Hydroxylation and Glycosylation of the Four Conserved Lysine Residues in the Collagenous Domain of Adiponectin

POTENTIAL ROLE IN THE MODULATION OF ITS INSULIN-SENSITIZING ACTIVITY*

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It has recently been shown that the fat-derived hormone adiponectin has the ability to decrease hyperglycemia and to reverse insulin resistance. However, bacterially produced full-length adiponectin is functionally inactive. Here, we show that endogenous adiponectin secreted by adipocytes is post-translationally modified into eight different isoforms, as shown by two-dimensional gel electrophoresis. Carbohydrate detection revealed that six of the adiponectin isoforms are glycosylated. The glycosylation sites were mapped to several lysines (residues 68, 71, 80, and 104) located in the collagenous domain of adiponectin, each having the surrounding motif of GXKGD(D). These four lysines were found to be hydroxylated and subsequently glycosylated. The glycosides attached to each of these four hydroxylated lysines are possibly glycosylgalactosyl groups. Functional analysis revealed that full-length adiponectin produced by mammalian cells is much more potent than bacterially generated adiponectin in enhancing the ability of subphysiological concentrations of insulin to inhibit gluconeogenesis in primary rat hepatocytes, whereas this insulin-sensitizing ability was significantly attenuated when the four glycosylated lysines were substituted with arginines. These results indicate that full-length adiponectin produced by mammalian cells is functionally active as an insulin sensitizer and that hydroxylation and glycosylation of the four lysines in the collagenous domain might contribute to this activity.

In addition to serving as an energy storage depot for triglycerides, adipose tissue is now recognized as an active endocrine organ that can secrete a variety of biologically active molecules (adipocytokines) in response to extracellular signals (1–4). Some of these adipocytokines, such as leptin, tumor necrosis factor-α, and resistin, have been shown to play critical roles in the regulation of systemic energy homeostasis, and altered expression and/or secretion of these adipocytokines may contribute to the causation of insulin resistance, type II diabetes, and its complications such as cardiovascular diseases.

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Adiponectin (also called ACRP30, adipQ, and GBP28) is a protein exclusively secreted by adipocytes and was originally cloned by four research groups using different approaches (5–8). Several recent studies suggest that adiponectin might be a critical, long sought after hormone that links obesity, insulin resistance, and type II diabetes (9–11). The adiponectin gene is located in chromosome 3q27, a susceptibility locus for type II diabetes and related metabolic syndromes (12–14). Circulating adiponectin is abundant in humans as well as rodents, with plasma circulating levels in the microgram/ml range, accounting for ~0.01% of the total plasma protein (15, 16). mRNA expression and the secretion level of adiponectin are dramatically decreased in a variety of animal models of insulin resistance as well as in obese humans and type II diabetic patients from different ethnic groups (15–20). Notably, treatment with the insulin-sensitizing peroxisome proliferator-activated receptor-γ agonist thiazolidinedione in several insulin-resistant animal models and human patients significantly increases the plasma concentration of adiponectin (21, 22). Thus, adiponectin correlates well with the insulin-sensitive state, and its absence is associated with insulin resistance, dyslipidemia, and hyperglycemia.

Evidence is accumulating that adiponectin or its synthetic analogs might be useful in the treatment of type II diabetes and other metabolic syndromes associated with insulin resistance. Lodish and co-workers (10) have recently reported that acute in vivo administration of truncated adiponectin decreases post-prandial plasma free fatty acid following a high fat meal and that chronic administration of this protein causes sustained weight loss in mice without affecting food intake. Scherer and co-workers (9) demonstrated that injection of recombinant adiponectin acutely abolishes hyperglycemia in several diabetic animal models, including ob/ob, non-obese diabetic, and streptozotocin-treated mice. Furthermore, Kadowaki and co-workers (11) showed that replenishment of adiponectin can restore insulin resistance in both high fat-fed and lipotropic mice, perhaps by increasing β-oxidation of fatty acid in muscle and thus decreasing muscular triglyceride content.

Mouse adiponectin is composed of 247 amino acid residues with an N-terminal collagenous domain and a C-terminal globular domain (7). The N-terminal collagenous domain is required for the high order multimerization of this protein (5). The three-dimensional structure of the C-terminal globular region is strikingly similar to that of tumor necrosis factor-α, even though there is no homology at the primary sequence level (23). The molecular mechanisms underlying the metabolic functions of adiponectin are largely unknown. Bacterially produced full-length recombinant adiponectin is inactive in restoring insulin sensitivity as well as in decreasing hyperglycemia (9, 11), indicating that post-translational modification might be critical for the insulin-sensitizing actions of adiponectin. Here,
we have shown that adiponectin secreted by adipocytes exists in multiple glycosylated isoforms. We also mapped the glyco-
sylation sites to several conserved lysines (residues 68, 71, 80, and 104) that are located in the collagenous domain of
adiponectin, with surrounding consensus sequences of
GXXGGE(D). These lysine residues are hydroxylated and sub-
sequently glycosylated. Furthermore, the important role of this
hydroxylsyl glycosylation was confirmed by mutational anal-
ysis, which showed that substitution of these lysine residues
with arginine significantly attenuated the ability of adiponec-
tin to enhance insulin’s inhibitory actions on hepatic glucose
production.

EXPERIMENTAL PROCEDURES

Materials—Dexamethasone, 3-isobutyl-1-methylxanthine, α-cyano-
4-hydroxycinnamic acid, collagenase, rat tail collagen type I, amino acid
standards, FLAG peptide, anti-FLAG M2 affinity gel, and the glucose
Trinder assay kit were purchased from Sigma. Human insulin (Ac-
trapid) was obtained from Novo Nordisk. The total cellular RNA extrac-
tion reagent (Trizol), tobacco etch virus (TEV) protease, mammalian
expression vector pcDNA3.1(+), and prokaryotic expression vector
pPROEX-HTb were from IntronVec. The QuikChange site-directed
mutagenesis kit was from Stratagene. BCA protein assay reagent was
from Pierce. The Immu-Blot kit for glycoprotein detection was from
Bio-Rad. FuGENE 6 transfection reagent, trypsin, Asp-N endoprotein-
ases, and the enhanced chemiluminescence detection system (ECL)
were from Roche Molecular Biochemicals. The Ni²⁺-nitrilotriacetic ac-
id-agarose column was from Qiagen Inc. All consumables for two-
dimensional gel electrophoresis, [1-3H]galactose, and [1-3H]glucose
were the products of Amersham Biosciences. All amino acid analysis
reagents and Cal Mix 2 calibration standards for mass spectrometer
were from Applied Biosystems.

Differentiation of 3T3-L1 Cells and Concentration of Proteins in the
Cell Culture Medium—3T3-L1 cells were maintained as subconfluent
cultures in Dulbecco’s modified Eagle’s medium (DMEM) 1 supple-
mented with 10% fetal calf serum. For differentiation, post-confluent
cells were induced by incubation with 0.25 µM dexamethasone, 0.5 mM
3-isobutyl-1-methylxanthine, and 10 µg/ml insulin for 2 days. This was
followed by incubation with 10 µg/ml insulin for 2 days. The cells were
then maintained in DMEM with 10% fetal calf serum for another 4
days.

To harvest proteins secreted by adipocytes, the cells at day 8 follow-
ing differentiation were washed three times with phosphate-buffered saline and then incubated with serum-free medium for another 4 h. The
medium was collected, centrifuged at 3000 × g for 10 min, filtered
through a 0.20-µm filter, and then concentrated and desalted using a
concentrator with a molecular mass cutoff of 5000 Da (Vivascience Ltd.). The proteins were quantitated using BCA reagent and stored at
-80 °C until used.

Two-dimensional Gel Electrophoresis, Immunoblotting, and Carbo-
hydrate Detection—The proteins secreted by either adipocytes or
3T3-L1 preadipocytes were separated by two-dimensional gel electrophoresis as described previously (24). The separated proteins were
stained with either silver or Coomassie Brilliant Blue R-250. For immu-
noblotting, proteins separated by two-dimensional gel electrophore-
sis were transferred to nitrocellulose membranes using a Multiphor II
Novablot electrophoretic transfer unit (Amersham Biosciences). The
membranes were blocked and then incubated with rabbit anti-adiponec-
tin polyclonal antibody (1:1000) overnight at 4 °C. After incubation with
horse-radish peroxidase-conjugated secondary antibody for 1 h at room
temperature, the bound antibodies were detected using the ECL detec-
tion kit. Glycoproteins were detected using the commercial Immu-Blot
kit according to the manufacturer’s instruction.

In-gel Trypsin Digestion and Reversed-phase High Performance Liq-
uid Chromatography (RP-HPLC)—Protein spots of interest separated
by two-dimensional gel electrophoresis were excised and subjected to
in-gel trypsin digestion as described previously (25). The extracted
tryptic peptide mixtures were fractionated by RP-HPLC on a Jupiter 5µ
C18 column (250 × 2.00 mm; Phenomenex Inc.). The prewarmed column
(37 °C) was washed for 7 min with 0.1% (v/v) trifluoroacetic acid, fol-
lowed by elution using a 50-min linear gradient of 8–36% acetonitrile at
a flow rate of 200 µl/min. Each fraction was collected manually and
subjected to further analysis as described below. 3H-Labeled glycopep-
tides were detected by liquid scintillation counting.

Amino Acid Sequencing and Amino Acid Analysis—Protein spots
separated by two-dimensional gel electrophoresis were transferred to
polyvinylidene difluoride membrane, stained with Coomassie Brilliant
Blue R-250, excised, and subjected to amino acid sequencing by Edman
degradation using a PerkinElmer Life Sciences Procise Model 492 pro-
tein sequencer. Internal amino acid sequences were obtained by se-
quencing the tryptic peptides following RP-HPLC fractionation.

For amino acid analysis, 5 µg of the tryptic peptides was vacuum-
dried and hydrolyzed in the gas phase with 6 M HCl and 1% phenol for

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1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s
medium; RP-HPLC, reversed-phase high performance liquid chroma-
tography; MALDI-TOF-MS, matrix-assisted laser desorption ioniza-
tion time-of-flight mass spectrometry.

FIG. 1. Endogenous adiponectin secreted by 3T3-L1 adipocytes exists as
eight isoforms. The medium from subconfluent 3T3-L1 preadipocytes (A) or
adipocytes at day 8 after induction of differ-
entiation (B) was collected and concen-
trated as described under “Experimental
Procedures.” 50 µg of proteins from each
sample was separated by two-dimen-
sional gel electrophoresis and visualized
by silver staining. The proteins preferen-
tially secreted by adipocytes are denoted
by numbered arrows. Secretory proteins
from adipocytes separated by two-dimen-
sional gel electrophoresis as described
above were detected using rabbit anti-adip-
onectin antibody (C) or the Immu-Blot
carbohydrate detection kit (D). Note that
all eight indicated proteins are immuno-
reactive with anti-adiponectin antibody.
Six of the eight isoforms (spots 3–8) of
adiponectin are glycosylated.
This treatment destroyed sugar residues, but still permitted detection and quantitation of hydroxylysine and hydroxyproline (26). Free amino acid residues were dissolved in 40 \mu l of 0.025% K_2EDTA, derivatized with phenyl isothiocyanate, separated on a Spheri-5 PTC 5 \mu m column (220 \times 2.1 mm), and analyzed with an Applied Biosystems Model 421 amino acid analyzer.

Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) Analysis—0.5 \mu l of the tryptic peptide mixtures or RP-HPLC-separated peptides was mixed with an equal amount of \alpha-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 60% acetonitrile and 0.3% trifluoroacetic acid), spotted onto the sample plates, and air-dried. Reflectron mass spectrometric analyses were performed on a Voyager DE PRO biospectrometry workstation (Applied Biosystems) using a pulsed laser beam (nitrogen laser, \lambda = 337 nm). All ion spectra were recorded in the positive mode with an accelerating voltage of 20.0 kV. The spectrometer was externally calibrated using Cal Mix 2 standard mixture.

Cloning, Recombinant Expression, and Purification of Adiponectin and Its Variants—Total RNA was obtained from 3T3-L1 adipocytes using Trizol reagent according to the manufacturer’s instructions. Reverse transcription-PCR was performed based on the mouse adiponectin nucleotide sequence (GenBankTM/EBI accession number U37222). Full-length adiponectin cDNA was then inserted into the pGEMT-easy vector, and its sequence was verified by DNA sequencing. The protein sequence was counted starting from the methionine residue.

For prokaryotic expression, the mouse adiponectin cDNA sequence was amplified using 5'-ATCGGGATCCGAAGATGACGTTACTACATCA-3' as the sense primer and 5'-TACGAATTCTCAGTTGGTATCATGGTAGAG-3' as the antisense primer. The BamHI/SalI fragment of the amplified DNA product was subcloned into the pPROEX-HTb plasmid, resulting in expression vector pPRO-His-Ad, which encodes full-length adiponectin with a His_6 tag at its N terminus. A similar strategy was used for the construction of prokaryotic expression vector pPRO-His-gAd, which expresses the His_6-tagged globular region of adiponectin (amino acid residues 110–247), except that the sense primer used was 5'-ATCGGGATCCCGCGGTAGATTGTACTACAC-3'. The expression of His-tagged full-length adiponectin or its globular region in BL21 cells was induced by the addition of 1 mM of isopropyl-\beta-D-thiogalactopyranoside to the growth medium. Full-length adiponectin or its globular region was purified from the bacterial lysates using the Ni^{2+}-nitrilo-
type I-coated 12-well plates in DMEM with 10% fetal bovine serum, 2 mM L-glutamine, 10 μM dexamethasone, and 10 μg/ml insulin at a density of 5 × 10^6 cells/well. The cells were allowed to adhere to the cell culture dishes for 24 h and then incubated overnight in DMEM with 5.5 mM glucose and no insulin or dexamethasone. Subsequently, the cells were stimulated with different concentrations of insulin and/or adiponectin for another 24 h. The medium was then replaced with 0.5 ml of glucose-free DMEM without phenol red and supplemented with 5 mM each alanine, valine, glycine, pyruvate, and lactate. After incubation for 6 h, the glucose level in the medium was measured using the glucose Trinder assay kit.

### RESULTS

**Adiponectin Secreted by Adipocytes Exists as Multiple Isoforms—**Two-dimensional gel electrophoresis analysis identified eight protein spots that were preferentially expressed and secreted by adipocytes, and not from undifferentiated 3T3-L1 preadipocytes (Fig. 1, A and B). N-terminal amino acid sequence analysis revealed that all of these proteins (spots 1–8) share an identical N-terminal sequence (EDDVTTTE), which unequivocally matches amino acid residues 18–25 of mouse adiponectin, a secretory protein expressed exclusively by adipocytes (5, 7). This sequenced fragment (EDDVTTTE) is located immediately after the hypothetical signal peptide cleavage site, suggesting that the heterogeneous isoforms of adiponectin are not caused by different protease cleavage during its secretion. The identities of these proteins as adiponectin were further confirmed by Western blot analysis, which showed that all eight proteins were immunoreactive to an antibody against mouse adiponectin (Fig. 1C). Two-dimensional gel electrophoresis separation of recombinant adiponectin produced by *E. coli* detected only a single spot (data not shown), suggesting that the existence of multiple isoforms of adiponectin produced by adipocytes is due to post-translational modification occurring during its secretion. Two-dimensional gel electrophoresis analysis of recombinant adiponectin transiently expressed and secreted by COS-7 cells also demonstrated multiple isoforms of this protein, a pattern similar to that in adipocytes (data not shown).

Carbohydrate-based detection of proteins separated by two-dimensional gel electrophoresis revealed that six isoforms of adiponectin (spots 3–8) derived from adipocytes are glycosylated (Fig. 1D), suggesting that glycosylation may at least partly contribute to the heterogeneity of adiponectin. Although there are two consensus N-linked glycosylation sites (Asn25 and Asn236), treatment with tunicamycin, an inhibitor of N-linked glycosylation (28), did not affect the glycosylation pattern (data not shown), thus excluding the possibility of N-linked glycosylation of adiponectin. A previous study using endoglycosidase H treatment also suggested that no N-glycosylation occurs on adiponectin (5). Furthermore, there were no potential serine and threonine residues predicted to be O-glycosylated using the NetOGlyc Version 2.0 prediction server (29), which produces network predictions of mucin-type O-glycosylation sites in mammalian proteins.

**Glycosylation of Adiponectin Occurs at Several Conserved Lysine Residues in the Collagenous Domain—**To further characterize the nature of the glycosylation and to map the glycosylation sites of adiponectin, tryptic peptide mixtures from each isoform of adiponectin derived from adipocytes, transiently transfected COS-7 cells, or *E. coli* were analyzed by MALDI-TOF-MS. Comparison of the mass spectra for these samples detected three prominent peptide fragments (with masses of 1679, 4260, and 4276 Da, respectively) that existed only in the six glycosylated isoforms, but not in the two unglycosylated isoforms or in adiponectin produced by *E. coli* (Fig. 2). Moreover, the masses of these three tryptic peptide fragments did not match any of the unmodified tryptic fragments of adi-
ponectin, indicating that glycosylation of adiponectin may occur in these three fragments.

To isolate these three peptide fragments, the tryptic peptide mixtures from all of the glycosylated isoforms were pooled and separated by RP-HPLC, and each fraction was analyzed by MALDI-TOF-MS (Fig. 3). This analysis found that fraction A, which eluted at 16.4% acetonitrile, contains the peptide with a mass of 1679 Da. The peptides with masses of 4276 and 4260 Da were detected in fractions B and C, which eluted at 18 and 18.4% acetonitrile, respectively. Amino acid sequence analysis identified the peptide with a mass of 1679 Da as KGEPGEAAAYYVR, a fragment corresponding to amino acid residues 104–115 of mouse adiponectin. The peptides with masses of 4276 and 4260 Da were derived from the same fragment (DGTPGKEKGKDAEGLLGPGETGDVGMTGAEGPR), which matches amino acid residues 62–95 of adiponectin. Notably, amino acid sequence analysis easily detected all of the amino acid residues in these three peptide fragments, except for the four lysine residues (lysine 104 in the peptide with a mass of 1679 Da and lysines 68, 71, and 80 in the peptides with masses of 4260 and 4276 Da). This result indicates that these lysine residues might be modified by hydrophilic groups such as carbohydrates so that the hydrophilic amino acid derivatives could not be efficiently extracted in nonpolar solvent by conventional liquid-phase sequencing. The conclusion that these four lysine residues are modified was supported by the observation that they were resistant to digestion with trypsin, a proteinase that specifically cleaves at the C terminus of either arginine or lysine residues. Interestingly, these four lysines (residues 68, 71, 80, and 104) are located in the collagenous domain of adiponectin, with surrounding motifs of G\(\times\)KGE(D). Sequence alignment revealed that these four lysines and their surrounding motifs are highly conserved across all of the available adiponectin sequences (Fig. 4).

To verify that these four lysines were modified, the three peptides purified above were further subjected to amino acid analysis following hydrolysis with 6 M HCl at 110°C for 24 h. The results showed the absence of lysine residues at the predicted positions in the elution gradient, although all of the other amino acid residues were detected with the expected molar ratios (Fig. 5). Further analysis of these spectra revealed that all of the lysine residues in these three peptides are hydroxylated. A hydroxylated proline residue was also detected in peptide B. This hydroxyproline was subsequently assigned to Pro\(^{94}\) (see below).

Because hydroxylation and subsequent glycosylation of hydroxylysine to form α-1,2-glucosylgalactosyl-O-hydroxylysine have previously been described in several secretory proteins with collagen-like domains (30, 31), we speculate that the same type of modification may occur on the four lysines (residues 68, 71, 80, and 104) in the three tryptic peptides isolated above. This speculation was supported by analysis of the MALDI-TOF-MS data for these three peptides (Fig. 3). For peptide A, the difference between the experimentally observed mass (1679 Da) and the calculated mass of KGEPGEAAAYYVR was 8 Da. This difference could be explained by a hydroxylysine residue. For peptides B and C, the differences were 58 and 42 Da, respectively. These differences could be explained by the addition of a carbohydrate, which is consistent with the MALDI-TOF-MS data. The conclusion that these four lysines are modified was further supported by analysis of the MALDI-TOF-MS data for these three peptides (Fig. 3). For peptide A, the difference between the experimentally observed mass (1679 Da) and the calculated mass of KGEPGEAAAYYVR was 8 Da. This difference could be explained by a hydroxylysine residue. For peptides B and C, the differences were 58 and 42 Da, respectively. These differences could be explained by the addition of a carbohydrate, which is consistent with the MALDI-TOF-MS data.

**Fig. 4.** The four modified lysines (residues 68, 71, 80, and 104) in the collagenous domain of adiponectin are conserved across all of the species investigated. The mouse, human, bovine, monkey, and dog adiponectin sequences relate to GenBank\textsuperscript{TM}/EBI accession numbers BAB22597, NP_004788, AAK58902, AAK92202, and AAL09702, respectively. The four modified lysines and their surrounding motifs are shaded.

**Fig. 5.** Amino acid analysis of the three tryptic peptides separated in Fig. 3. Peptide A (with a mass of 1679 Da), peptide B (with a mass of 4276 Da), and peptide C (with a mass of 4260 Da) were digested with 6 M HCl at 110°C for 24 h and subjected to amino acid analysis as described under “Experimental Procedures.” Trace a, amino acid standard; trace b, hydroxyproline standard; trace c, hydroxysine standard; trace d, hydrolysate of peptide A; trace e, hydrolysate of peptide B; trace f, hydrolysate of peptide C. The arrows and star indicate the peaks of hydroxysine (OH-K) and hydroxyproline (OH-P), respectively.
Da) and the theoretical mass (1339 Da) is 340 Da, which is exactly the mass of a glucosylgalactosylhydroxyl group. The experimentally observed mass for peptide C differs from its predicted mass by 1020 Da, an expected mass for three glucosylgalactosylhydroxyl groups that may attach to the three lysines (residues 68, 71, and 80) in peptide C. The experimentally observed mass of peptide B (4276 Da) differs from its theoretical mass by 1036 Da, which is the expected size for three glucosylgalactosylhydroxyl groups plus another hydroxyl group.

The assumption that each lysine in peptides B and C has an attached glycoside group of 340 Da was further supported by digestion of these two peptides with endoproteinase Asp-N, which specifically cleaves at the N terminus of asparagine.
MALDI-TOF-MS analysis showed that the experimentally observed mass for the fragment containing Lys^{68} is 340 Da larger than its theoretical mass, whereas the actual mass for the fragment containing Lys^{68} and Lys^{71} differs from its theoretical mass by 680 Da (Fig. 6). This result also indicated that an extra hydroxylation in peptide B occurred at proline 94. Hydroxylation of proline 94 was also verified by amino acid analysis and amino acid sequencing.

To further verify that the glycosides attached to the four lysine residues are modified by glucosylgalactosyl groups, COS-7 cells transiently expressing FLAG-tagged adiponectin were radiolabeled with [3H]galactose or [3H]glucose. The tryptic peptide mixtures from unlabeled or radiolabeled adiponectin were separated by RP-HPLC as described in the legend to Fig. 3 to obtain peptides A–C. The fractions containing each peptide were subjected to liquid scintillation counting.

MALDI-TOF-MS analysis showed that the experimentally observed mass for the fragment containing Lys^{68} is 340 Da larger than its theoretical mass, whereas the actual mass for the fragment containing Lys^{68} and Lys^{71} differs from its theoretical mass by 680 Da (Fig. 6). This result also indicated that an extra hydroxylation in peptide B occurred at proline 94. Hydroxylation of proline 94 was also verified by amino acid analysis and amino acid sequencing.

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**Substitution of the Four Lysines (Residues 68, 71, 80, and 104) in the Collagenous Domain of Adiponectin Attenuates Its Insulin-sensitizing Activity**—To investigate whether the glycosylation of the four hydroxylated lysines affects the biological activities of adiponectin, we generated a construct (pcDNA-Ad(K→R)-F) encoding an adiponectin variant in which the four lysine residues have been replaced with arginines. SDS-PAGE analysis revealed that wild-type adiponectin secreted by COS-7 cells migrated as three bands with slightly different molecular masses (Fig. 8). The upper two bands, which accounted for ~85% of the total adiponectin, were glycosylated. In contrast, the Lys-to-Arg adiponectin variant consisted mainly of a single unglycosylated band that migrated with a lower apparent molecular mass than the two major glycosylated bands of wide-type adiponectin. This result further confirms that glycosylation of adiponectin mainly occurs at the four lysine residues in the collagenous domain.

Two recent studies have demonstrated that adiponectin can enhance the action of insulin to inhibit hepatic glucose production (9, 32). Consistent with these reports, our results showed that insulin at a concentration of 50 μM did not significantly affect glucose production in primary rat hepatocytes (Fig. 9, upper panel). Half-maximal suppression by insulin was observed at a concentration of 200 μM. The ability of subphysiological concentrations of insulin to suppress hepatic glucose production was significantly enhanced by adiponectin produced by mammalian cells. In the presence of 20 μg/ml adiponectin, 50 μM insulin dramatically decreased glucose production by 40 ± 3%. A concentration dependence study revealed that the EC_{50} of adiponectin is at a level of ~4 μg/ml (Fig. 9, lower panel), a concentration within the physiological range of adiponectin (15, 16). Compared with wild-type adiponectin, the insulin-sensitizing ability of the Lys-to-Arg adiponectin variant on hepatic gluconeogenesis was significantly attenuated. In the presence of the adiponectin variant at 4 μg/ml, 50 μM insulin showed no significant effect on glucose production and caused only a 13 ± 1% decrease in the presence of this protein at 20 μg/ml. Bacterially generated full-length adiponectin (Fig. 9) and the globular region (data not shown) were biologically ineffective in enhancing the hepatic action of insulin to suppress gluconeogenesis.

**DISCUSSION**

Although several recent reports independently demonstrated an antidiabetic role of adiponectin, it is still controversial as to which form of adiponectin is functionally active. Studies by Lodish and co-workers (10) and Kadowaki and coworkers (11) found that a truncated fragment corresponding to the globular domain of adiponectin is effective in decreasing hyperglycaemia and restoring insulin resistance, whereas bacterially produced full-length adiponectin shows no activity. Although these findings are certainly of pharmacological interest, their physiological relevance remains uncertain. The preponderance of plasma adiponectin exists as a full-length protein with an apparent molecular mass of 30 kDa (5, 6). We were unable to detect any proteolytic fragments of adiponectin in...
Our two-dimensional gel electrophoresis analysis revealed that adiponectin secreted by adipocytes is extensively modified into multiple isoforms with different pI values and molecular masses and that this heterogeneity can be explained at least partly by glycosylation (Fig. 1). Comparison of the mass spectra of the unglycosylated and glycosylated isoforms allowed us to identify the four conserved lysines (residues 68, 71, 80, and 104) in the collagenous domain as potential glycosylation sites (Fig. 2). The conclusion that these four lysines are glycosylated was further supported by the following evidence. First, these four lysines were not able to be identified during sequencing and were also resistant to trypsin cleavage, indicating that they may well be modified. Second, amino acid analysis revealed that all four lysines were hydroxylated (Fig. 5). Third, the glycosylation of adiponectin was substantially decreased following substitution of these four lysines with arginines (Fig. 8) or following treatment with \( \alpha,\alpha'-\text{dipyridyl} \), a hydroxylase inhibitor (data not shown). Notably, hydroxyllysyl glycosylation of these four sites was detected in all six major glycosylated isoforms, which account for >85% of the total adiponectin secreted by adipocytes, suggesting that glycosylation is one of the major post-translational modifications occurring in adiponectin.

Hydroxylation of lysine and subsequent glycosylation with galactose and glucose to form \( \alpha,1,2\)-glucosylgalactosyl-O-hydroxylysine have been previously observed in several secretory proteins with collagen-like domains, including complement component C1q and pulmonary surfactant proteins (30). Although the exact carbohydrate composition remains to be further defined, we have obtained evidence suggesting that the glycosides attached to the four lysines are possibly glucosylgalactosyl groups. Mass spectrometric analysis indicated that the mass of the glycoside group at lysines 80 and 104 is 340 Da, an expected size for a glucosylgalactoses-hydroxyd residue (Fig. 6). Radiolabeling experiments also revealed that the glycosides contain both \( ^{3}H \)galactose and \( ^{3}H \)glucose (Fig. 7).

Mutational analysis revealed that substitution of the four conserved lysines with arginines in the collagenous domain significantly attenuated the ability of subphysiological concentrations of adiponectin to enhance the hepatic action of insulin to suppress glucose production (Fig. 9). This result indicates that these four lysines in the collagenous domain are critically involved in the insulin-sensitizing action of adiponectin and also suggests that hydroxylation or the glycosides attached to these residues might be functionally important. The mechanisms by which these four hydroxyllysine residues or their attached glycosides in the collagenous domain enhance the insulin-sensitizing effect of adiponectin remain to be defined. The insulin-sensitizing ability of the adiponectin variants in which only one of the four lysine residues was replaced with arginine was much lower than that of wild-type adiponectin, but significantly higher than that of the variant with mutations at all four sites (data not shown), suggesting that the hydroxylinesines or the attached glycosides might function in a cooperative manner. Hydroxylation and glycosylation might be critical for the three-dimensional structure required for the full biological activity of the adiponectin molecule. The lack of hydroxylation and/or glycosylation might destabilize the collagen-like stalk and thus interfere with the formation of high order complexes. It is also possible that hydroxyl or carbohydrate groups directly participate in ligand-receptor interaction. These possibilities are currently under investigation in our laboratory.

In summary, this study demonstrates that the four lysines (residues 68, 71, 80, and 104) in the collagenous domain of adiponectin are critical for its insulin-sensitizing activity with
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respect to inhibition of hepatic glucose production. These four lysine residues were found to be hydroxylated and glycosylated, thus emphasizing the important role of post-translational modifications in the biological activities of adiponectin. The existence of multiple isoforms of adiponectin secreted by adipocytes (Fig. 1) implies that there might be other post-translational modifications that could also be functionally relevant. Indeed, a recent study suggested the presence of disialic acid residues as modifying groups in adiponectin (33). Further characterization of other post-translational modifications occurring in adiponectin will shed new light on the molecular mechanisms underlying its biological activities.

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REFERENCES

1. Bradley, R. L., Cleveland, K. A., and Cheatham, B. (2001) Recent Prog. Horm. Res. 56, 329–358
2. Frühbeck, G., Gomez-Ambrosi, J., Muruzabal, F. J., and Burrell, M. A. (2001) Am. J. Physiol. 280, E827–E847
3. Kim, S., and Moustaïd-Moussa, N. (2000) J. Nutr. 130, 3110S–3115S
4. Steppan, C. M., and Lazar, M. A. (2002) Trends Endocrinol. Metab. 13, 18–23
5. Scherer, P. E., Williams, S., Fogliano, M., Baldini, G., and Lodish, H. F. (1995) J. Biol. Chem. 270, 26746–26749
6. Nakano, Y., Tohe, T., Choi-Miura, N. H., Mazda, T., and Tomita, M. (1996) J. Biochem. (Tokyo) 120, 803–812
7. Hu, E., Liang, P., and Spiegelman, B. M. (1996) J. Biol. Chem. 271, 10697–10703
8. Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y., and Matsubara, K. (1996) Biochem. Biophys. Res. Commun. 221, 286–289
9. Berg, A. H., Combs, T. P., Da, X., Brownlee, M., and Scherer, P. E. (2001) Nat. Med. 7, 947–953
10. Frueh, J., Tsao, T. S., Javorschi, S., Ebets-Reed, D., Erickson, M. R., Yen, F. T., Bihain, B. E., and Lodish, H. F. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2005–2010
11. Maemura, T., Kanon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsubo, M., Nakamura, Y., Gavrilova, O., Vinson, C., Reitman, M. L., Kagechiha, H., Shudo, K., Yoda, M., Nakano, Y., Tohe, T., Nakanishi, K., Shimomura, I., Tomita, M., Prugel, P., and Kadowaki, T. (2001) Nat. Med. 7, 941–946
12. Saito, K., Tohe, T., Minoshima, S., Asakawa, S., Sumiya, J., Yoda, M., Nakano, Y., Shimizu, N., and Tomita, M. (1999) Gene (Amst.) 239, 67–73
13. Takahashi, M., Arita, Y., Yamagata, K., Matsuzawa, Y., Okutomi, K., Horie, M., Shimomura, I., Hotta, K., Kuriyama, H., Kihara, S., Nakamura, T., Yamashita, S., Funahashi, T., and Matsuzawa, Y. (2000) Int. J. Obes. 24, 861–868
14. Das, K., Lin, Y., Widen, E., Zhang, Y. H., and Scherer, P. E. (2001) Biochem. Biophys. Res. Commun. 280, 1120–1129
15. Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyao, K., Kuriyama, H., Nishida, M., Yamashita, S., Okubo, K., Matsuba, K., Muraguchi, M., Ohmoto, Y., Funahashi, T., and Matsuzawa, Y. (1999) Biochem. Biophys. Res. Commun. 257, 79–83
16. Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwashashi, K., Kuriyama, H., Ouchi, N., Maeda, K., Nishida, M., Kihara, S., Sakai, N., Nakajima, T., Hasegawa, K., Muruguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Hanafusa, T., and Matsuzawa, Y. (2000) Arte- riolel. Thromb. Vasc. Biol. 20, 1595–1599
17. Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R. E., and Tataranni, P. A. (2001) J. Clin. Endocrinol. Metab. 86, 1930–1935
18. Statnick, M. A., Beavers, L. S., Connor, L. J., Corominola, H., Johnson, D., Hammond, C. D., Rafaello-Pfahil, R., Seng, T., Suter, T. M., Sluka, J. P., Ravussin, E., Gadski, R. A., and Caro, J. F. (2000) Int. J. Exp. Diabetes Res. 1, 81–88
19. Hotta, K., Funahashi, T., Bokkin, N. L., Ortmeier, H. K., Arita, Y., Hansen, B. C., and Matsuzawa, Y. (2001) Diabetes 50, 1126–1133
20. Yang, W. S., Lee, W. J., Funahashi, T., Tanaka, S., Matsuzawa, Y., Chao, C. L., Chen, C. L., Tsu, T. Y., and Chuaung, L. M. (2001) J. Clin. Endocrinol. Metab. 86, 3815–3819
21. Combatsiaris, T. P., Tanen, M., Berger, J., Zhang, B., Doebber, T., Surwii, R., Petro, A., Scherer, P. E., and Moller, D. E. (2001) Diabetes 50, A271–A272
22. Maeda, N., Takahashi, M., Funahashi, T., Kihara, S., Nishizawa, H., Kishida, K., Nagaretani, H., Matsuda, M., Komuro, R., Ouchi, N., Kuriyama, H., Hotta, K., Nakamura, T., Shimomura, I., and Matsuzawa, Y. (2001) Diabetes 50, 2094–2099
23. Shapiro, L., and Scherer, P. E. (1998) Curr. Biol. 8, 353–358
24. Wang, Y., Xu, A., Ye, J., Kraegen, E. W., Tse, C. A., and Cooper, G. J. (2001) Diabetes 50, 1821–1827
25. Wang, Y., Xu, A., Pearson, R. B., and Cooper, G. J. (1999) FEBS Lett. 462, 25–30
26. Johnson, R. G., Stollery, J., Keeley, F. W., and Herbert, M. A. (1980) Clin. Orthop. Relat. Res. 152–208
27. Leffert, L. H., Koch, K. S., Moran, T., and Williams, M. (1979) Methods Enzymol. 58, 536–544
28. Wu, G. C., Lai, H. L., Chin, Y. W., Chu, Y. T., and Cheng, Y. (2001) J. Biol. Chem. 276, 35450–35457
29. Hansen, J. E., Lund, O., Tolstrup, N., Gooley, A. A., Williams, K. L., and Brunak, S. (1998) Glycoconj. J. 15, 115–130
30. Colley, K. J., and Baenziger, J. U. (1997) J. Biol. Chem. 272, 10290–10295
31. Shinkai, H., and Yonemasu, K. (1979) Biochim. Biophys. Acta 579, 1120–1129
32. Combs, T. P., Berg, A. H., Obici, S., Scherer, P. E., and Rossetti, L. (2001) J. Clin. Invest. 108, 1875–1881
33. Satoh, C., Yaukawa, Z., Honda, N., Matsuda, T., and Kitajima, K. (2001) J. Biol. Chem. 276, 28849–28856
