin, the other, a COOH-terminal fragment of human nucleolin) encompassing RGG boxes (Fig. 4A) have been shown to be a good substrate for PRMTs, especially type I PRMTs (13,22). As expected, all fragments tested were methylated by both PRMTs (Fig. 4B and C).

Arginine residues in EWS were asymmetrically dimethylated by PRMT8. As PRMT8 is highly similar to PRMT1, one would speculate that PRMT8 asymmetrically dimethylates arginine residues in EWS. To confirm directly whether

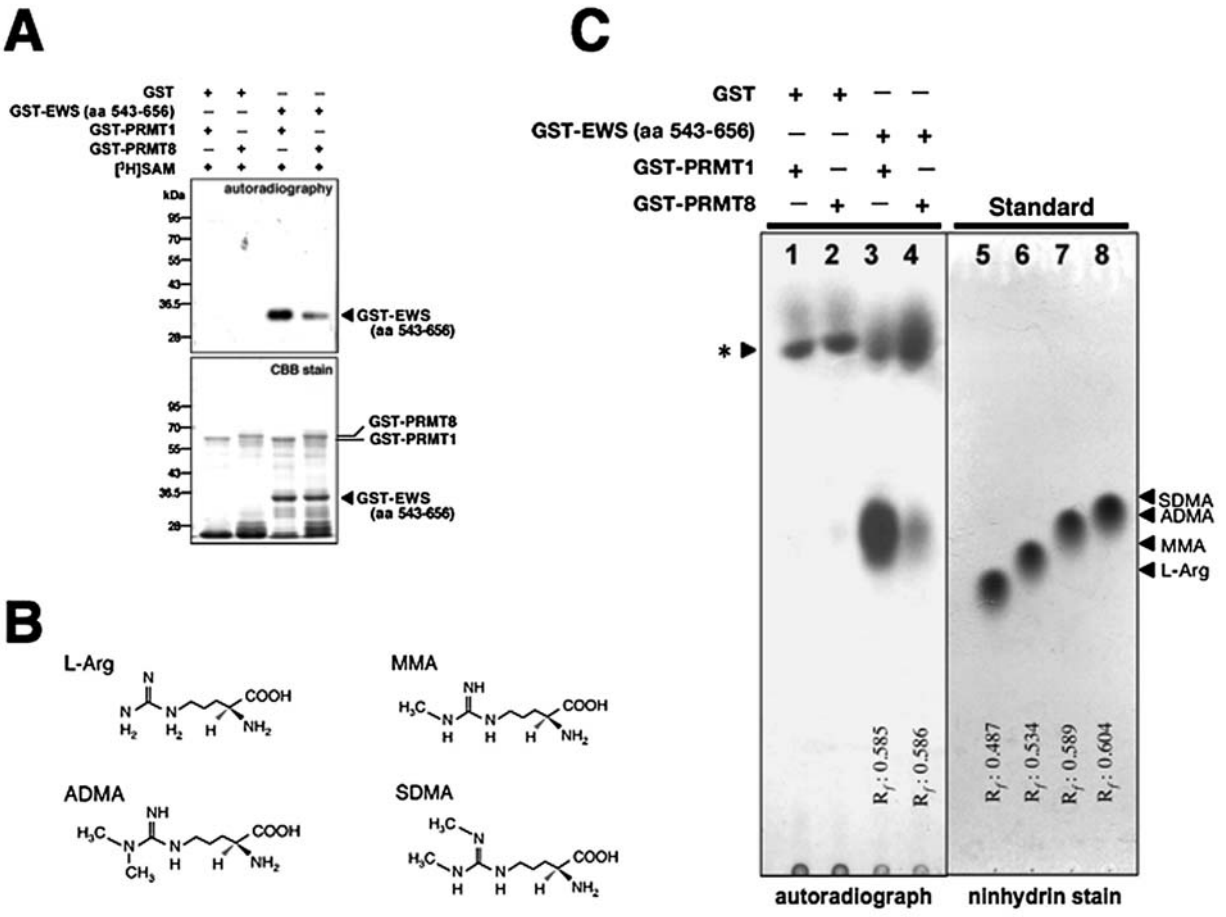

Figure 5. PRMT1 and PRMT8 catalyze the formation of ADMA from EWS (aa 543-656) including RGG3 as a substrate. (A) *In vitro* methylation assay using GST-PRMT1 or GST-PRMT8 and GST-EWS (aa 543-656) or GST under the conditions described in Fig. 1C. (B) Chemical structures of post-translational modifications of arginine residues in proteins. SDMA, symmetricω-N^G, ω-N'^G-dimethylarginine; ADMA, asymmetric ω-N^G, ω-N^G-dimethylarginine; MMA, ω-N^G-monomethylarginine; L-Arg, L-arginine. (C) TLC analysis of hydrolyzed [^{3}H]methyl GST-EWS (aa 543-656) synthesized by GST-PRMT1 and 8 are shown. *In vitro* methylation assay using GST-PRMT1 or GST-PRMT8, and GST-EWS (aa 543-656) or GST under the conditions described in Fig. 1C as in (B). Lanes 5, 6, 7 and 8 show the bands of standard L-arginine, MMA, ADMA, and SDMA, respectively, stained with ninhydrin as described in Materials and methods. Exposure time was 24 days. R_f value, distance traveled by the compound/distance traveled by the solvent front. Asterisk shows the position of excessive free *S*-adenosyl-L-[methyl-^{3}H]methionine.

ADMA is synthesized by PRMT1 and PRMT8, *in vitro* methylation assay was performed with GST-EWS (aa 543-656) including the RGG3 as a substrate (Fig. 5A). The aliquot of each reaction mixture was acid-hydrolyzed and the lysate was analyzed using TLC. Positions of standard amino acids (each structure is illustrated in Fig. 5B) were visualized by ninhydrin staining, and the radiolabeled arginine products were exposed to autoradiography film. In the cases of both PRMT1 and PRMT8, addition of GST-EWS (aa 543-656) stimulated the formation of ADMA (Fig. 5C).

Discussion

Previously, it was shown that dimethylarginine residues exist in EWS *in vitro* and *in vivo* (8,9,23,24). In this study, we first screened the eight known PRMTs for their ability to methylate EWS by using *in vitro* methylation assay. GST pull-down assay showed that some of type I PRMTs physically associated with EWS. Among these type I subfamily members, only PRMT8 had a comparable activity to PRMT1, the most dominant methyltransferase *in vitro*. The interaction between EWS and PRMT8 occurred through the COOH-terminal region of EWS including the RGG3 and NH_2-terminal region of PRMT8 encompassing the AdoMet binding domain. Furthermore, arginine residues in the COOH-terminal region of EWS including RGG3 were asymmetrically dimethylated by PRMT8.

In addition to PRMT1 and 8, PRMT2, 3, 4 and 6 also bound to EWS *in vitro* as shown in Fig. 1A. It has been reported that EWS RGG boxes were methylated not only by PRMT1 but also by PRMT3 *in vitro* with a lower activity than PRMT1 (24), and these observations were also confirmed in this study (Fig. 1C). PRMT4 (also known as CARM1) displays a higher degree of specificity and does not methylate RGG box (14). Although PRMT6 (15) was previously thought to behave like PRMT1 and 3 *in vitro*, a recent study showed that PRMT6 is more specific than PRMT1 or 3 in its recognition of substrate (25). The type II methyltransferases PRMT5 (16) and 7 (17) had low affinity with EWS and they did not methylate EWS. PRMT2 has been previously reported as an inactive enzyme (18), similarly no methylation activity was detected in the present assay.

Recently, Pahlich *et al* (26) identified the interaction between PRMT8 and EWS protein, and its dimethylation. In the present study, we found that PRMT8 bound to and asymmetrically methylated EWS through the COOH-terminal portion by using a series of EWS fragments (Fig. 2A), and we also suggested that the major target arginine residues for methylation exist in RGG3 (Fig. 2B and C).

While PRMT8 and PRMT1 are extremely similar, a recent study reported that the NH_2-terminal ~60 aa region is suppressive for the full activation of PRMT8 activity (21). We thus attempted to map the EWS-interacting and catalytic domains of PRMT8. As shown in Fig. 3B, PRMT8 (aa 1-213), an NH_2-terminal fragment including the AdoMet binding domain associated with EWS at levels comparable to those of full-length PRMT8. However, PRMT8 (aa 1-213) completely abolished its enzymatic activity (Fig. 3C). PRMT8 (aa 1-213) contains a region that corresponds to the NH_2-terminal helices αY and αZ of PRMT1, which forms one of the substrate binding 'acidic' surfaces (27) and the primary structure around this region is completely conserved between PRMT1 (aa 37-56) and PRMT8 (aa 84-103). According to X-ray crystallographic study, the dimerization of PRMT1 is essential for AdoMet binding and enzymatic activity to form an 'acidic ring' structure (27,28). The PRMT1 dimer is formed through the mutual 'dimerization arm' located in the COOH-terminal 'body' [corresponding to PRMT8 (aa 214-394)] and the outer surface of AdoMet binding domain existing in the NH_2-terminal 'head' [corresponding to PRMT8 (aa 1-213)] of PRMT1. Since neither PRMT8 (aa 1-213) nor PRMT8 (aa 214-394) functions alone as a methyltransferase, PRMT8 may require dimerization to express its enzymatic activity.

We also observed an additional methylated polypeptide in full-length GST-PRMT8 containing lanes (Fig. 3C, also evident in Figs. 2C and 4C), which are present even in incubations lacking substrates, and a calculated molecular mass of 68 kDa. This indicated that GST-PRMT8 is capable of methylating itself. While PRMT1 and PRMT8 significantly methylated substrates including RGG boxes derived from nucleolin and EWS, PRMT8 had lower activity than PRMT1 when the fibrillarin-derived RGG box was used as methyl-accepting substrate as previously reported (21) (Fig. 4B and C). This result implicates that substrate-specificity of PRMT8 is slightly different from that of PRMT1. On the basis of the above results, the arginine residues in the COOH-terminal portion of EWS were also expected to convert to ADMA. To determine the specific products of the methylation reaction catalyzed by PRMT1 and PRMT8, reaction products were acid-hydrolyzed to their amino acid components, and fractionated by their hydropathy on a high-resolution TLC. By using this technique, we determined that PRMT8 did not catalyze the formation of SDMA, but did catalyze the formation of ADMA from EWS *in vitro* (Fig. 5C).

Arginine methylation is an emerging regulator of protein function; however, the practical consequences of this modification and its mode of regulation remain largely unknown. It has been shown that MMA and ADMA, but not SDMA, derived from the proteolysis of methylated proteins inhibit the activity of eNOS, leading to atherosclerosis (29). As a high correlation has been reported between the amounts of MMA and ADMA in the blood and the incidence of cardiovascular diseases, these free methylarginines derived from proteins methylated by PRMT8, the brain specific PRMT, may be involved in the occurrence of neuronal diseases through the inhibition of nNOS.

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