CARM1 Regulates Proliferation of PC12 Cells by Methylating HuD

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HuD is an RNA-binding protein that has been shown to induce neuronal differentiation by stabilizing labile mRNAs carrying AU-rich instability elements. Here, we show a novel mechanism of arginine methylation of HuD by coactivator-associated arginine methyltransferase 1 (CARM1) that affected mRNA turnover of p21cip1/waf1 mRNA in PC12 cells. CARM1 specifically methylated HuD in vitro and in vivo and colocalized with HuD in the cytoplasm. Inhibition of HuD methylation by CARM1 knockdown elongated the p21cip1/waf1 mRNA half-life and resulted in a slow growth rate and robust neuritogenesis in response to nerve growth factor (NGF). Methylation-resistant HuD bound more p21cip1/waf1 mRNA than did the wild type, and its overexpression upregulated p21cip1/waf1 protein expression. These results suggested that CARM1-methylated HuD maintains PC12 cells in the proliferative state by committing p21cip1/waf1 mRNA to its decay system. Since the methylated population of HuD was reduced in NGF-treated PC12 cells, downregulation of HuD methylation is a possible pathway through which NGF induces differentiation of PC12 cells.

Hu proteins have been identified as target antigens in the sera of patients with paraneoplastic encephalomyelitis, an autoimmune disease associated with small-cell lung cancer and neuroblastoma (20, 64). The four members of the Hu protein family have been identified as RNA-binding proteins (RBPs) that show homology to the Drosophila melanogaster ELAV protein (27, 28, 42, 52, 56). These mammalian Hu/ELAV proteins, with the exception of HuR, are expressed exclusively in neurons (20, 45, 52). Hu family proteins share the characteristic of three RNA recognition motif domains (RRMs), with a hinge region intervening between the second and the third RRMs (27, 28, 56). In previous reports, Hu proteins had been reported to bind to long poly(A)+ tails (1, 46), but recent studies demonstrated that they recognize AU-rich elements (AREs) which reside in the 3′-untranslated regions (3′-UTRs) of some labile mRNA species (33, 40, 42, 53, 54) and determine their stability or translational efficiency (6, 7, 23, 29, 37).

HuD, one of the Hu family proteins, has been shown to bind to AREs found in the 3′-UTRs of several mRNAs, including c-fos (14), tau (9), GAP43 (15), and p21cip1/waf1 (30), and to the U-rich element found in the p27 mRNA 5′-UTR (36). Previously, it was reported that overexpression of HuD induces neuronal differentiation in PC12 cells, cortical primary culture neurons, and retinoic acid-induced teratocarcinoma cell lines (5). On the other hand, antisense-mediated knockdown of HuD resulted in the inhibition of neurite extension in PC12 cells (48) and HuD-deficient mice exhibited a larger population of dividing stem cells in the adult subventricular zone (3). These findings indicated that HuD is required for neuronal differentiation processes, including growth arrest and cell fate acquisition of neural stem/progenitor cells, and possibly for sprouting and regeneration of mature neurons.

Given that HuD-bound gene products are involved in cell cycle arrest (p21cip1/waf1 and p27), neurite outgrowth (GAP43 and tau), and functional differentiation (choline acetyltransferase) (21), HuD is presumed to induce the neuronal cell shape by exerting a protective effect on these ARE-containing labile mRNAs by antagonizing ARE-mediated mRNA decay. In the case of nerve growth factor (NGF)-induced differentiation of PC12 cells, NGF alters the RNA binding property of HuD towards AREs in the course of differentiation. However, there is no evidence revealing how HuD-ARE interactions are regulated under the NGF signal transduction pathway. We note that HuR, a ubiquitously distributed Hu protein, was arginine methylated by coactivator-associated arginine methyltransferase 1 (CARM1) in the myeloid cell line when the cells were stimulated by lipopolysaccharide (41). However, functional differences between methylated and unmethylated HuR have not yet been elucidated. Since the four mammalian Hu proteins (HuR, HuB, HuC, and HuD) are quite akin to each other in amino acid sequence (27, 52), we explored the possibility that HuD is also methylated at the corresponding arginine residue to HuR.

RBPs are the major substrate group for protein arginine methyltransferases (PRMTs) (8, 26, 34, 37, 43, 45, 47, 49, 50, 57, 60, 61, 67). Most RBPs contain GAR domains, which consist of a repetition of RGG or RXR (X is an aliphatic residue) (11, 58) and are the canonical targets for type I PRMTs that catalyze the formation of asymmetric NG,NG-dimethylarginine residues (37, 49). Type I enzymes PRMT1 and PRMT3 favor GAR domains as their substrates (18, 25, 66), and especially PRMT1 has a promiscuity to methylate arginine residues encompassed by GAR domains (65, 66). On the other hand, another type I enzyme, CARM1, methylates a narrow spectrum of proteins, histone H3 (12, 44), p300/CBP (69), PABP1, and TARPP (39), all of which lack GAR-like domains around the arginine residues. HuR also lacks the canonical GAR domain but instead has an alanine residue 2 residues N-terminal...
before the methylated arginine, which is common to most of the CARM1 substrates (41).

In this report, we first demonstrated that HuD is also an in vivo and in vitro substrate for CARM1 by 1H labeling and immuno-detection of the methylarginine residue of which Arg236 is mapped as the methylated residue by CARM1. Though CARM1 was so far reported to reside predominantly in the cell nuclei, in PC12 cells CARM1 distribution ranges from the nuclei to the cytoplasm, including the cell peripheries, and CARM1 is colocalized with HuD in the cytoplasm. To examine the biological significance of HuD methylation by CARM1, we established CARM1-depleted PC12 cell lines and investigated the effect of CARM1 loss on HuD-regulated gene expression. In a series of CARM1-depleted cell lines, methylated HuD was completely lost, with the total HuD level being unchanged, and the p21^cip1/waf1 protein levels was remarkably increased compared with levels in parental PC12 cells. Further, we demonstrated that unmethylated HuD bound more p21^cip1/waf1 mRNA than did methylated HuD and led to prolongation of p21^cip1/waf1 mRNA half-life. This phenomenon was repro-duced in the PC12 cells overexpressing R236K methylation-resistant HuD. p21^cip1/waf1 cyclin-dependent kinase inhibitor has been shown to inhibit the proliferation of PC12 cells and accelerate neurite outgrowth in response to NGF (22, 71, 72). As anticipated, these cells exhibited a slower growth rate in the growth medium and accelerated neuritogenesis in response to NGF than did the parental and mock-transfected PC12 cells. These findings indicated that CARM1 negatively regulates neuronal differentiation of PC12 cells by methylating HuD to prevent p21^cip1/waf1 mRNA from entering into the decay pathway. The overlapped distribution of CARM1 with BrdU-positive cells in the subventricular zone of the adult mouse generalizes the inhibitory role of CARM1 for the differentiation of neural progenitor/precursor cells as well as PC12 cells.

MATERIALS AND METHODS

Chemicals and antibodies. We used the following antibodies: anti-PRMT1 monoclonal antibody (MAb) (Abcam Ltd., Cambridge, United Kingdom), anti-CARM1 polyclonal antibody (PAb) (Upstate Biotech, Charlottesville, VA), anti-PRMT3 MAb (Upstate Biotech), anti-tau MAb (Upstate Biotech), anti-PRMT1 MAb (Abcam Ltd.), anti-mono- and dimethylarginine (anti-M/DMA) MAb (Abcam Ltd.), anti-p21^cip1/waf1 MAb (Santa Cruz Biotech, Inc., Santa Cruz, CA), anti-GAP43 PAb (Santa Cruz Biotech), rabbit anti-HuD PAb (Santa Cruz Biotech), goat anti-HuD PAb (Santa Cruz Biotech), anti-β-tubulin PAb (Santa Cruz Biotech), anti-hemagglutinin (HA) MAb (Sigma-Aldrich, St. Louis, MO), anti-actin MAb (C4; Chemicon International Inc., Temecula, CA), Alexa Fluor 488-conjugated anti-mouse/rabbit immunoglobulin G (IgG) antibody (Molecular Probes Inc., Eugene, OR), and horseradish peroxidase (HRP)-conjugated anti-mouse/rabbit immunoglobulin G (IgG) antibody (Cell Signaling Tech., Beverly, MA). Recombinant histone H3 and H4 proteins were purchased from Upstate Biotech. The reagents used in this work were NGF (Upstate Biotech) and actinomycin D (Sigma-Aldrich). Recombinant His6-CARM1 was generated by insertion of the sense and antisense stem region of CARM1 shRNA which corresponds to 229 to 247 nucleotides with 4% paraformaldehyde. The brains were postfixed overnight in the same

Immunocytochemistry. PC12 cells were fixed in 4% formaldehyde, permeabilized, and blocked in 0.02 M PBS containing 0.3% Triton X-100 and 5% bovine serum albumin (BSA) for 30 min at room temperature (RT). They were incubated overnight at 4°C with anti-CARM1 antibody (1:100) and anti-HuD antibody (1:100). For fluorescence immunocytochemistry, the preparations were incubated for 1 h with Alexa Fluor 488-labeled goat anti-rabbit IgG antibody (Molecular Probes Inc.). Tetramethyl rhodamine isothiocyanate-labeled phalloidin (1:100; Sigma-Aldrich) was used to detect F-actin in PC12 cells. When necessary, DAPI (4,6-diamidino-2-phenylindole) (Wako Pure Chemical Industries, Osaka, Japan) was used to stain the nuclei. Samples were examined under a confocal laser-scanning microscope (Zeiss Axioplan2 LSM510). Enzyme immunodetection was conducted with a Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) by following the manufacturer’s instructions. Anti-CARM1 antibody (RayBiotech) was incubated for 12 h at 4°C and washed with 1% bovine serum albumin-TBS (pH 7.5), and then the secondary antibody (goat anti-rabbit IgG–H+L, Molecular Probes Inc.) was incubated for 90 min at RT. After the washing steps, the preparations were incubated with the nuclear-specific dye DAPI (0.1 μg/ml) for 30 min at RT. The preparations were then washed with phosphate-buffered saline (PBS) (50 μg/g of body weight), and 2 h later they were deeply anesthetized.

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histone H4 (3 μg each) was incubated with 1 μg of His-PRMTTs in the presence of 1μCi of [3H]AdoMet. As reported, histone H4 was methylated chiefly by His6-PRMT1 (left panel, lanes 1 and 2) and histone H3 predominantly by His6-CARM1 (left panel, lanes 3 and 4). GST-HuDwt was a specific substrate for His6-CARM1, not His6-PRMT1 (left panel, lanes 5 and 6). The methylated band indicated by an arrow was a histone H3 dimer. The right panel shows Coomassie staining of the gel identical to that shown in the left panel. Each of the substrates (asterisks) and enzymes (arrowheads) are indicated. Lane M is a molecular size marker (in kilodaltons). (B) In vitro methylation assay of GST-HuD mutants. GST-HuD, -R236K, -R238K, and GST alone were methylated by His6-PRMT1 or His6-CARM1. GST-HuD, histone H3, or GST-R238K to similar extents (left panel, lanes 2 and 4, arrowheads), while GST-R236K harboring a mutation at R236 (corresponding to R237 of HuR) was not 3H-labeled by CARM1 (left panel, lane 3). The faster-migrating bands are presumed to be degradative products of the HuD portion. GST alone was incubated with CARM1. 3H-labeled bands were found in GST-HuDwt and GST-R238K to similar extents (left panel, lanes 2 and 4, arrowheads), while GST-R236K harboring a mutation at R236 (corresponding to R237 of HuR) was not 3H labeled by CARM1 (left panel, lane 3). The faster-migrating bands are presumed to be degradative products of the HuD portion. GST alone was also found to undergo methylation (left panel, lane 1). Input of the recombinant proteins was verified by Coomassie staining of the gel shown in the left panel (right panel). GST and GST-tagged proteins are indicated by asterisks and CARM1 by the arrow. (C) CARM1-methylated GST-HuD was blotted with anti-M/DMA. GST-HuDwt and GST-R236K were incubated with recombinant CARM1 (left panel, lanes 3 and 4) and PRMT1 (left panel, lane 5). Input of GST-HuD proteins was verified by Coomassie staining (right panel). (D) HDu harboring the R236K mutation was methylation defective. Cell extracts from HA-HuD- and HA-R236K-expressing PC12 cells were immunoprecipitated with anti-HA antibody, and the precipitated proteins were analyzed by immunoblotting with anti-M/DMA antibody (upper panel) or anti-HA antibody (lower panel). Only HA-HuD was detectable with anti-HA antibody, while HA-R236K was not, as expected.

FIG. 1. CARM1-methylated Arg236 of HuD in vitro. (A) Recombinant histones H3 and H4 and GST-tagged HuDwt were methylated by either His6-PRMT1 or His6-CARM1. GST-HuD, histone H3, or GST-R238K to similar extents (left panel, lanes 2 and 4, arrowheads), while GST-R236K harboring a mutation at R236 (corresponding to R237 of HuR) was not 3H-labeled by CARM1 (left panel, lane 3). The faster-migrating bands are presumed to be degradative products of the HuD portion. GST alone was also incubated with CARM1. 3H-labeled bands were found in GST-HuDwt and GST-R238K to similar extents (left panel, lanes 2 and 4, arrowheads), while GST-R236K harboring a mutation at R236 (corresponding to R237 of HuR) was not 3H labeled by CARM1 (left panel, lane 3). The faster-migrating bands are presumed to be degradative products of the HuD portion. GST alone was also found to undergo methylation (left panel, lane 1). Input of the recombinant proteins was verified by Coomassie staining of the gel shown in the left panel (right panel). GST and GST-tagged proteins are indicated by asterisks and CARM1 by the arrow. (C) CARM1-methylated GST-HuD was blotted with anti-M/DMA. GST-HuDwt and GST-R236K were incubated with recombinant CARM1 (left panel, lanes 3 and 4) and PRMT1 (left panel, lane 5). Input of GST-HuD proteins was verified by Coomassie staining (right panel). (D) HDu harboring the R236K mutation was methylation defective. Cell extracts from HA-HuD- and HA-R236K-expressing PC12 cells were immunoprecipitated with anti-HA antibody, and the precipitated proteins were analyzed by immunoblotting with anti-M/DMA antibody (upper panel) or anti-HA antibody (lower panel). Only HA-HuD was detectable with anti-HA antibody, while HA-R236K was not, as expected.
Analysis of mRNA stability. For the mRNA half-life assay, actinomycin D (Sigma-Aldrich) was added to media at 3 μg/ml of the final concentration, which was designated as 0 h. Total RNA was extracted by Iogen reagent (Nippon Gene Co., Ltd., Tokyo, Japan) at each time point and used for Northern blot analysis. The signal of p21\(^{\text{cip1/waf1}}\) mRNA at each time point was then evaluated by a densitometric program and normalized to that of GAPDH mRNA. The values were plotted on a logarithmic scale, and the time period required for the densitometric values to undergo a reduction to one-half of the initial abundance was calculated.

Nuclear run-on assay. Assays were performed as described previously (21), with minor modifications. Briefly, 5 μg of PCR-amplified DNAs corresponding to the indicated genes (enhanced green fluorescent protein, p21\(^{\text{cip1/waf1}}\), GAPDH, and GAPDH) was denatured and blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nuclei prepared from 2 × 10\(^5\) PC12 cells were isolated from each treatment group by using lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 0.5% NP-40, pH 7.4), and nascent mRNAs were labeled in vitro transcription buffer (100 mM KCl, 2.5 mM MgCl\(_2\), 2.5 mM rATP, 2.5 mM rGTP, 2.5 mM rUTP) containing 500 μCi [\(\alpha\)-\(32\)P]UTP (Amersham Bioscience Corp.) for 30 min at 30°C. Radiolabeled RNA was then isolated using Iogen reagent (Nippon Gene Co., Ltd.) and hybridized onto the blotted membrane as shown for the Northern blot analysis.

IP followed by RT-PCR. Fifty-percent-confluent PC12 cells plated on a 15-cm-diameter plastic dish were transiently transfected with 15 μg of pC-HuDwt and pC-R236K by using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, the cells were incubated for 48 h with or without NGF and lysed by immunoprecipitation (IP) buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 1.5 mM MgCl\(_2\), 250 mM KCl, 2 mM DTT, 1 mM EDTA, 1% Triton X-100, and 1,000 U/ml RNase inhibitor, plus protease inhibitor tablet, pH 7.4). HA-tagged proteins were immunopurified by incubation for 2 h at 4°C with protein G Sepharose beads (Amersham Bioscience Corp.) that had been pre-coated with 20 μg of either IgG or anti-HA. The immunoprecipitates were washed five times with NT2 buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1 mM MgCl\(_2\), and 0.05% NP-40, pH 7.4). The RNAs in the precipitates were extracted using the Iogen reagent and reverse transcribed by Ready-To-Go RNA Isolation kit (Amersham Bioscience Corp.) for 30 min at 30°C. Radiolabeled RNA was then isolated using Iogen reagent (Nippon Gene Co., Ltd.) and hybridized onto the blotted membrane as shown for the Northern blot analysis.

Recombinant protein. Escherichia coli BL21 cells (Novagen, Madison, WI) were transformed by pCold-PRMT1, while JM110 dam methylase-deficient cells (Toyobo, Osaka, Japan) were transformed by the pCold-CARM1 vector. The transformed cells were propagated at 37°C up to an optical density at 280 nm of 0.5, followed by an incubation of 15 h with vigorous agitation in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The collected cells were sonicated in lysis buffer (50 mM Tris-HCl, 250 mM NaCl, 10 mM guanidine containing 10 mg/ml lysozyme, pH 8.0), sonicated at 12,000 rpm for 20 min. The supernatants were incubated with Ni-nitrilotriacetic acid His-bind resin (Novagen) for 6 h at 4°C. The gels were washed four times in wash buffer (50 mM Tris-HCl, 250 mM NaCl, 20 mM guanidine, pH 8.0) and eluted by elution buffer (50 mM Tris-HCl, 250 mM NaCl, 100 mM guanidine, pH 8.0). The BL21 strains transformed with pcGEX-HuDwt, R236K, and R238K were cultured on a large scale at 37°C in the presence of 1 mM IPTG. The collected cells were sonicated in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl\(_2\), 1 mM DTT, 1 mM EDTA, 1% Triton X-100, plus protease inhibitor cocktail, pH 7.5), and centrifuged at 12,000 × g for 20 min. The supernatants were used for in vitro transcription.

CARM1, PRMT1, PRMT3, and actin. CARM1 shRNA did not affect PRMT1 and PRMT3 protein levels (right column). (C) Endogenous HuD was arginine methylated in the native PC12 cells but not in the CARM1- cells. Whole-cell extracts from parental and CARM1- PC12 cells were loaded directly (lower panel) or immunoprecipitated with either anti-M/DMAP antibody or control IgG (upper panel). These samples were analyzed by immunoblotting with anti-HuD antibody. Anti-M/DMAP antibody precipitated endogenous HuD from mock-transfected PC12 cell lysate (lane 2) but not from the lysate of CARM1- cell line 15 (lane 3). The fraction precipitated with control IgG1 contained no HuD immunoreactivity (lane 1). CTR, control empty vector-expressing cells; siRNA, CARM1 shRNA-expressing cells.
were incubated with glutathione Sepharose (Amersham Bioscience Corp.) for 6 h, washed four times with PBS containing 0.5% Triton X-100, and eluted by 20 mM reduced glutathione (Nacalai Tesque, Kyoto, Japan) in 50 mM Tris-HCl (pH 8.0).

In vitro protein methylation assay. In vitro methylation of recombinant wild-type HuD and mutated forms (R236K and R238K) tagged with glutathione S-transferase (GST) was performed as follows. A total of 3 μg of each substrate (H3 histone, H4 histone, GST-tagged wild-type HuD [GST-HuDwt], GST-R236K, and GST-R238K) was incubated with 1 μg of His$_6$-tagged PRMT1 or CARM1 in reaction buffer (50 mM Tris-HCl, 1 mM DTT, pH 7.4) in the presence of 1 μCi of [$^3$H]Adenosylmethionine (AdoMet) (Amersham Bioscience Corp.) for 1 h at 37°C. For the cold assay, the reactions were performed in reaction buffer containing 10 mM AdoMet (Sigma-Aldrich).

RESULTS

HuD undergoes in vitro and in vivo arginine methylation by CARM1. There is a poor understanding of the mechanism by which HuD is regulated to antagonize ARE-mediated mRNA decay. Li et al. reported that HuR, one of the Hu family proteins, is methylated specifically by CARM1 in vitro and in vivo (41). The methylated arginine residue of HuR and the surrounding amino acid sequence are conserved among all other Hu proteins (28, 52). Accordingly, we examined whether HuD might also be methylated by CARM1 at its homologous arginine residue. GST-HuDwt was incubated with recombinant His$_6$-tagged PRMT1 and CARM1 in the presence of [$^3$H]AdoMet. $^3$H incorporation was observed with GST-HuDwt incubated with CARM1 (Fig. 1A, lane 6) but not with PRMT1, which is predominantly responsible for the type I arginine methylation in mammalian cells (Fig. 1A, lane 5) (65). To confirm the activities of the prepared His$_6$-tagged CARM1 and PRMT1, we incubated them with their respective known substrates, histones H4 and H3 (4, 44, 62). Both of the recombinant enzymes were competent to incorporate $^3$H into their specific substrates (Fig. 1A, lanes 1 to 4). The $^3$H-labeled, slower-migrating band of the histone H3 lane on the fluorographic image was presumably its previously reported dimerized product (Fig. 1A, lane 4) (41). To map the CARM1-methylated arginine residue on HuD, we performed mutational analysis of recombinant HuD. Since Arg$^{236}$ of HuDwt corresponds to arginine-methylated Arg$^{217}$ of HuR (41), we replaced either Arg$^{236}$ or Arg$^{238}$ of GST-HuD with a lysine residue (R236K and R238K). As anticipated, the Arg$^{236}$ mutation exhibited methylation resistance to CARM1 activity against HuDwt (Fig. 1B, lane 3), whereas the Arg$^{238}$ mutation was methylated to a level comparable to that for HuDwt (Fig. 1B, lane 4). It is important to note that the HuD (R236K) mutant still contains many other arginine residues. GST alone did not undergo any modification by CARM1 (Fig. 1B, lane 1), demonstrating that CARM1 action was targeted to the HuD portion of the fusion recombinant protein. Therefore, only the Arg$^{236}$ residue serves as a target for CARM1-mediated arginine methylation.

To further examine whether the $^3$H-labeled proteins are derived from arginine dimethylation by CARM1, we performed Western blot analysis on the in vitro methylated products. Anti-M/DMA antibody recognized GST-HuDwt incubated with CARM1 (Fig. 1C, lane 3), but faint immunoreactivity was observed with CARM1-incubated GST-R236K (Fig. 1C, lane 4). This was comparable to results with GST-HuDwt incubated with PRMT1 (Fig. 1C, lane 5), which is presumed to be a nonspecific signal. Anti-
FIG. 4. CARM1 depletion in PC12 cells exclusively induces p21cip1/waf1 expression. (A) Protein expression patterns of HuD-regulating genes in the wild-type (W), mock-transfected (M), and CARM1−/− PC12 cells, which were all cultured under the same growth condition. The whole-cell lysates of these cells were blotted with anti-CARM1, -PRMT1, -PRMT3, -p21cip1/waf1, -GAP43, -tau, -p27, and -actin antibodies. Note that p21cip1/waf1 protein levels significantly increased in CARM1−/− clones 15, 16, and 33. These cell lines also represented a slight increment of basal protein level of GAP43. GAPDH served to monitor equal loading and transfer of samples. (B) Effects of exogenous HA-HuD and HA-R236K on inducing p21cip1/waf1 expression. PC12 cells were transiently transfected with HA-HuD or HA-R236K, and total lysates were blotted with anti-p21cip1/waf1, -GAP43, and -actin antibodies. The p21cip1/waf1 protein level was significantly increased in HA-R236K-expressing cells (top panel, lane 3). The same levels of HA-tagged proteins were monitored by anti-HA blotting. (C) Upregulation of p21cip1/waf1 mRNA in CARM1−/−
asymmetric dimethylarginine antibody, which specifically recognizes asymmetrically methylated arginine residues on the Sam68 RGG repeat (18), did not detect the methylated GST-HuD (data not shown). These results suggested that the observed [3H] incorporation to GST-fused HuD by CARM1 was attributable to the methylation of Arg236 of HuD, but we could not determine whether the detected dimethylarginine residue was mono- or dimethylated and, if dimethylated, whether it was symmetrically or asymmetrically methylated.

To reconfirm Arg236 of HuD as a methyltransferase target in PC12 cells, HA-tagged HuD harboring the Arg236-to-Lys mutation (HA-R236K) was transiently expressed in the cells and immunoprecipitated with an anti-M/ADMA antibody. The precipitated proteins from HA-tagged HuDwt (HA-HuDwt)-expressing cells were blotted with anti-HA antibody, while no immunoreactivity was detected with the HA-R236K-expressing cells (Fig. 1D). This result demonstrated that Arg236 of HuD is a major methylation site by CARM1 in PC12 cells.

To determine whether endogenous HuD is methylated by CARM1 in vivo, we established PC12 cell clones in which endogenous CARM1 expression was stably suppressed by RNA interference. Reduction of CARM1 in the stable cell lines was confirmed by immunocytochemistry (Fig. 2A) and Western blot analysis (Fig. 2B). In these clones, CARM1 expression was completely extinct, with PRMT1 and PRMT3 levels being unaffected (Fig. 2B). By use of immunocytochemistry, the dense staining in the nuclei of the parent PC12 cells was abolished in all cell lines, though faint immunoreactivities in the cytoplasm remained (Fig. 2A, panel c). The cytoplasmic CARM1 immunoreactivities are discussed below, because the observation that CARM1 resides in the cytoplasm of PC12 cells as well as in the nuclei (Fig. 2A, panels a and c) argues against previous studies demonstrating its nuclear localization (24, 70). To see whether CARM1 loss affects methylation of HuD in PC12 cells, precipitated HuD with anti-M/ADMA antibody from CARM1−/− cells was compared with that from native PC12 cells. Though the total HuD level in the CARM1−/− cells was equivalent to that in the native cells (Fig. 2C, lower panel), HuD immunoreactivity precipitated with anti-M/ADMA antibody from the CARM1−/− cells was significantly decreased, comparable to that precipitated with the control mouse IgG (Fig. 2C, lane 3).

Subcellular localization of CARM1 in PC12 cells. In terms of subcellular localization, previous reports showed that CARM1 was localized predominantly in the nuclei of HeLa cells (24) and mouse embryonic fibroblast cells (70). In spite of no evidence for the cytoplasmic existence of CARM1, its so-far-reported substrates, PABP1 and TARPP, are both cytoplasmic proteins (2, 35), and HuD also resides in the cytoplasm of PC12 cells and induces neuronal differentiation (31, 32). A recent report demonstrated that in C2C12 myoblasts CARM1 localizes not only in the nuclei but also in the sarcoplasm (13).

To consolidate the subcellular localization of CARM1 in PC12 cells, immunocytochemistry was done with HRP-diaminobenzidine tetrahydrochloride (DAB) and fluorescence-labeling methods. In HeLa cells, immunoreactivity was restricted to the nuclei by both methods, as previously reported, with almost no significant staining in the cytoplasm (Fig. 3A, left panels). In contrast, in the PC12 cells, unambiguous staining was identified in the cytoplasm as well as in the nuclei by both methods (Fig. 3A, middle panels). To confirm the specificity of the immunoreactivity, anti-CARM1 antiserum that was adsorbed with either recombinant GST-CARM1 or GST was applied to the PC12 cell preparations. DAB-stained signal with anti-CARM1 antibody was absorbed with GST-CARM1 (Fig. 3A, right panels) but not with GST (data not shown) in HeLa cells and PC12 cells, ruling out the possibility that the cytoplasmic staining was due to artifacts.

We further examined whether HuD and CARM1 colocalize in the cytoplasm of PC12 cells. As shown in Fig. 3B, HuD and CARM1 immunoreactivities overlapped in the cytoplasm, especially in the region centered around the nuclei rather than in the cell peripheries (Fig. 3B, panels a to c). When the cells were immunostained with another anti-HuD antibody, the co-localization was reproduced (Fig. 3C). When the CARM1−/− cells were immunostained, weak cytoplasmic immunoreactivity of CARM1, which did not overlap with that of HuD, was observed (Fig. 3B, panels d and f). Since the subcellular localization of HuD was not affected in the presence or absence of CARM1, Arg236 methylation of HuD does not act on its subcellular localization (Fig. 3B, compare panels b and c).

CARM1 depletion induces p21\(^{cip1/waf1}\) expression by prolongation of the mRNA half-life. To elucidate the functional significance of HuD methylation, we examined the effect of unmethylated HuD on the growth and differentiation state of PC12 cells by using CARM1-depleted clones (no. 15, 16, and 33). We first analyzed the protein levels of p21\(^{cip1/waf1}\), GAP43, tau, and p27, whose mRNA half-lives are regulated by HuD. The lysates from mock-transfected cells and three methylation-defective clones cultured in the growth media were blotted with each of the above antibodies. Inhibited methylation of HuD had no effect on tau and p27 expression levels (Fig. 4A).
GAP43 expression level did not change in two clones but was significantly increased in clone no. 33 (Fig. 4A). However, p21cip1/waf1 protein levels dramatically increased in all of the CARM1-depleted clones (Fig. 4A). To examine whether it was the unmethylated population of HuD that induced p21cip1/waf1 expression, we biased the total population of HuD toward the unmethylated population by overexpressing methylation-resistant HuD (HA-R236K) in the native PC12 cells. The overexpression of HA-R236K considerably promoted p21cip1/waf1 expression compared with the level achieved with HA-HuDwt (Fig. 4B, top panel). However, GAP43 protein levels were unchanged in the HA-R236K- and HA-HuDwt-expressing cells (Fig. 4B). Thus, the relative increases in the unmethylated population of HuD by two different methods induced a common effect on p21 protein expression. Therefore, we next explored how unmethylated HuD induces the rises in p21cip1/waf1 protein.

When the transcript levels of p21cip1/waf1, GAP43, and tau were compared between mock-transfected and CARM1− cell line no. 15, only p21cip1/waf1 mRNA had a 3.26-fold increase in clone no. 15 cultured under the growth condition (Fig. 4C, top panel, compare left and middle lanes). Other transcripts examined were not so affected by the loss of CARM1 (Fig. 4C), while NGF administration upregulated all three of the transcripts to the same extent (Fig. 4C, compare the left and right lanes in each panel). To examine if the induction of p21cip1/waf1 mRNA in CARM1− cells resulted from transactivation of the gene, we performed a nuclear run-on assay. The nuclear extract from CARM1− cells labeled an amount of p21cip1/waf1 transcript comparable to (or rather less than) that from the parental cell (Fig. 4D, compare left and middle panels), which was different from the case of the NGF-treated native cells, whose nuclear extract could activate the transcription of both of the genes (Fig. 4D, right panel). This result raised the possibility that the rise in basal p21cip1/waf1 transcript level could be due to an enhancement of the protective effect of HuD on the transcript, not to its transactivation.

Given that HuD was shown to bind to p21cip1/waf1 mRNA and protect it from ARE-mediated decay, we next assayed the decay rate of p21cip1/waf1 transcript under the condition where the total transcription level was halted by actinomycin D. In the parental cell line, NGF treatment markedly elongated the half-life of p21cip1/waf1 mRNA from an apparent half-life of 2.4 h for the untreated group to that of 8.7 h for the NGF-treated group (Fig. 4E, top and bottom panels, and F). As expected, the CARM1-defective condition elongated the average half-life to 8.5 h, similarly to the NGF treatment (Fig. 4E, middle panel, and F). These observations indicated that the propagation of unmethylated HuD in CARM1− cells can protect p21cip1/waf1 mRNA from the degradation pathway to elevate its expression.

Methylation state of HuD regulates its complex formation with p21cip1/waf1 mRNA. We further tested whether an RNA-protein complex formation between HuD and p21cip1/waf1 transcript would be regulated in a methylation-dependent manner. The association with PC12 cells was analyzed by PCR-based detection of the p21cip1/waf1 mRNA in immunoprecipitated HuD. Template RNA for RT-PCR was extracted from the precipitated fractions with anti-HA antibody from either HA-HuDwt- or HA-R236K-expressing cells. The densitometric values of the amplified products of p21cip1/waf1 were normalized to those of GAPDH. No amplified product was gained from the precipitated mRNA with control IgG1 from HA-HuD-expressing cells. Control IgG1 did not yield the amplified product of p21cip1/waf1 (top panel, lane 1) from HA-HuD-expressing cells. HA-R236K precipitated with anti-HA antibody yielded 5.8 times more PCR product of p21cip1/waf1 than HA-HuD (top panel, lanes 2 and 3). Differences (n-fold) are indicated between the blots. NGF treatment propagated the amplified product of p21cip1/waf1 from HA-HuD to the extent of HA-R236K (top panel, lane 4). Background detection of GAPDH-amplified product from each of the precipitated materials served to monitor equal use of material in each IP (middle panel). (B) NGF downregulated the methylated population of HuD. Whole-cell extracts from the untreated PC12 cells and the cells treated with NGF for 3 days were loaded directly (lanes 3 and 4) or subjected to IP with anti-M/DMA antibody (lanes 1 and 2), and they were blotted with anti-HuD antibody. Antiactin antibody blotting of the total lysates confirmed the same protein load (lanes 5 and 6).

FIG. 5. Unmethylated HuD exhibited a higher binding capacity for p21cip1/waf1 mRNA. (A) Detection by RT-PCR of p21 mRNA in materials that were precipitated with anti-HA antibody from HA-HuD- and HA-R236K-expressing cells. Control IgG1 did not yield the amplified product of p21cip1/waf1 (top panel, lane 1) from HA-HuD-expressing cells. HA-R236K precipitated with anti-HA antibody yielded 5.8 times more PCR product of p21cip1/waf1 than HA-HuD (top panel, lanes 2 and 3). Differences (n-fold) are indicated between the blots. NGF treatment propagated the amplified product of p21cip1/waf1 from HA-HuD to the extent of HA-R236K (top panel, lane 4). Background detection of GAPDH-amplified product from each of the precipitated materials served to monitor equal use of material in each IP (middle panel). (B) NGF downregulated the methylated population of HuD. Whole-cell extracts from the untreated PC12 cells and the cells treated with NGF for 3 days were loaded directly (lanes 3 and 4) or subjected to IP with anti-M/DMA antibody (lanes 1 and 2), and they were blotted with anti-HuD antibody. Antiactin antibody blotting of the total lysates confirmed the same protein load (lanes 5 and 6).
p21cip1/waf1 mRNA than did the wild type. Interestingly, when HA-HuD-expressing cells were treated with NGF, p21cip1/waf1 was amplified from the HA-precipitated mRNA to a level comparable to that for HA-R236K-expressing cells (Fig. 5A, lane 4). Then we investigated how the in vivo methylation state of endogenous HuD alters during the course of NGF-induced differentiation of PC12 cells. At 3 days after NGF treatment, HuD protein precipitated with anti-M/DMA antibody was extinguished, with the total HuD level almost unchanged (Fig. 5B). These data indicated that NGF may reduce the Arg236 methylation level of HuD during neuronal differentiation to enhance the direct or indirect interaction of HuD with p21cip1/waf1 mRNA.

Inhibited methylation of HuD decreases growth rate and facilitates neurite outgrowth following NGF treatment. To characterize the phenotype of CARM1 cells, we compared these cells with the wild-type and mock-transfected cells in growth and neuritogenic activity. When we first examined the growth rates of CARM1 clones, they all exhibited much slower proliferative rates than did wild-type and mock-transfected clones (P < 0.01) (Fig. 6A). In addition, CARM1 clones had a tendency to extend their neurites in response to NGF. Some of the CARM1 clones extended neurites with lengths more than twice the diameter of their cell body, even in the growth media (Fig. 6B and C). In the case of the parental PC12 clone, less than 10% of the cells displayed prominent neurite outgrowth 12 to 24 h after NGF treatment (Fig. 6B). By contrast, about 12 and 31% of CARM1 cells developed neurites at 12 h and 24 h after NGF administration, respectively (Fig. 6B and C). The optimal neurite lengths of CARM1 clones at 48 h after NGF administration were larger than those of parental cells at 72 h, which amounted to the average lengths of CARM1 clones at 24 h. Together with the fact that p21cip1/waf1 can inhibit proliferation and accelerate neurite outgrowth in response to NGF (72), it is possible that the lowered level of methylated HuD in the CARM1 cells can promote p21cip1/waf1 expression by the protective effect of unmethylated HuD on its transcripts to make a differentiated phenotype for the cells.

CARM1 in the adult mouse brain partially colocalizes with BrdU-positive cells. The above-described data raised the possibility that CARM1 maintains the cells in the proliferative state. To investigate the correlation between CARM1 and cell proliferation, BrdU-labeled adult mouse brain was doubly stained with anti-CARM1 and anti-BrdU antibodies. While BrdU-positive cells were densely distributed along the ventric-

FIG. 6. Effects of CARM1 loss on proliferation and NGF-induced neurite outgrowth. (A) Comparison of levels of proliferation in parent, mock-transfected, and CARM1-depleted cells. PC12 cells were plated on 6-well dishes at a density of 1 × 10⁵ cells per well. The cells were then cultured in growth medium and counted by a hemocytometer at every 24-h interval. Cell numbers represent the mean values from triplicate experiments. CARM1-defective cells (no. 15, 16, and 33) showed slower growth rates than parental and mock-transfected cells. (B) CARM1 depletion promoted a susceptibility to NGF-induced neurite outgrowth. Parental PC12 cells and CARM1 cells were plated on collagen-coated, 4-well-chamber slides at a density of 1 × 10⁴ cells per well. Microscopic images of the parent and CARM1 PC12 cells (no. 15 and 33) are shown. Cultures were fixed at the indicated times after the onset of NGF exposure and stained with tetramethyl rhodamine isothiocyanate-labeled phalloidin (red). (C) Quantification of the results shown in panel B. The cells that had at least one neurite with a length covering two cell bodies in diameter was counted as neurite bearing. The error bars represent standard deviations from triplicate results. Approximately 200 cells were counted for each sample.
ular zone (VZ) (Fig. 7b), CARM1 distribution ranged from the VZ to the ventricular border of the subventricular zone (Fig. 7a), in which the arrested cells start radial migration from the VZ. More than 60% of BrdU-positive cells displayed CARM1 immunoreactivity (Fig. 7c) in the VZ, suggesting that most of the CARM1-expressing cells have the competence to proliferate. Thus, CARM1 was involved in the proliferation of neural cell precursors.

**DISCUSSION**

Aletta’s group reported that protein methyltransferase inhibitors completely blocked NGF-induced neurite extension of PC12 cells without affecting their viability and proliferative activity and that the required methyltransferase activity for neurite extension might be due chiefly to asymmetric arginine dimethylation and not to methylation of nucleotides or other amino acid residues (16, 17). Among the substrates for arginine methyltransferases, we noted RBPs, some of which proved to be involved in neuronal differentiation. Fortunately, one of the RBPs, HuR, was shown to be methylated in vitro and in vivo by CARM1 arginine methyltransferase (41). HuR belongs to the Hu/ELAV RBP family and regulates ARE-mediated mRNA decay by competing with AUF decay-promoting factors for ARE-containing mRNA (38, 63). The alignment of the amino acid sequence around the dimethylated arginine residue of HuR displays a high sequence homology with that of the other mammalian Hu members (28, 52). Then we focused on a neuron-specific Hu family protein, HuD, as the methylated amino acid residues (16, 17). Among the substrates for arginine methyltransferases, we noted RBPs, some of which proved to be involved in neuronal differentiation. Fortunately, one of the RBPs, HuR, was shown to be methylated in vitro and in vivo by CARM1 arginine methyltransferase (41). HuR belongs to the Hu/ELAV RBP family and regulates ARE-mediated mRNA decay by competing with AUF decay-promoting factors for ARE-containing mRNA (38, 63). The alignment of the amino acid sequence around the dimethylated arginine residue of HuR displays a high sequence homology with that of the other mammalian Hu members (28, 52). Then we focused on a neuron-specific Hu family protein, HuD, as the methylated substrate and examined whether or how its methylation regulates the turnover of the bound mRNAs in PC12 cells.

Using an in vitro protein methylation assay, we showed that HuD is a specific substrate for CARM1. [3H]-labeled HuD was blotted with an antibody against mono- and dimethylarginine, which was used to demonstrate in vivo methylation of HuR (41). The anti-M/DNA antibody immunoprecipitated endogenous HuD from the lysate of native PC12 cells but not that of CARM1- cell lines that we established (Fig. 2C). It also precipitated exogenous HuD from HuD WT-transfected PC12 cell lysate but not that from R236K-transfected cells (Fig. 1D). These data demonstrate that Arg236 is a major CARM1 methylation site, both in vitro and in vivo, which corresponds to Arg227 of HuR. Since the antibody towards methylarginine residues used in this study was incapable of sufficiently identi-
transcript level and decay rate (Fig. 4A, C, and E). We inferred that RNP complex components other than HuD might be different among p21cip1/waf1 and GAP43 transcripts and that those of p21cip1/waf1 are influenced primarily by methylation of HuD. Since the methylation state of HuD could possibly affect the turnover of unknown transcripts, we attempted to make a catalogue of transcripts with differential binding characters to methylated and unmethylated HuD by use of a microarray-based method.

To investigate whether HuD is the sole mediator for producing such gene expression patterns in CARM1− cells, we introduced an excess amount of the methylation-resistant HuD mutant R236K into native PC12 cells (Fig. 4B). The overexpression of naked HuD remarkably induced p21cip1/waf1 protein without affecting the GAP43 protein level, demonstrating depletion (Fig. 4B). Note that CARM1 different among p21cip1/waf1 and GAP43 transcripts and that RNP complex components other than HuD might be transcript level and decay rate (Fig. 4A, C, and E). We inferred that NGF-induced p21cip1/waf1 mRNA may be one of the major reasons that methylated HuD was extinct 3 days after NGF treatment with PC12 cells, even when total HuD was kept at the steady-state level (Fig. 5B). This observation indicated that NGF could produce the same effect on HuD as CARM1 depletion, in keeping with our result that NGF induced p21cip1/waf1 transcript to an extent similar to that induced by CARM1 depletion (Fig. 4B). Note that CARM1− cells exhibited a slow growth rate (Fig. 6A) and accelerated neurite extension (Fig. 6B and C), which also occurs in the NGF-treated PC12 cells; thus, it is possible that the loss of methylated HuD and the resultant rise in p21cip1/waf1 mRNA may be one of the major processes for the NGF signaling pathway. To explain how NGF decreases the methylated population of HuD with the total amount being unchanged, we assume that, once methylated, HuD is enzymatically demethylated at the onset of NGF signaling. Recently, monomethyl-arginine residues on H3 and H4 histone proteins were shown to be methyl-deaminated to citrulline by protein arginine demethylase 4 (PADI4) (19, 68). However, since this kind of reaction has been shown to act on monomethylatedarginine, a novel system might be required for elimination or oxidation of the methyl group from arginine-methylated HuD. Altogether, the methylated state of HuD determines the p21cip1/waf1 expression level, and the inhibited methyltransferase activity of CARM1 can arrest the growth of PC12 cells to represent a partially differentiated cell shape by propagating unmethylated HuD. Accordingly, our results are inconsistent with previous reports that protein arginine methylation is required for NGF-induced differentiation of PC12 cells (16). In fact, these reports evaluated the whole effect of protein methylation by using methyltransferase inhibitors with broad spectra, whereas we focused on the effect of selective inhibition of CARM1. Compared with PRMT1, CARM1 activity covers a limited species of proteins (39). This is why our results are discrepant with the general inhibition of arginine methylation that aborted NGF-induced neuritogenesis of PC12.

In conclusion, our data demonstrated that CARM1 methylates Arg56 of HuD in vitro and in vivo and that the methylation of HuD raised the vulnerability of p21cip1/waf1 transcripts to direct PC12 cells to the proliferative state, whereas unmethylated HuD endowed the cells with differentiated phenotypes in an NGF-independent manner. Our final observation, that CARM1-expressing cells were localized in the VZ of the lateral ventricle of the adult mouse brain and overlapped with BrdU-positive cells (Fig. 7), supports the notion that CARM1 keeps the cells in the proliferative state.

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