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Identification of Amino-Terminal Region of Adiponectin as a Physiologically Functional Domain

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Abstract Adiponectin is an abundant adipose-specific protein, which acts as an anti-diabetic, anti-atherogenic, and anti-inflammatory adipokine. Although recent advances in the field of adiponectin have been made by the identification of adiponectin receptors and by the understanding about relationship between its multimerization and functions, detailed molecular background remains unclear. Our established anti-human adiponectin antibodies, ANOC 9103 and ANOC 9104, blocked some adiponectin functions such as the growth inhibition of B-lymphocytes on stromal cells and the inhibition of acetylated LDL uptake in macrophages, suggesting that they may recognize important functional regions of adiponectin. As a result of epitope mapping based on the ability to bind to the deleted adiponectin mutants, we identified that these antibodies recognize amino-terminal region of adiponectin before the beginning of the collagen-like domain. Notably, a peptide fragment (DQETTTQGPGVLLPLPKGACTGWMA) corresponding to amino acid residues 17–41 of human adiponectin could bind to restricted types of cells and block adiponectin-induced cyclooxygenase-2 gene expression and prostaglandin E_2 production in MS-5 stromal cells. Moreover, the deletion of its amino-terminal region reduced the abilities to inhibit not only collagen-induced platelet aggregation but also diet-induced hepatic steatosis. These data indicate that amino-terminal region of adiponectin is a physiologically functional domain and that a novel receptor, which recognizes amino-terminal region of adiponectin, may exist on some types of cells. Further investigations will contribute to the understanding of molecular mechanisms about adiponectin functions as well as to the designing of novel strategies for the treatment of patients with insulin-resistance, vascular dysfunction, and chronic inflammation. J. Cell. Biochem. 98: 194–207, 2006.

Key words: adiponectin; adipokine; epitope; functional domain; receptor; Cox-2

Adiponectin, also known as ACRP30 and apM1, belongs to the soluble defense collagen family, which shares homology with complement C1q as well as surfactant protein A, surfactant protein D, and mannose-binding lectin [Scherer et al., 1995; Maeda et al., 1996; Gil-Campos et al., 2004; Matsuzawa, 2005]. This molecule has four putative domains such as a signal peptide at N-terminus, a hypervariable region with no interspecific homology, a collagen-like domain, and a C-terminal globular domain. Recent solution of the adiponectin crystal structure also indicated additional high similarity to tumor necrosis factor (TNF)-α [Shapiro and Scherer, 1998]. Although adiponectin is expressed and secreted exclusively in adipose tissue, it is abundant in plasma and has anti-inflammatory, anti-atherogenic, and anti-diabetic properties [Fasshauer et al., 2004; Gil-Campos et al., 2004; Matsuzawa, 2005; Trujillo and Scherer, 2005]. Circulating adiponectin concentrations are low in human subjects and animal models with obesity and/or insulin-resistance [Arita et al., 1999; Hotta et al.,

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2000; Yamauchi et al., 2001]. Longitudinal studies analyzing rhesus monkeys have demonstrated that adiponectin levels are negatively correlated with body weight, fat content, and insulin levels as well as the decline toward diabetic state [Hotta et al., 2001]. Most importantly, adiponectin-deficient mice exhibited early onset of diet-induced insulin-resistance [Maeda et al., 2002]. On the other hand, adiponectin levels are significantly lower in patients with coronary artery diseases than in control subjects [Ouchi et al., 1999], suggesting a possible association between reduced adiponectin levels and vasculopathic states. Adiponectin was also reported to protect ob/ob obese mice and apo-E-deficient mice from developing atherosclerosis [Yamauchi et al., 2003b]. Adiponectin-deficient mice display more severe vascular neointimal thickening after vascular cuff-induced injury of an artery than wild-type mice [Matsuda et al., 2002]. In addition, adiponectin can suppress the activation of transcription factors involved in inflammation, such as nuclear factor-kappa B (NF-κB) in vascular endothelium [Ouchi et al., 2000]. This inhibitory effect of adiponectin is accompanied by the accumulation of cAMP, and is blocked by a protein kinase A (PKA) inhibitor, suggesting that adiponectin modulates inflammatory responses through cross-talk between cAMP-PKA and NF-κB pathways in endothelial cells [Ouchi et al., 2000]. Additional anti-inflammatory effects of adiponectin include the reduction of phagocytic activity and TNF-α secretion in macrophages as well as the suppression of leukocyte colony formation [Yokota et al., 2000].

Adiponectin produced in bacteria or embryonic cells forms a variety of multimers. Adiponectin transgenic mice expressing a truncated analog of adiponectin revealed that the globular domain of adiponectin not only raised lipid clearance and lipoprotein lipase activity but also enhanced insulin sensitivity [Combs et al., 2004]. In contrast, only hexameric and larger isoforms can activate NF-κB transcription factor in a manner dependent upon phosphorylation and degradation of inhibitor of nuclear factor-kappa B (IκB)-α factor [Tsao et al., 2003]. Thus, multimerization of adiponectin regulates some biological activities of adiponectin. Recently, two receptor forms for adiponectin, AdipoR1 and AdipoR2, were identified [Yamauchi et al., 2003a]. They have seven transmembrane domains although they are structurally very different from G-protein-coupled receptors. Expression or suppression of these receptors has indicated that they activate unique sets of signals, which account for the increased insulin sensitivity. In addition to AdipoR1 and AdipoR2, adiponectin can recognize several cytokines and T-cadherin [Arita et al., 2002; Hug et al., 2004; Wang et al., 2005]. Notably, adiponectin strongly suppressed proliferation and migration of human aortic smooth muscle cells through direct binding to platelet-derived growth factor-BB [Arita et al., 2002]. Thus, there are several molecular mechanisms how adiponectin delivers its biological activities.

Because of complex regulation and a wide range of biological activities, neither the physiological role of the receptors nor the signal transduction pathways have yet been fully elucidated. We have established anti-human adiponectin monoclonal antibodies (Ab), ANOC 9103 and ANOC 9104, by immunizing adiponectin-deficient mice with recombinant human adiponectin [Arita et al., 1999]. They are known to block some biological activities of adiponectin. As summarized in Table I, ANOC 9103 reverses the inhibition of the proliferation of B-lymphocyte progenitors on stromal cells by adiponectin [Yokota et al., 2003]. ANOC 9104 inhibits the reduction of leukocyte colony formation by adiponectin [Yokota et al., 2000].

TABLE I. Anti-Adiponectin Abs, ANOC 9103 and ANOC 9104, Block Some Physiological Activities of Adiponectin

Adiponectin functions, which are blocked by anti-adiponectin Abs	Abs	References
Reduction of CFU-GM[a] colony formation	ANOC 9104	Yokota et al. [2000]
Suppression of TNF-α-induced Iκ-B-α phosphorylation	ANOC 9104	Ouchi et al. [2000]
Suppression of class A macrophage scavenger receptor expression	ANOC 9104	Ouchi et al. [2001]
Growth inhibition of B-lymphocytes on stromal cells	ANOC 9103	Yokota et al. [2003]

[a]CFU-GM: colony-forming units-granulocyte-macrophage.

In the present study, we have performed a series of epitope mapping studies for ANOC 9103 and ANOC 9104 Abs to identify functional domains of adiponectin. Both ANOC 9103 and ANOC 9104 recognize N-terminal regions of adiponectin before the beginning of the collagen-like domain. We have also evaluated roles of the Ab-recognizing regions in adiponectin functions.

MATERIALS AND METHODS

Reagents

Production and purification of recombinant human adiponectin were performed as previously described [Arita et al., 1999]. Murine Abs against human adiponectin, ANOC 9103, ANOC 9104, and ANOC 9132, were raised by immunizing adiponectin-deficient mice with recombinant human adiponectin, and the specific recognition of each Ab was verified by Western blotting [Arita et al., 1999]. Two adiponectin fragments were synthesized by Sigma Genosys (Ishikari, Japan). The peptide sequences were designed as "DQETTTQGPGVLLPLPKGACTGWMA" corresponding to amino acid residues (AA) 17–41 of human adiponectin for fragment-1 as well as "ACTGWMAGIPGHPGHNGAPGRDGRD" corresponding to AA 35–59 for fragment-2. Lipopolysaccharide (LPS) derived from *Escherichia coli* 055:B5 (phenol extracted and chromatographically purified by gel filtration) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). Interleukin (IL)-1 β was purchased from R&D systems (Minneapolis, MN).

Mice and Cells

C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). Adiponectin-deficient mice were generated as described previously [Maeda et al., 2002]. All mice were maintained at the Institute for Experimental Animals, Osaka University School, and male mice were approximately 8–12 week of age at the time of use. A murine bone marrow stromal cell line, MS-5, was maintained in α-MEM medium supplemented with 10% fetal calf serum (FCS) (Flow, Aurora, OH). A THP-1 human monocytic leukemia line, a Nalm6 human B-cell leukemia line, and an ONHL-1 human B-lymphoma line, were cultured in RPMI1640 medium supplemented with 10% FCS. A Ba/F3 murine pro-B cell line was cultured in RPMI1640 medium supplemented with 10% FCS in the presence of IL-3 (Kirin Brewery Ltd., Tokyo, Japan). A MKN45 human gastric cancer line, a 293 T human embryonic line, and a C2C12 murine myoblast line were cultured in DMEM medium supplemented with 10% FCS. Human umbilical vein endothelial cells (HUVEC) were purchased from KURABO (Osaka, Japan), and maintained in HuMedia-EG2 (KURABO).

Epitope Mapping for ANOC 9103 and ANOC 9104

To produce the deletion mutants of human adiponectin, adiponectin cDNAs corresponding to AA 17–244, AA 37–244, AA 107–244, AA 17–188, AA 17–120, AA 17–46, AA 54–244, AA 72–244, AA 90–244, or AA 26–244 were amplified with polymerase chain reactions (PCR), respectively. Each amplified fragment was digested with *Bam*HI and *Eco*RI, and cloned into the pGEX-6P-1 plasmid (Amersham, Piscataway, NJ). *E. coli* strain JM109 was transformed with the constructed plasmids, and the synthesis of recombinant deletion mutants of human adiponectin was induced by isopropylthio-β-D-galactoside. The bacterial cells were pelleted and suspended in 50 mM Tris-HCl (pH 8.0) containing 0.1% Tween 20, and then sonicated. After centrifugation, each lysate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilion; Millipore Corp., Bedford, MA). After blocking of residual binding sites on the filter, immunoblotting was performed with the appropriate anti-human adiponectin Abs, followed by the reaction with horse radish peroxidase-labeled horse anti-mouse Ig (Cell Signaling, Beverly, MA). Immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (DuPont NEN, Boston, MA).

Enzyme-Linked Immunosorbent Assay (ELISA)

To detect the binding of anti-human adiponectin Abs to adiponectin fragments, either adiponectin fragment-1 or fragment-2 (5 μg/ml) was coated on 96-well microtiter plates overnight at 4°C. After the plates were blocked with PBS containing 0.05% Tween 20 and 2% bovine serum albumin (BSA), 100 μl of ANOC 9103 or ANOC 9104 (2 μg/ml) was then added to each well at room temperature for 1 h. After washing, the bound Abs were detected with biotinylated goat Ab against mouse IgG (Vector, Burlingame, CA) and avidin-biotin-alkaline

phosphatase complex (Vector). Enzyme-substrate reaction was performed with the ELISA amplification system (Sanko Jyunyaku, Tokyo, Japan) according to the manufacturer's instructions. To determine the concentration of PGE_2, cultured MS-5 supernatants were subjected to an enzyme immunoassay kit (Cayman Chemical Co, Ann Arbor, MI). The concentration of TNF-α in mouse serum was also evaluated with a Cytoscreen ELISA kit (Biosource, Kamarilo, CA).

Flow Cytometry

The binding capacity of adiponectin fragments corresponding to the epitopes of ANOC 9103 or ANOC 9104 to bind to several types of cells were analyzed with flow cytometry analysis as described previously [Oritani et al., 2000]. Briefly, incubations with fluorescein isothiocyanate (FITC)-labeled fragments as well as washing steps were accomplished on ice in Hank's balanced solution containing 1% BSA and 0.1% sodium azide. The stained cells were analyzed with FACSort (Becton Dickinson, Mountain View, CA).

Measurement of Cox-2 mRNA

MS-5 in a confluent state were preincubated for 24 h in α-MEM medium, and then exposed to recombinant human adiponectin in the presence of the indicated Abs or fragments for 4 h. The cells were harvested, and total RNA was prepared with RNA-TRIzol extraction (GIBCO, Grand Island, NY), followed by the treatment with DNase. cDNA was produced with the use of the ThermoScript reverse transcription-PCR system (Invitrogen, Carlsbad, CA). Real-time PCR was performed on an ABI-Prism 7700 using the Master Mix SYBR Green kit (PE-Applied Biosystems, Norwalk, CT) according to the manufacturer's instructions. Primers used in this study were: 5′-TGGTGCCTGGTCTGATGATG-3′ and 5′-GGATGCTCCTGCTTGAGTATGTC-3′ for Cox-2 as well as 5′-CAAAAGCCACCCCCACTCCTAAGA-3′ and 5′-GCCCTGGCTGCCTCAACACCTC-3′ for β-actin.

Preparation and Administration of Adenovirus

Adenovirus producing the full-length murine adiponectin (Ad-fAdipo) or β-galactosidase (Ad-βgal) were prepared by using the Adenovirus Expression Vector kit (Takara, Kyoto, Japan) [Okamoto et al., 2002]. To prepare adenovirus producing the N-terminal region-truncated adiponectin (Ad-delAdipo), cDNAs of signal sequence (nt 86–136), and murine adiponectin coding sequence without N-terminal region (nt 203–829) were connected by a newly synthesized *Bam*HI site. The constructed cDNA fragment was cloned into the Transfer Vector. After the vector was linealized with *Pme*I digestion, in vivo homologous recombination with pAdEasy-1 was performed in bacteria. After selecting, the recombinant was linealized with *Pac*I digestion, and transfected into QBI-293A cells. Stocks of Ad-delAdipo were amplified and purified with sequential centrifugation in CsCl step gradients [Kanegae et al., 1994]. Each step was performed with a kit of AdEasy vector system (Quantum Biotechnologies, Carlsbad, CA) according to the manufacturer's instructions. To produce the indicated adenovirus-derived proteins, Ad-fAdipo, Ad-delAdipo, or Ad-βgal (1×10^8 plaque-forming units/head) was injected into tail veins of adiponectin-deficient mice. Each protein produced by adenovirus was monitored by Western blot analysis. An equal aliquot of plasma was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilion; Millipore Corp.). After blocking of residual binding sites on the filter, immunoblotting was performed with a rabbit polyclonal antibody against murine adiponectin, followed by the reaction with horse radish peroxidase-labeled goat anti-rabbit Ig (Promega, Madison, WI). Immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (DuPont NEN).

Platelet Aggregation Study

Platelet aggregation assays were performed as described previously [Kato et al., 2005]. Briefly, murine whole blood was collected by cardiac puncture into 0.1 volume of 3.8% sodium citrate solution. Murine whole blood was centrifuged at 200*g* for 10 min to obtain platelet-rich plasma (PRP) or 750*g* for 10 min to obtain platelet-poor plasma (PPP). PRP was obtained from adiponectin-deficient mice and PPP was from adiponectin-deficient mice injected Ad-fAdipo, Ad-delAdipo, or Ad-βgal. PRP and PPP were mixed at a concentration of 3.0×10^8/ml of platelets. Platelet aggregation was initiated by the addition of 15 μl of collagen to 135 μl of the

mixture. Platelet aggregation was monitored using a model PAM-6C platelet aggregometer (Mebanix, Tokyo, Japan).

Mouse Hepatic Steatosis Model

Adiponectin-deficient mice were fed a choline-deficient L-amino acid (CDAA)-defined diet for 14 days after Ad-fAdipo-, Ad-delAdipo-, or Ad-βgal-injection. Mice were given free access to food and water. On day 14, the mice were killed, and liver samples were collected. The liver was immediately embedded in Optimal Cutting Temperature (OCT; Sakura Finetechnical Co. Ltd., Tokyo, Japan) compound and frozen in liquid nitrogen. We then prepared tissue slices (5 μm). For histological analyses, the three sections from each mouse were stained with Oil Red O. The steatosis area was quantified by using an image analyzing computer software (IPLab Spectrum; Signal Analytics, Vienna, VA).

RESULTS

Identification of the ANOC 9103- and ANOC 9104-Recognition Sites

We have reported that anti-human adiponectin Abs, ANOC 9103 and ANOC 9104 blocked some biological activities of adiponectin as listed in Table I. To determine the functional adiponectin domains for these activities, we examined their epitopes with Western blot analysis for a series of the deletion mutants of human adiponectin (Figs. 1A and 2A). AA 1–18 of human adiponectin corresponds to a signal peptide, AA 19–41 to a hypervariable region, AA 42–107 to a collagen-like domain, and AA 108–244 to a globular domain, respectively. In a series of N-terminal deletion of human adiponectin, ANOC 9103 bound to Adipo (17–244) and Adipo (37–244), but not to Adipo (107–244) (Fig. 1B). ANOC 9104 bound to Adipo (17–244), but not to Adipo (37–244) or Adipo (107–244). In a series of C-terminal deletion of human adiponectin, ANOC 9103 bound to Adipo (17–244), Adipo (17–188), and Adipo (17–120), but not to Adipo (17–46) (Fig. 1C). ANOC 9104 bound to Adipo (17–244), Adipo (17–188), and Adipo (17–120) as well as Adipo (17–46). These data suggest that ANOC 9103 recognizes AA 47–106 of human adiponectin and that ANOC 9104 recognizes AA 17–36 of human adiponectin. To clarify their epitopes in detail, Western blot analysis for additional deletion mutants was performed as shown in Figure 2. ANOC 9103 bound to Adipo (37–244), but not to Adipo (54–244), Adipo (72–244), or Adipo (90–244) (Fig. 2B). With regard to the epitope of ANOC 9104, it bound to adipo (17–244), but not to Adipo (26–244) or Adipo (37–244) (Fig. 2C).

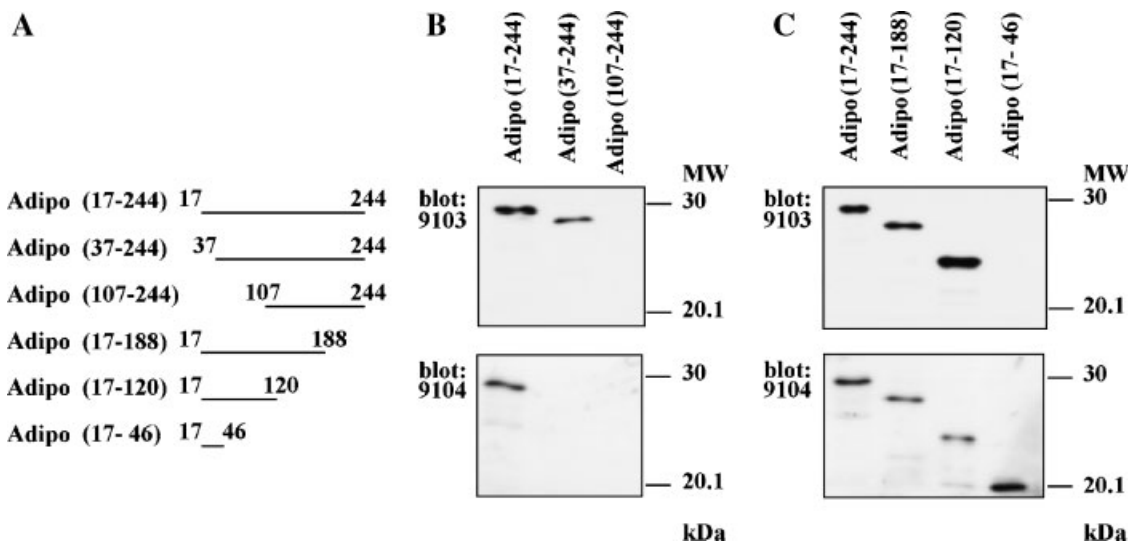

Fig. 1. Binding of ANOC 9103 and ANOC 9104 to the deletion mutants of human adiponectin. **A**: Amino acid residues of human adiponectin mutants lacking N-terminal or C-terminal regions are schematically shown. **B**, **C**: Proteins of the adiponectin mutants with the deletion of N-terminal (B) or C-terminal (C) regions were produced and prepared as described in the Materials and Methods. Each lysate (derived from 0.03 μl of bacterial culture) was subjected to Western blot analysis with anti-human adiponectin Ab, ANOC 9103 or ANOC 9104. The binding of Abs was detected with horse radish peroxidase-labeled anti-mouse Ig, followed by the enhanced chemiluminescence detection system. Similar results were observed in three independent experiments.

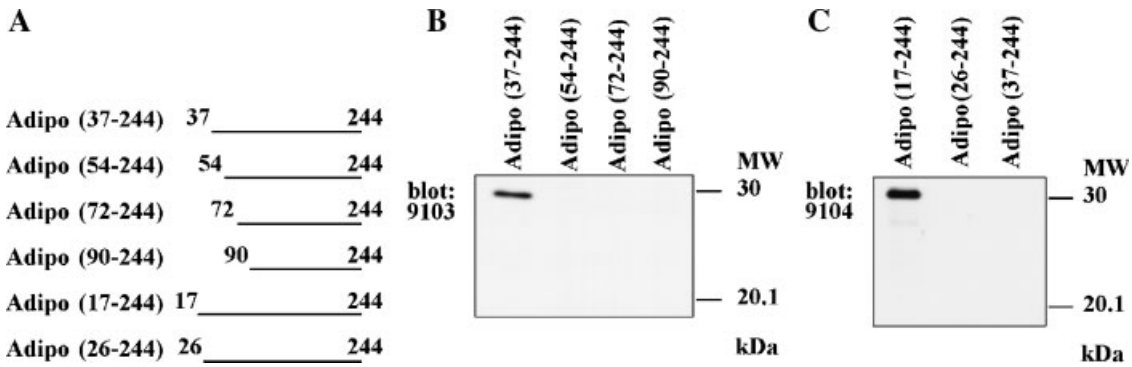

Fig. 2. Identification of the ANOC 9103- and ANOC 9104-recognition sites. **A**: Amino acid residues of human adiponectin with gradual deletions between AA 37 and 90 or between AA 17 and 37 are schematically shown. **B**, **C**: Each lysate (derived from 0.03 μl of bacterial culture) containing the indicated truncated form of human adiponectin was prepared as described in the Materials and Methods, and subjected to Western blot analysis with ANOC 9103 (B) or ANOC 9104 (C). The binding of Abs was detected with horse radish peroxidase-labeled anti-mouse Ig, followed by the enhanced chemiluminescence detection system. Similar results were observed in three independent experiments.

Taken together our results for epitope mapping, ANOC 9103 recognizes AA 47–53 of human adiponectin, in addition, ANOC 9104 recognizes AA 17–25 of human adiponectin. Another anti-human adiponectin Ab, ANOC 9132 bound to AA 107–244 of human adiponectin (data not shown), suggesting that it recognizes the globular domain. The Ab-recognition sites were confirmed with an ELISA technique. Fragment-1 (DQETTTQGPGVLLPLPKGACTGWMA) represents AA 17–41, and fragment-2 (ACTGWMAGIPGHPGHNGAPGRDGRD) represents AA 35–59 of human adiponectin, respectively. Each fragment was coated on plates, and the binding of ANOC 9103 or ANOC 9104 Ab to the fragments was detected by biotinylated anti-mouse IgG, followed by the avidin-biotin-alkaline phosphatase complex reaction. As shown in Figure 3, ANOC 9103 bound to fragment-2, but not to fragment-1. Conversely, ANOC 9104 bound to fragment-1, but not to fragment-2. In either ELISA, ANOC 9132 did not bind to fragment-1 or fragment-2. Therefore, ANOC 9103 specifically recognizes AA 47–53 of human adiponectin, which correspond to the starting portion of the collagen-like region, in addition, ANOC 9104 recognizes AA 17–25 of human adiponectin, which correspond to the hypervariable region.

Recognition of Cells by the Fragments Corresponding to the 9103- and 9104-Epitopes

The binding capacity of FITC-labeled fragment-1 and fragment-2 to several types of cells was evaluated with flow cytometry analysis (Table II). Both fragment-1 and fragment-2 bound to the cell surface of MS-5 stromal cells. Fragment-2 significantly bound to a THP-1 monocytic leukemia line, while the binding of fragment-1 was detected faintly. Interestingly, the binding of both fragments was enhanced when differentiation of THP-1 cells into macrophages were induced by PMA. HUVEC and a C2C12 myoblast line showed higher reactivity with fragment-2 than with fragment-1. Their recognition was influenced neither by the activation state of HUVEC nor by the differentiation state of C2C12 cells. In the case of a 293 T embryonic line, a MKN45 gastric cancer line, a Nalm6 B-cell leukemia line, an ONHL-1 B-lymphoma line, and a Ba/F3 pro-B cell line, the binding of fragment-2, but not fragment-1 was detected. Therefore, fragment-1 binds to

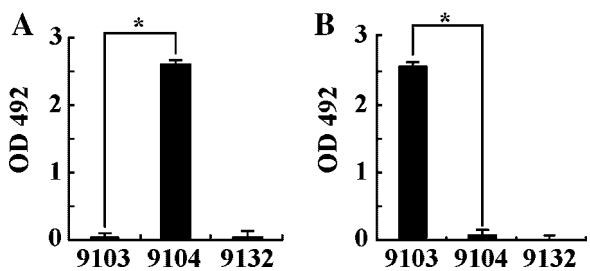

Fig. 3. Recognition of adiponectin fragments by ANOC 9103 and ANOC 9104. Five microgram per milliliter of either fragment-1 corresponding to AA 17–41 of human adiponectin (**A**) or fragment-2 corresponding to AA 35–59 of human adiponectin (**B**) was coated on 96-well microtiter plates overnight. The binding of ANOC 9103, ANOC 9104, and ANOC 9132 (2 μg/ml) to each fragment was evaluated with ELISA, and expressed as OD492. The results represent mean ± SD of triplicate samples. Similar results were obtained in two independent experiments. $^*P < 0.01$ by Student' *t*-test.

TABLE II. Binding Capacity of Adiponectin Fragments to Several Types of Cells

Cells	Origin	Δ Mean fluorescence[d]	
		Fragment-1	Fragment-2
MS-5	Bone marrow stroma	1.62	1.69
THP-1	Monocytic leukemia	1.98	3.59
THP-1 with PMA[a]		4.68	8.29
C2C12	Myoblast	1.01	6.46
C2C12 with horse serum[b]		1.16	6.66
293T	Embryonic	0.26	5.51
MKN45	Gastric cancer	0.50	4.06
Ba/F3	Pro-B lymphocyte	0.32	1.51
Nalm-6	B-cell leukemia	0.22	2.57
ONLH-1	B-lymphoma	0.53	5.35
HUVEC	Umbilical vein endothelial cells	2.10	4.82
HUVEC with IL-1 β[c]		1.58	4.52

[a]Differentiation of THP-1 cells into macrophages was induced by the stimulation with PMA (50 ng/ml) for 24 h.
[b]Differentiation of C2C12 cells into myocytes was induced by exchanging medium.
[c]Activation of HUVEC was induced by the stimulation with IL-1 β (10 ng/ml) for 24 h.
[d]Δ Mean fluorescence was calculated as [fluorescence intensity of the staining with FITC-fargment-1 or FITC-fragment-2)−(fluorescence intensity of the control staining]. Data are shown as mean in at least two independent staining results.

the restricted type of cells, in contrast, fragment-2 to a variety of cells.

Fragment-1 Inhibits Adiponectin-Induced Cox-2 Gene Expression and Prostanoid Production In Vitro

As we reported previously, adiponectin inhibited not only the production of B-lymphocytes but also the formation of fat cells in long-term bone marrow cultures [Yokota et al., 2002, 2003]. These inhibitory effects of adiponectin were mainly mediated through the induction of Cox-2 in stromal cells. Because ANOC 9103 blocked adiponectin-induced growth inhibition of B-lymphocytes on MS-5 stromal cells, we analyzed influences of fragment-1 and fragment-2 on adiponectin functions in this culture system. As shown in Figure 4A, the treatment of MS-5 cells with adiponectin induced Cox-2 gene expression approximately threefold when the induction was evaluated with Real-time PCR. Both ANOC 9103 and ANOC 9104 blocked this adiponectin function, while ANOC 9132, anti-globular adiponectin Ab did not (Fig. 4A,C). Notably, the adiponectin function was also blocked by the addition of fragment-1, but not fragment-2 (Fig. 4B,C: 41.9 ± 14.9 % inhibition with fragment-1 and 1.7 ± 8.6 % inhibition with fragment-2), and the inhibition by fragment-1 was dependent on its concentration (Fig. 4D). Expression of Cox-2 mediates the synthesis of prostanoids including PGE_2 [Goetzl et al., 1995]. We next evaluated the inhibitory effects of fragment-1 and fragment-2 on the synthesis of PGE_2 induced by adiponectin. When the concentrations of PGE_2 in culture supernatants were analyzed with ELISA, MS-5 cells treated with adiponectin began to produce significant levels of PGE_2 (Fig. 4E: 96 pg/ml in Experiment 1 and 126 pg/ml in Experiment 2). Fragment-1 significantly inhibited PGE_2 secretion induced by adiponectin, but fragment-2 did not (<1 pg/ml with fragment-1, 150 pg/ml with fragment-2 in Experiment 1 as well as 8.5 pg/ml with fragment-1, 154 pg/ml with fragment-2 in Experiment 2). Therefore, the addition of fragment-1, which contains the epitope of ANOC 9104, reverses adiponectin-induced Cox-2 gene expression and PGE_2 production in MS-5 stromal cells.

Adiponectin Lacking its N-Terminal Region Inhibits LPS-Induced TNF-α Secretion In Vivo

To examine physiological roles of N-terminal domain of adiponectin including the ANOC 9104-recognition site, we prepared adenovirus producing murine adiponectin lacking the N-terminal domain (Ad-delAdipo) as well as full-length of murine adiponectin (Ad-fAdipo). The product of Ad-delAdipo was composed of AA 40–247 of murine adiponectin whose deleted N-terminal region corresponds to the human sequence of fragment-1. Adenovirus producing β-gal (Ad-βgal) was used as a negative control. Each protein produced by adenovirus was monitored with Western blot analysis (Fig. 5).

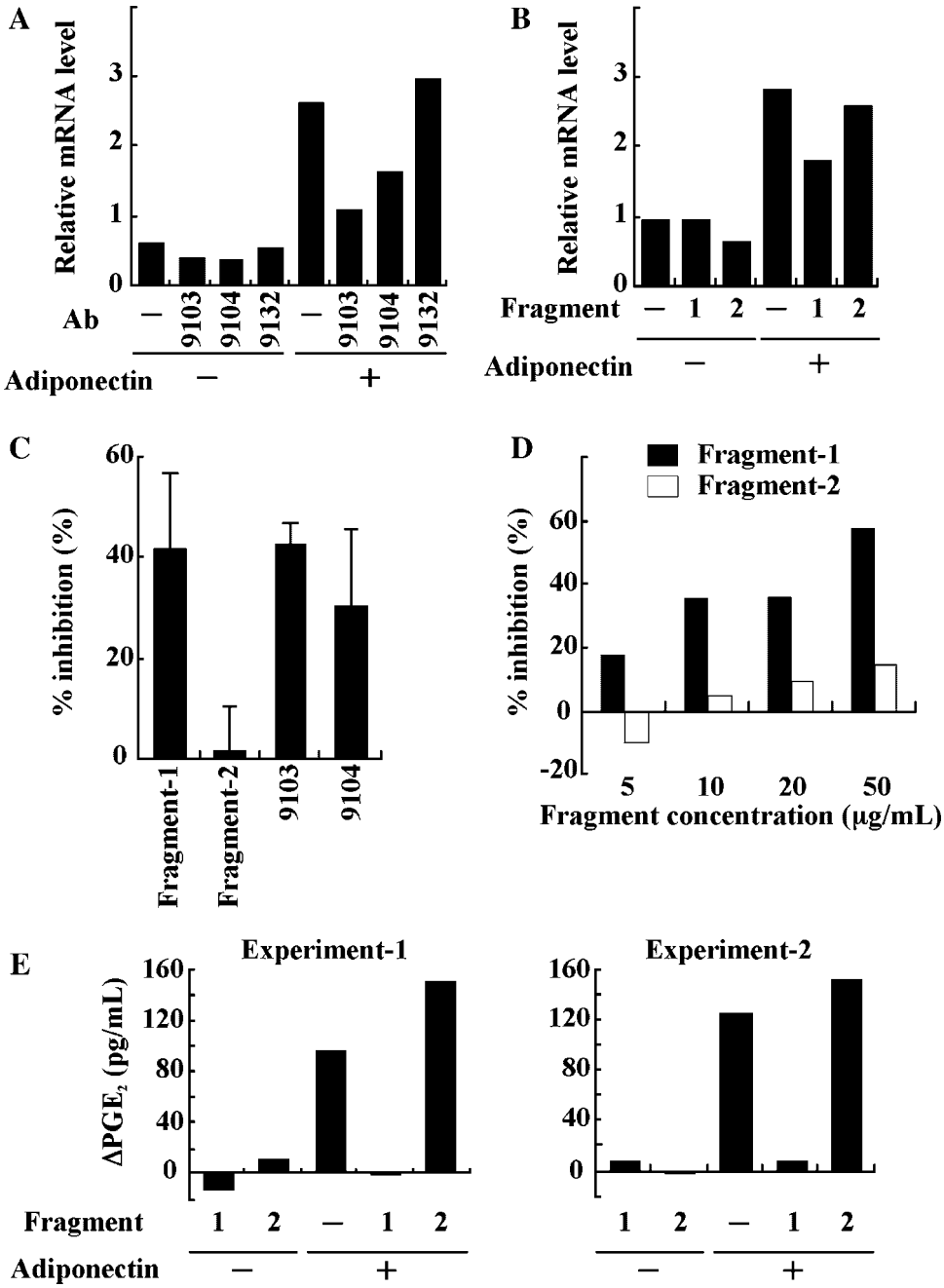

Fig. 4. Effects of adiponectin fragments on adiponectin-induced Cox-2 gene expression and prostanoid production in MS-5 cells. **A**–**C**: MS-5 cells in a confluent condition were preincubated for 24 h in α-MEM medium, and then exposed to recombinant human adiponectin (10 μg/ml) in the presence of the indicated Abs (30 μg/ml) or fragments (10 μg/ml) for 4 h. Total RNAs were prepared with TRIzol extraction, and Cox-2 mRNA levels were measured by real-time quantitative RT-PCR. The mRNA levels of Cox-2 were divided by those of β-actin, a standard control gene, and normalized. The relative mRNA expressions of Cox-2 in the presence of anti-adiponectin Abs (A) or adiponectin fragments (B) are shown. The results represent mean of duplicate samples. Data are representative of four independent experiments. The percentage inhibition was calculated as [1 − (mRNA level with Abs or fragments)/(mRNA levels with control] × 100, and the data are shown as mean ± SD percentages of inhibition in four independent experiments (C). **D**: MS-5 cells in a confluent condition were preincubated for 24 h in α-MEM medium, and then exposed to recombinant human adiponectin (10 μg/ml) in the presence of the indicated concentrations of adiponectin fragments for 4 h. The percentage inhibition by fragment-1 (closed column) or by fragment-2 (open column) is shown. The results represent mean of duplicate samples. Data are representative of three independent experiments. **E**: MS-5 cells in a confluent condition were preincubated for 24 h in α-MEM medium, and then exposed to recombinant human adiponectin (10 μg/ml) in the presence of fragment-1 or fragment-2 (10 μg/ml) for 24 h. Supernatant was then collected from each culture, and subjected to ELISA for PGE_2. The increase of PGE2 from control cultures without adiponectin or fragments is shown from two independent experiments.

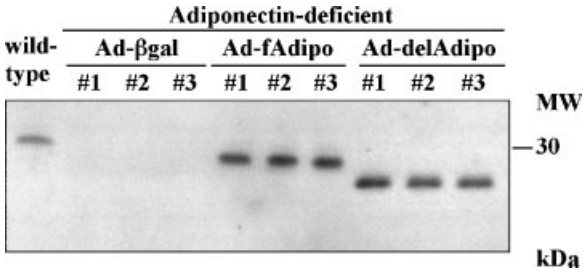

Fig. 5. Adiponectin proteins produced by adenovirus. Ad-fAdipo, Ad-delAdipo, or Ad-βgal (1×10^8 plaque-forming units/head) was injected into tail veins of adiponectin-deficient mice. On day 5, plasma samples were obtained. Ten microliters of diluted plasma sample (dilute 500-fold with PBS for adenovirus-injected mice and 50-fold with PBS for wild-type mice) was subjected to Western blot analysis with anti-murine adiponectin Ab. The binding of Ab was detected with horse radish peroxidase-labeled anti-rabbit Ig, followed by the enhanced chemiluminescence detection system.

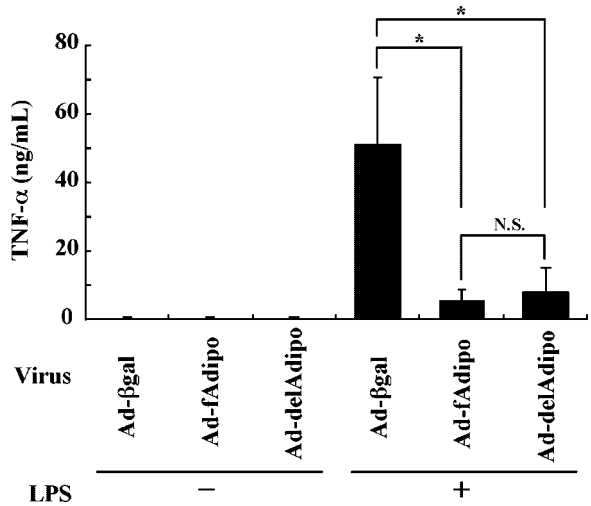

Fig. 6. In vivo effects of murine adiponectin lacking its N-terminal region on TNF-α production after LPS-injection. Ad-fAdipo, Ad-delAdipo, or Ad-βgal (1×10^8 plaque-forming units/head) was injected into tail veins of adiponectin-deficient mice. On day 5, LPS (1 μg/head) was injected intraperitoneally, and blood samples were obtained after 1 h of the LPS-injection. Serum TNF-α levels were measured with ELISA. The data indicate mean ± SD from 10 mice of each group. $^*P < 0.01$ and N.S. not significant by Student' *t*-test.

Adiponectin-deficient mice display high expression of TNF-α mRNA in adipose tissue and high concentrations of TNF-α in plasma [Maeda et al., 2002]. Moreover, the supplementation of plasma adiponectin decreases TNF-α concentration in adiponectin-deficient mice [Maeda et al., 2002]. We measured serum concentration of TNF-α of adiponectin-deficient mice treated with Ad-fAdipo, Ad-delAdipo, or Ad-βgal after LPS-injection. Although serum TNF-α levels of mice treated with Ad-βgal were significantly elevated, those of Ad-fAdipo- as well as Ad-delAdipo-treated mice were not elevated (Fig. 6). Therefore, N-terminal region of adiponectin is not required for the inhibition of LPS-induced TNF-α secretion in vivo.

Adiponectin Lacking its N-Terminal Region Shows Reduced Activities to Inhibit Collagen-Induced Platelet Aggregation

We recently found that adiponectin inhibited collagen-induced platelet aggregation (Kato, unpublished observation). We used PPP obtained from whole blood of adiponectin-deficient mice treated with Ad-fAdipo, Ad-delAdipo, or Ad-βgal as a source of adiponectin. At a low concentration of collagen (2.5 μg/ml), full-length adiponectin greatly inhibited platelet aggregation, but the inhibitory effect of N-terminal-truncated adiponectin was limited (Fig. 7A). Figure 7B summarizes the results of five independent experiments, and the inhibition of platelet aggregation by full-length adiponectin was always greater than that by N-terminal-truncated adiponectin. Therefore, N-terminal region of adiponectin plays a role in the inhibition of platelet aggregation.

Adiponectin Lacking its N-Terminal Region Shows Reduced Activities to Inhibit CDAA-Defined Diet-Induced Hepatic Steatosis In Vivo

Xu and his colleagues reported that administration of mice with recombinant adiponectin alleviated steatohepatitis induced by chronic consumption of high-fat ethanol-containing food [Xu et al., 2003]. We employed a CDAA-defined diet model experiment to induce hepatic steatosis without alcohol consumption [Koteish and Diehl, 2001; Jin et al., 2005]. The hepatic steatosis was evaluated after 14 days of CDAA-defined diet in adiponectin-deficient mice treated with Ad-fAdipo, Ad-delAdipo, or Ad-βgal. As shown in Figure 8A, the accumulation of fat in liver was greatly reduced by full-length adiponectin, and partly by N-terminal-truncated adiponectin. When the degree of fat accumulation in liver was evaluated with the area of red spots in the liver sections, mean percentages of the steatosis areas were calculated as 59.1 ± 16.3% for mice expressing β-gal, 29.7 ± 7.1 % for mice expressing full-length adiponectin, and 43.0 ± 9.4 % for mice expressing N-terminal-truncated adiponectin, respectively (Fig. 8B).

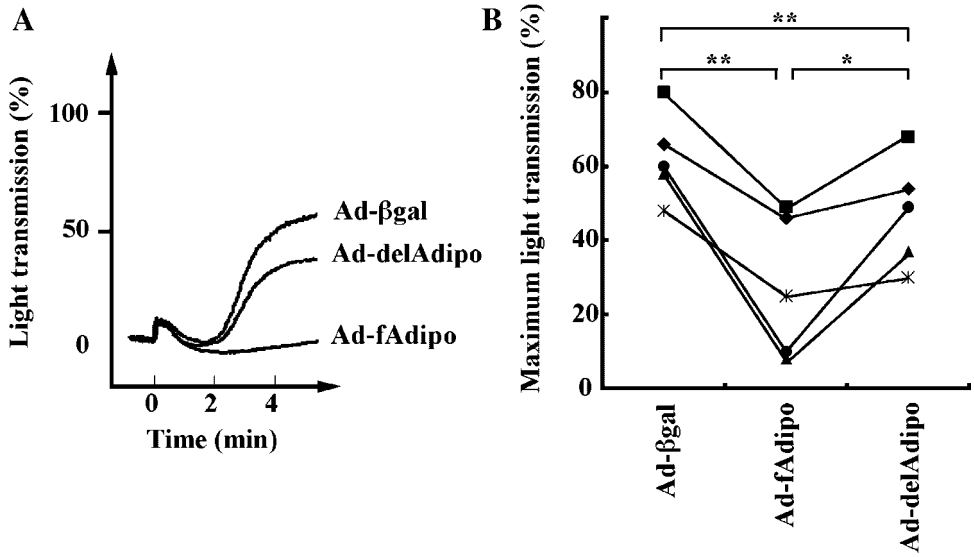

Fig. 7. Effects of murine adiponectin lacking its N-terminal region on platelet aggregation induced by collagen. **A**: Ad-fAdipo, Ad-delAdipo, or Ad-βgal (1×10^8 plaque-forming units/head) was injected into tail veins of adiponectin-deficient mice. On day 5, PPP was obtained from the adenovirus-injected mice as a source of adiponectin. PRP was obtained from adiponectin-deficient mice without adevovirus-injection as a source of platelets. The PPP and PRP were mixed at a concentration of 3.0×10^8/ml of platelets. Platelet aggregation was initiated by the addition of 2.5 μg/ml of collagen under stirring condition and monitored by aggregometer. **B**: PPP from Ad-fAdipo-, Ad-delAdipo-, or Ad-βgal-injected adiponectin-deficient mice and PRP from adiponectin-deficient mice without adevovirus-injection were mixed at a concentration of 3.0×10^8/ml of platelets. Platelet aggregation was initiated by the addition of 2.5 μg/ml of collagen under stirring condition and monitored by aggregometer. $^{**}P < 0.01$ and $^{*}P < 0.05$ by Student' *t*-test.

Therefore, N-terminal region of adiponectin plays a role in the alleviation of diet-induced accumulation of fat in liver.

DISCUSSION

In a series of our experiments, anti-human adiponectin Abs, ANOC 9103 or ANOC 9104, have blocked several biological activities of adiponectin in vitro. Adiponectin inhibits the uptake of acetylated LDL by human monocyte-derived macrophages in a dose dependent manner [Ouchi et al., 2001]. Adiponectin inhibits the colony formation of granulocyte-macrophage-colony forming units [Yokota et al., 2000]. These adiponectin functions were abrogated by the addition of ANOC 9104. Adiponectin-induced Cox-2 gene expression and PGE_2 production in stromal cells were significantly blocked by both ANOC 9103 and ANOC 9104 as shown in Figure 4. Moreover, adiponectin-binding to cells was partially inhibited by ANOC 9103 and ANOC 9104 (unpublished observation). Their blocking capacities have suggested that ANOC 9103 and ANOC 9104 may recognize important functional regions on adiponectin molecule. In the present study, we identified the ANOC 9103- and ANOC 9104-recognition sites with an epitope mapping based on the ability to bind to the deleted adiponectin mutants. ANOC 9103 recognizes AA 47–53 of human adiponectin, which correspond to the starting portion of the collagen-like region, and ANOC 9104 recognizes AA 17–25, which correspond to the hypervariable domain. Structurally, the adiponectin protein in its most basic form is a homotrimer of 30 kDa subunits [Tsao et al., 2003]. The trimer exhibits a "ball-and-stick" structure where the globular domain forms the ball and the collagen-like domain forms the stick [Tsao et al., 2003]. There is also a small ball-like structure, which represents the N-terminal region of adiponectin upstream of the collagen-like domain, on the other side of the stick [Tsao et al., 2003]. The trimers are connected into a larger multimer by disulfide bonds, and the hexamer shows two trimers lying adjacent to each other in parallel head-to head fashion [Tsao et al., 2003]. The high molecular weight of adiponectin shows a "bouquet-like" high order structure [Shapiro and Scherer, 1998]. Taken together with our results of epitope mapping, both ANOC 9103 and ANOC 9104 recognize the N-terminal region of

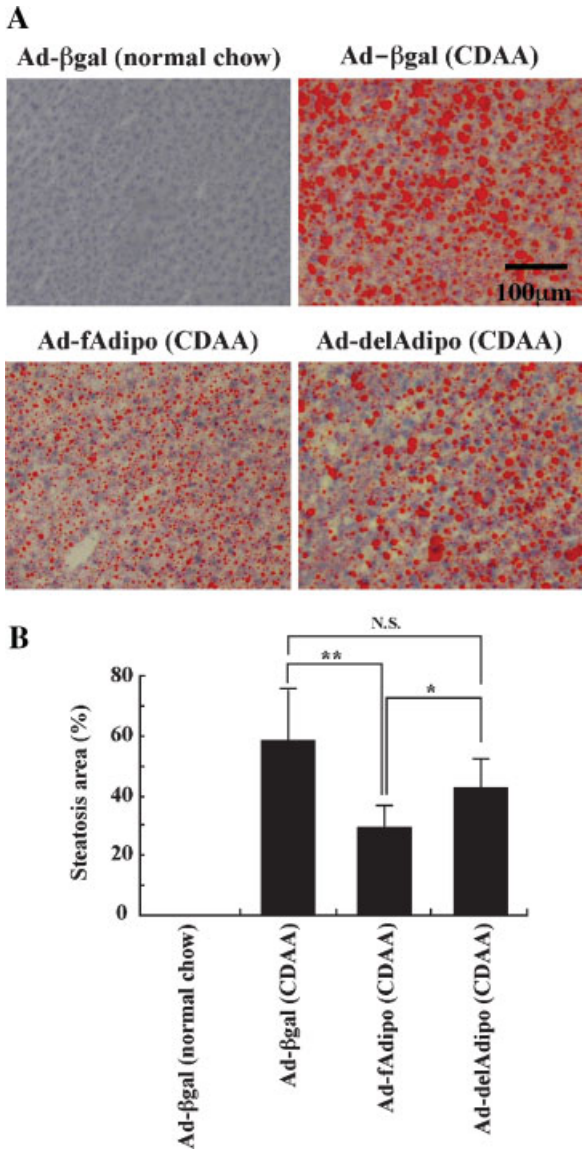

Fig. 8. In vivo effects of murine adiponectin lacking its N-terminal region on CDAA-defined diet-induced hepatic steatosis. After adiponectin-deficient mice were injected with Ad-fAdipo, Ad-delAdipo, or Ad-βgal (1×10^8 plaque-forming units/head), the mice were fed a CDAA-defined diet for 2 weeks. On day 14, their liver samples were collected and subjected to histological analysis with Oil Red O staining. A series of representative photomicrographs (at magnitude ×4) of the liver sections from each adenovirus-injected mouse are shown (**A**). The red spotted areas, Oil Red O-positive areas, were quantified with an image analyzing computer software, and expressed as the percentage of the total area of the specimen (**B**). The data indicate mean ± SD from five mice of each group. $^{**}P < 0.01$, $^{*}P < 0.05$, and N.S. not significant by Student' *t*-test.

adiponectin before the beginning of the collagen-like domain, which corresponds to the small ball in the trimer as well as the root of the rigid stick of a bouquet-like structure in high molecular weight form of adiponectin.

A peptide fragment-1 (AA 17–41), which corresponds to the epitope of 9104, can bind to the surface of several types of cells such as a MS-5 stromal cell line. It is noteworthy that the treatment of MS-5 cells with fragment-1 inhibited adiponectin-induced Cox-2 gene expression and PGE_2 production. These facts are likely to suggest that the N-terminal region of adiponectin including the hypervariable region is a functional domain to induce Cox-2 gene expression and PGE_2 production and that MS-5 cells may express some receptors, which recognize the N-terminal region of adiponectin. Both ANOC 9103 and ANOC 9104 could inhibit the stimulatory effect of adiponectin on Cox-2 gene expression. However, only fragment-1 showed the same effect. These results between Abs and fragments were not consistent. One possibility is that ANOC 9103 can block the binding of adiponectin to the receptor even if ANOC 9103 recognizes near the receptor-binding site because the ANOC 9103-recognition site exists by the ANOC 9104-recognition site and because antibodies are larger than peptide fragments. AdipoR1 is a high-affinity receptor for globular adiponectin as well as a low-affinity receptor for full-length adiponectin, and is abundantly expressed in skeletal muscle [Yamauchi et al., 2003a]. AdipoR2 is an intermediate-affinity receptor for full-length and globular adiponectin, and is mainly expressed in liver [Yamauchi et al., 2003a]. Both AdipoR1 and AdipoR2 mediate the increment of AMP kinase and peroxisomal proliferator-activated receptor (PPAR) activities, resulting in the increased fatty-acid oxidation and glucose uptake, which accounts for the increased insulin sensitivity [Yamauchi et al., 2003a]. Moreover, transgenic mice expressing globular adiponectin significantly upregulated insulin sensitivity [Combs et al., 2004]. Thus, the globular domain of adiponectin is thought to be required for the binding to AdipoR1 and AdipoR2. On the other hand, the collagen-like domain has been known to be important for multimerization of adiponectin, which determines its affinity to the receptors [Pajvani et al., 2003; Tsao et al., 2003; Waki et al., 2003]. Our results suggesting some physiological roles of the N-terminal region of adiponectin including the hypervariable region are very exciting because there is little information about this region.

Platelets initially adhere to the injured vascular surface and/or the exposed subendotherial

materials [Fuster et al., 1992]. The activated platelets then aggregate to each other, followed by next steps of thrombogenesis such as the hemostatic plug formation and the pathologic thrombus formation. Thus, our result that adiponectin inhibits the homotypic aggregation of platelets is one of the important mechanisms how adiponectin displays anti-thrombogenic activities in vivo. On the other hand, Xu and his colleagues reported that circulating adiponectin levels decreased by chronic consumption of high-fat ethanol-containing food and that administration of those mice with recombinant adiponectin dramatically reduced hepatomegaly and steatohepatitis [Xu et al., 2003]. Our results also show that replenishment of adiponectin with an adenovirus expression system improves CDAA-defined diet-induced hepatic steatosis in adiponectin-deficient mice. Thus, adiponectin alleviates both alcoholic and non-alcoholic hepatic steatosis in vivo. Interestingly, our constructed truncated-form of adiponectin lacking its N-terminal region showed less activity to inhibit the collagen-induced platelet aggregation and the diet-induced accumulation of fat in liver than full-length of adiponectin. It is possible that unknown receptors, which recognize the N-terminal region of adiponectin may be expressed on the surface of hepatocytes and platelets, and that they may attribute to some adiponectin functions in vivo. However, the interpretation about our in vivo experiments is complicated. Recent studies have revealed that the recognition of AdipoR1 and AdipoR2 as well as the binding to cytokines is dependent on state of multimerization of adiponectin [Pajvani et al., 2003; Tsao et al., 2003; Waki et al., 2003]. T-cadherin, a novel adiponectin-receptor recognizes only hexameric or high molecular weight forms of adiponectin [Hug et al., 2004]. Thus, multimerization of adiponectin seems to determine its affinity to the receptors. Recently, a cysteine residue lying at the N-terminal region of adiponectin was shown to play an essential role in assembling high molecular weight form of adiponectin [Pajvani et al., 2003; Tsao et al., 2003]. The delAdipo proteins lack the cysteine residue because it lies at the Ab-recognition sites. Thus, N-terminal-truncated adiponectn may loose the ability to assemble high molecular weight complex. Indeed, our Western blot analysis under the non-reducing condition showed that the N-terminal-truncated adiponectn proteins were detected predominantly as low molecular weight forms (data not shown). Thus, the possibility also exists that the impaired activities of N-terminal-truncated adiponectin may result from the failure of multimerization. We do not know either is the case because molecular mechanisms for these in vivo adiponectin functions are unclear. Further analysis will clarify in vivo roles of N-terminal region of adiponectin.

Recent studies have suggested the role of adipose tissue in the development of a systemic inflammatory state, which contributes to obesity-associated vasculopathy and cardiovascular risk [Berg and Scherer, 2005]. Although the mechanisms of adiponectin to exhibit anti-atherogenic effects are largely unknown, adiponectin seems to regulate low-grade inflammation in vivo. Indeed, an inverse relationship was observed between adiponectin and C-reactive protein in plasma of patients with coronary artery diseases [Ouchi et al., 2003]. In the present study, we showed that N-terminal region of adiponectin is important to mediate signals for Cox-2 induction. In an animal model of carrageenin-induced pleurisy, Cox-2 is pro-inflammatory during early phase of inflammation, but aids resolution of inflammation at the later phase by generating an alternative set of anti-inflammatory prostaglandins [Gilroy et al., 1999]. In addition, several clinical trials have suggested that Cox-2 inhibitors may lead to increased cardiovascular events [Mukherjee et al., 2001]. Thus, one possible mechanism how adiponectin exhibits anti-atherogenic effects may be to keep base-line expression of Cox-2 in vivo, because adiponectin is abundant. Our results also propose the existence of a possible receptor, which recognizes the N-terminal region of adiponectin and mediates signals for Cox-2 induction in MS-5 cells. In this situation, our designated fragment-1 will be a powerful tool to isolate the novel adiponectin receptor with an expression cloning based on the binding capacity. Further analysis will facilitate the understanding of molecular mechanisms of adiponectin and the designing of novel strategies to treat patients with vascular-dysfunctional and/or chronic inflammatory diseases.

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