0888-8809/06/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 20(7):1673–1687
Copyright © 2006 by The Endocrine Society
doi: 10.1210/me.2005-0390

Adiponectin Multimerization Is Dependent on Conserved Lysines in the Collagenous Domain: Evidence for Regulation of Multimerization by Alterations in Posttranslational Modifications

Ayanthi A. Richards, Tim Stephens, Hayley K. Charlton, Alun Jones, Graeme A. Macdonald, Johannes B. Prins, and Jonathan P. Whitehead

Centre for Diabetes and Endocrine Research (A.A.R., T.S., H.K.C., G.A.M., J.B.P., J.P.W.), School of Medicine, University of Queensland, Princess Alexandra Hospital, Brisbane, Queensland 4102, Australia; and Institute for Molecular Bioscience (A.J.), University of Queensland, Brisbane, Queensland 4072, Australia

Adiponectin is a secreted, multimeric protein with insulin-sensitizing, antiatherogenic, and antiinflammatory properties. Serum adiponectin consists of trimer, hexamer, and larger high-molecular-weight (HMW) multimers, and these HMW multimers appear to be the more bioactive forms. Multimer composition of adiponectin appears to be regulated; however, the molecular mechanisms involved are unknown. We hypothesize that regulation of adiponectin multimerization and secretion occurs via changes in posttranslational modifications (PTMs). Although a structural role for intertrimer disulfide bonds in the formation of hexamers and HMW multimers is established, the role of other PTMs is unknown. PTMs identified in murine and bovine adiponectin include hydroxylation of multiple conserved proline and lysine residues and glycosylation of hydroxylysines. By mass spectrometry, we confirmed the presence of these PTMs in human adiponectin and identified three additional hydroxylations on Pro71, Pro76, and Pro95. We also investigated the role of the five modified lysines in multimer formation and secretion of recombinant human adiponectin expressed in mammalian cell lines. Mutation of modified lysines in the collagenous domain prevented formation of HMW multimers, whereas a pharmacological inhibitor of prolyl- and lysyl-hydroxylases, 2,2′-dipyridyl, inhibited formation of hexamers and HMW multimers. Bacterially expressed human adiponectin displayed a complete lack of differentially modified isoforms and failed to form *bona fide* trimers and larger multimers. Finally, glucose-induced increases in HMW multimer production from human adipose explants correlated with changes in the two-dimensional electrophoresis profile of adiponectin isoforms. Collectively, these data suggest that adiponectin multimer composition is affected by changes in PTM in response to physiological factors. (*Molecular Endocrinology* 20: 1673–1687, 2006)

THE PLASMA PROTEIN adiponectin is an adipocyte-secreted peptide hormone with demonstrated antidiabetic, antiatherogenic, and antiinflammatory properties (reviewed in Ref. 1). Adiponectin is secreted, and exists in the circulation, as a series of multimers ranging from homotrimers to disulfide-dependent hexamers and high-molecular-weight (HMW) multimers (2). Early reports demonstrated that circulating total adiponectin levels were reduced in states of obesity, insulin resistance, and diabetes. More recently, it has emerged that HMW multimers are the more bioactive species (3) and that the ratio of HMW to total adiponectin, rather than total levels alone, provides the closest correlation with measures of insulin sensitivity, particularly hepatic insulin sensitivity (3–6). Furthermore, a number of naturally occurring mutations in adiponectin have been identified in patients with type 2 diabetes. In transient transfection studies, several of these mutant forms of adiponectin exhibit defective formation and/or secretion of HMW multimers (2, 7). Reduced serum levels of HMW adiponectin are also associated with coronary artery disease (8), whereas the vascular protective effects of adiponectin were shown to be restricted to the HMW component (8).

First Published Online February 23, 2006

Abbreviations: BFA, Brefeldin A; BMI, body mass index; CHO, Chinese hamster ovary; CSL, core-specific lectin; 2DE, two-dimensional electrophoresis; DTT, dithiothreitol; ER, endoplasmic reticulum; HA, hemagglutinin; HEK, human embryonic kidney; HMW, high molecular weight; IPTG, isopropyl-β-D-thiogalactopyranoside; LC, liquid chromatography; LMW, low molecular weight; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS, mass spectrometry; PTM, posttranslational modification; RT, room temperature; WT, wild type.

Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

A number of agents are now recognized regulators of adiponectin expression and multimer composition. Thiazolidinediones, a class of peroxisome proliferator activated receptor γ agonists commonly used in antidiabetic therapy, increase adipocyte expression of adiponectin [via a peroxisome proliferator activated receptor γ response element (9)] and simultaneously increase the proportion of circulating HMW multimers (3). Testosterone, although not affecting intracellular production of adiponectin or multimer composition, appears to selectively retard secretion of HMW multimers from adipocytes (10). This is in keeping with the observed sexual dimorphism in circulating adiponectin levels and multimer composition, with males having reduced levels of total and HMW adiponectin (11, 12). What remains to be elucidated are the molecular mechanisms underlying the regulation of adiponectin multimer composition and secretion.

The adiponectin protein consists of three structural domains (Fig. 1A); the N-terminal variable region (of greatest amino acid sequence divergence between species), the collagenous domain (named so because of its sequence and structural homology with collagen), and the C-terminal globular (receptor-binding/effector) domain (13). Biochemical analyses of adiponectin multimers have revealed the importance of two kinds of molecular interaction that are integral to their structure (see Fig. 1B). First, the protein forms homotrimers via the noncovalent interactions of the collagenous domains in a triple helix. Hydrophobic interactions between globular domains may also contribute to trimer stability and help to initiate triple helix formation (14). Early biochemical studies demonstrated that temperatures over 70 C resulted in breakdown of intratrimer interactions (2). Second, intermolecular disulfide bonds, involving C_{39} in the variable

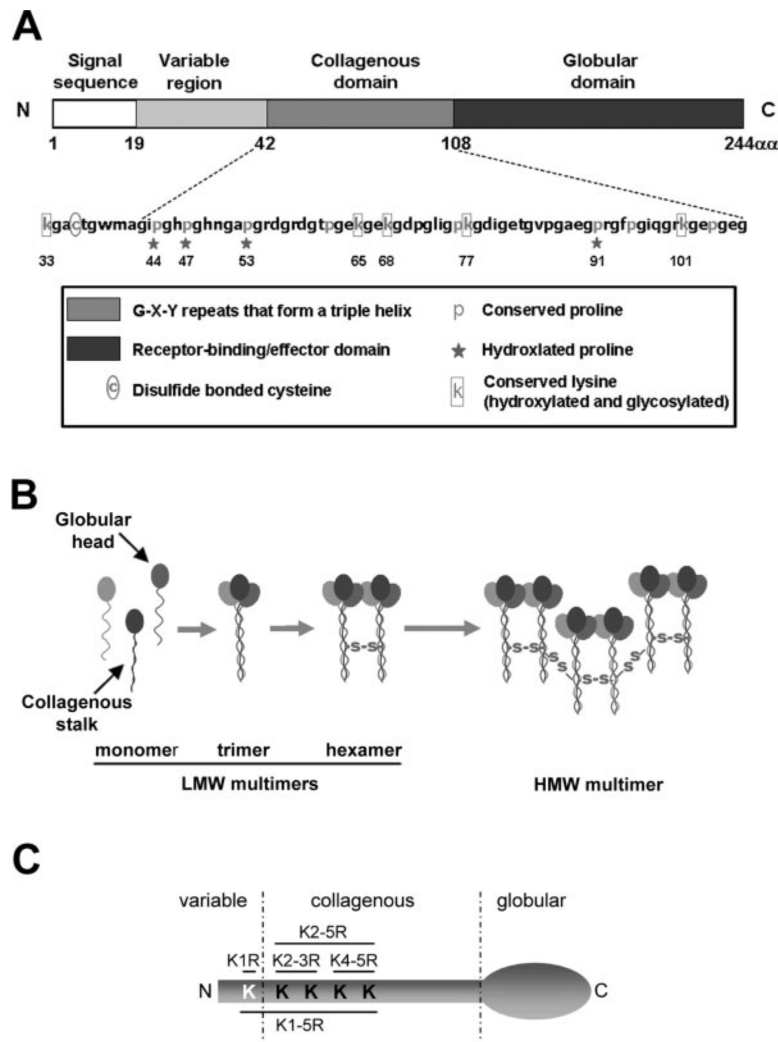


Fig. 1. Structure of Human Adiponectin

A, Structural domains of human adiponectin. Known PTMs are indicated. B, Schematic representation of adiponectin multimers showing triple helix and disulfide bond interactions. C, Conserved, hydroxylated and glycosylated lysine residues (K) in the variable and collagenous domains of human adiponectin that were substituted with arginines. Nomenclature of the various mutants (K1R, K2–3R, K4–5R, and K1–5R) is shown. N, Amino terminus; C, carboxyl terminus, $\alpha\alpha$, amino acids.

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

domain of the murine protein (C_{36} of human adiponectin), provide the intertrimer links within hexamers and HMW species. Amino acid substitution of this Cys residue produces a protein unable to form multimers larger than trimer, and reduction of disulfide bonds in wild-type (WT) adiponectin similarly collapses hexamer and HMW species to trimer (2, 11, 15). Furthermore, boiling of purified trimers, hexamers, and HMW multimers, resulting in denaturation of each to their composite monomers and dimers, revealed that hexamers and HMW species, unlike trimers, are composed almost exclusively of disulfide-linked dimers, whereas trimers displayed a 1:1 ratio of monomer to dimer (15).

Adiponectin also undergoes posttranslational modifications (PTMs) other than disulfide bonds. The presence of an α2,8-linked disialic acid moiety (Neu5Aca2→8Neu5Aca2→3Gal) has been reported, although the site(s) and structural and functional significance of this modification have not been determined (16). Wang *et al.* (17, 18) identified four conserved prolines in the collagenous domain that undergo hydroxylation, and five conserved lysines (one in the variable region and four in the collagenous domain) that are hydroxylated and subsequently glycosylated (see Fig. 1A). The same investigators showed that conservative amino acid substitution of the collagenous domain lysines in murine adiponectin (17) produced a variant that was no longer glycosylated and was largely unable to enhance insulin action in hepatocytes. In collagen, proline hydroxylation and lysine hydroxylation and glycosylation are known to have an important structural role in triple helix formation, secretion, and stability (19). This, together with the selective requirement of HMW forms for the hepatic effects of adiponectin, prompted us to examine the role of proline hydroxylation and lysine hydroxylation/glycosylation in multimerization of human adiponectin.

In the current study, we have employed site-directed mutagenesis and pharmacological approaches in combination with biochemical analyses to assess the role of PTMs, particularly lysine hydroxylation/glycosylation and proline hydroxylation, in adiponectin multimerization and secretion. Our findings indicate an essential requirement for lysine hydroxylation and glycosylation in the formation of HMW multimers, and provide the first evidence that regulation of HMW multimer production by adipocytes may occur via alterations in PTM of adiponectin.

RESULTS

Mass Spectrometry (MS) Analysis of Purified Recombinant Human Adiponectin

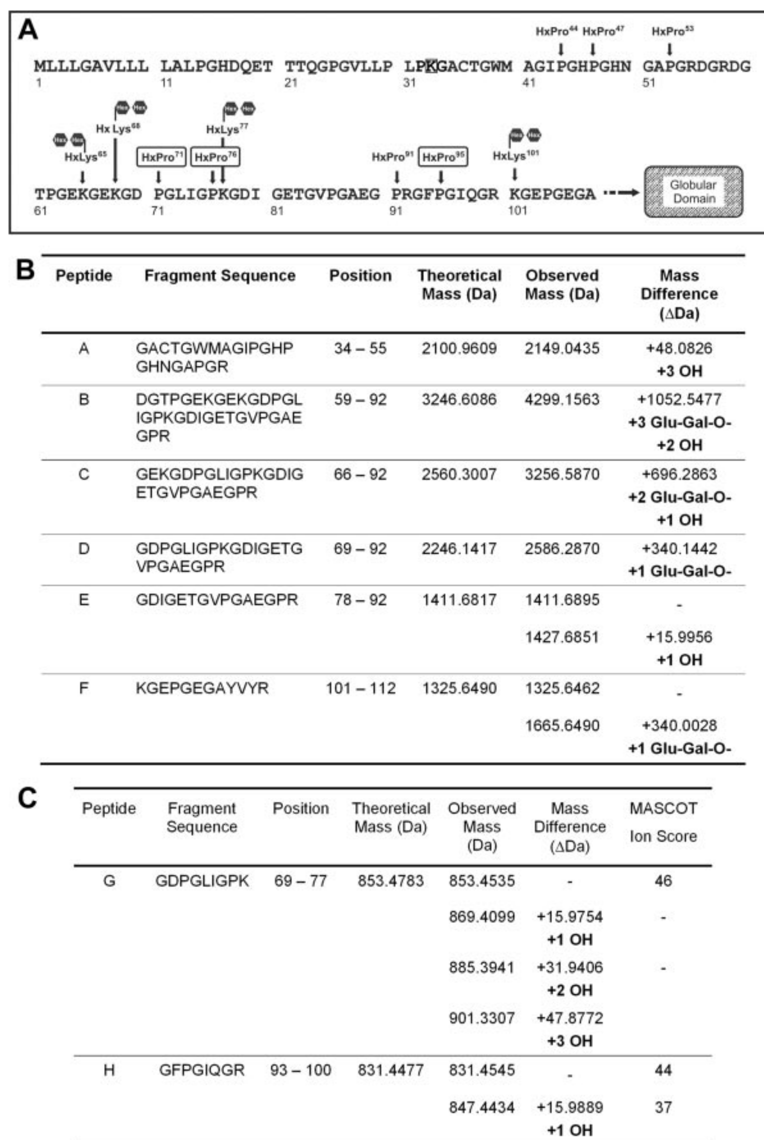
To directly investigate the PTMs in human adiponectin, and compare them with those identified in the mouse and bovine proteins, we subjected tryptic digests of purified human embryonic kidney (HEK)-produced recombinant adiponectin to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), MALDI-TOF-TOF, and liquid chromatography (LC)/MS/MS analysis. An adiponectin mass fingerprint was produced spanning over 74% of the protein sequence. Comparison of the experimentally derived mass spectrum with the predicted spectrum revealed several modified peptides with mass changes of +16 and +340 Da corresponding to the addition of a hydroxyl or a glucosylgalactosyl-hydroxyl moiety, respectively. Using this approach, we have been able to confirm the presence of all of the hydroxy-proline and glucosylgalactosyl hydroxy-lysine residues previously identified in mouse and bovine adiponectin (17, 18), except for modification of Lys33, which was not observed, probably due to poor representation of the N terminus of the protein in the peptide mass fingerprint (Fig. 2, A and B). Importantly, we observed peptides resulting from differential cleavage at Lys65, Lys68, and Lys77 (Fig. 2B; peptides B, C, D, and E). Trypsin cleavage occurs at C-terminal peptide bonds of lysine and hydroxylysine but not glucosylgalactosyl hydroxy-lysine residues (20). Thus, our fingerprint data provide clear evidence of differential glycosylation of Lys65, Lys68, and Lys77. Lys101 in peptide F was also variably glycosylated (Fig. 2B).

In addition to the previously described modifications, MALDI-TOF analysis suggested the addition of hydroxyl groups at Pro71, Pro76, and Pro95 (Fig. 2C). MS/MS sequencing demonstrated that Pro95, situated within the peptide GFPGIQGR, was present in both the unmodified and hydroxylated form (Fig. 2C, peptide H). Residues Pro71 and Pro76 are located within the peptide GDPGLIGPK (Fig. 2C, peptide G). Analysis of the fingerprint data for this peptide revealed masses of 853 Da (base), 869 Da (base + 16 Da), 885 Da (base + 32 Da), and 901 Da (base + 48 Da), and MALDI-TOF-TOF peptide sequencing confirmed the identity of the base peptide. Together, these data suggest that peptide GDPGLIGPK is differentially hydroxylated at Pro71, Pro76, and Lys77.

Competent Expression and Assembly of Human Adiponectin Multimers in CHO and HEK Cells

Waki *et al.* (2) first characterized the multimer composition of adiponectin from mouse and human sera by SDS-PAGE of non-reduced and non-heat-denatured samples. These analyses clearly demonstrated the existence of multiple HMW species of human adiponectin compared with a single HMW band in mouse serum. Subsequent studies have focused on the murine protein, and have often employed the well-characterized 3T3-L1 murine adipocyte cell line. The lack of an analogous human adipocyte cell line necessitated the creation of a surrogate expression system that would also be easily amenable to genetic manipulation. WT human adiponectin cDNA was cloned from adipose tissue mRNA by RT-PCR and stably transfected into

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022



B

Peptide	Fragment Sequence	Position	Theoretical Mass (Da)	Observed Mass (Da)	Mass Difference (ΔDa)
A	GACTGWMAGIPGHP GHNGAPGR	34 – 55	2100.9609	2149.0435	+48.0826 **+3 OH**
B	DGTPGEKGEKGDPGL IGPKGDIGETGVPGAE GPR	59 – 92	3246.6086	4299.1563	+1052.5477 **+3 Glu-Gal-O- +2 OH**
C	GEKGDPGLIGPKGDIG ETGVPGAEGPR	66 – 92	2560.3007	3256.5870	+696.2863 **+2 Glu-Gal-O- +1 OH**
D	GDPGLIGPKGDIGETG VPGAEGPR	69 – 92	2246.1417	2586.2870	+340.1442 **+1 Glu-Gal-O-**
E	GDIGETGVPGAEGPR	78 – 92	1411.6817	1411.6895	-
				1427.6851	+15.9956 **+1 OH**
F	KGEPGEGAYVYR	101 – 112	1325.6490	1325.6462	-
				1665.6490	+340.0028 **+1 Glu-Gal-O-**

C

Peptide	Fragment Sequence	Position	Theoretical Mass (Da)	Observed Mass (Da)	Mass Difference (ΔDa)	MASCOT Ion Score
G	GDPGLIGPK	69 – 77	853.4783	853.4535	-	46
				869.4099	+15.9754 **+1 OH**	-
				885.3941	+31.9406 **+2 OH**	-
				901.3307	+47.8772 **+3 OH**	
H	GFPGIQGR	93 – 100	831.4477	831.4545	-	44
				847.4434	+15.9889 **+1 OH**	37

Fig. 2. Mass Spectroscopy of Human Adiponectin

A, Schematic of the collagenous domain of human adiponectin showing all modifications identified by MS analysis of tryptic fragments. In addition to the four known hydroxy-proline residues (44, 47, 53, and 91), three additional proline residues (71, 76, and 95) were found to be similarly modified (*boxed*). Additionally, the four known glycosylated hydroxy-lysines in the collagenous domain were identified, but not that in the variable domain (*framed*). B, Identification, by peptide mass fingerprinting, of known modifications that are conserved in human adiponectin. Peptides A and E demonstrate hydroxylation of prolines 44, 47, 53, and 91, as shown previously for mouse and bovine adiponectin. Peptides C, D, and E are all derivatives of peptide B that result from differential glycosylation of hydroxylysines 65, 68, and 77. Peptide F also demonstrates variable glycosylation of hydroxylysine 101. C, Identification of three novel hydroxyprolines in human adiponectin. The unmodified form of peptide G was identified by its mass fingerprint and then confirmed by sequencing using MALDI-TOF-TOF. Mass fingerprints were also identified for variants of this peptide containing one, two, or three hydroxyl groups (most probably at Pro71, Pro76, and Lys77). Proline 95 in peptide H was similarly identified to be variably hydroxylated. Peptide sequence data were scored using the MASCOT ion score algorithm to produce a standardized probabilistic measure of confidence.

both HEK and Chinese hamster ovary (CHO) cell lines. HEK cells have been used previously for adiponectin production (3, 11), whereas CHO cells were demonstrated to efficiently produce the core-specific lectin (CSL) or mannose-binding protein, a closely related, multimeric plasma protein secreted by hepatocytes (21, 22). Analysis of secreted adiponectin from CHO and HEK stable cell lines revealed comparable multimer profiles (Fig. 3A, lanes 3 and 4). A C-terminally hemagglutinin (HA)-tagged construct was also generated, and the tagged protein was demonstrated to form similar multimers to untagged adiponectin (Fig. 3A, lanes 4 and 5). Comparison of secreted adiponectin from CHO (lane 3) and HEK (lane 4) stable lines with conditioned media

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

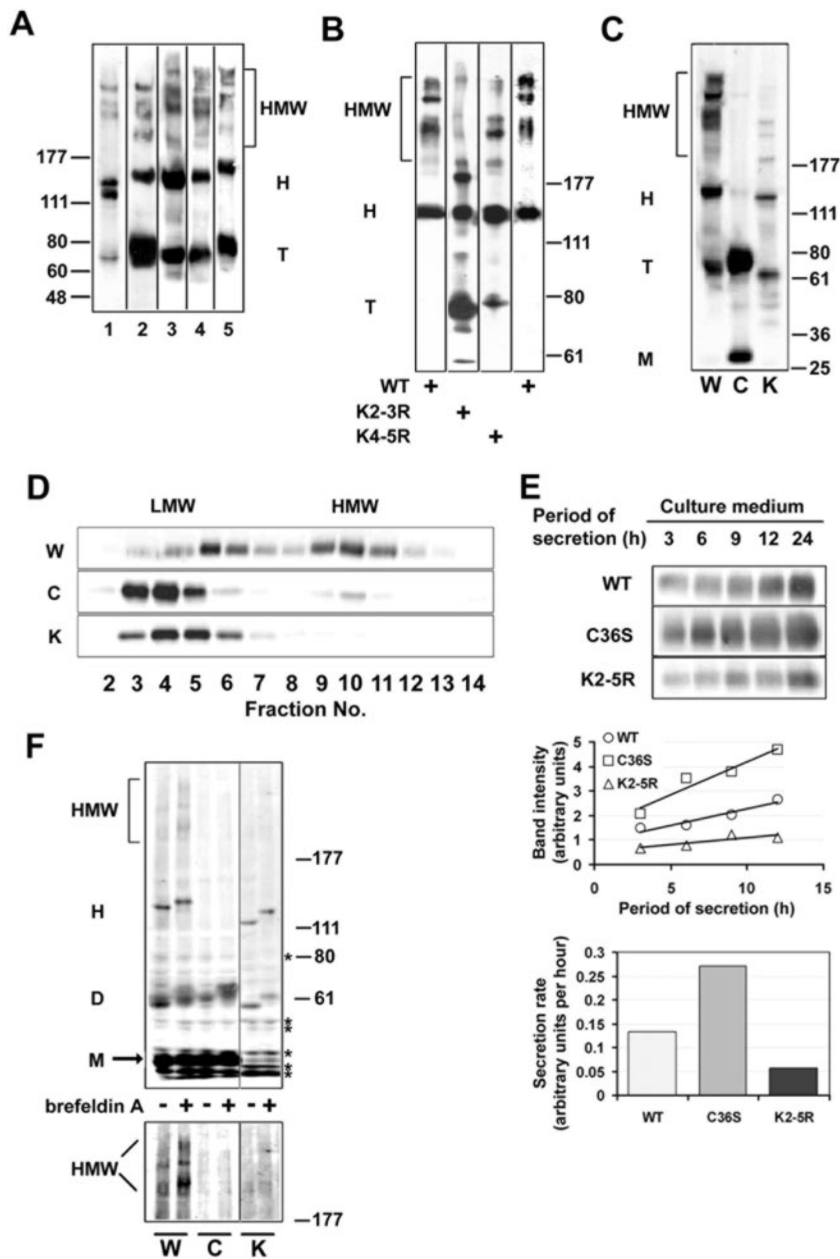


Fig. 3. Conservative Substitution of Hydroxylated and Glycosylated Lysine Residues in the Collagenous Domain Impairs HMW Multimer Formation and Secretion

A, Comparison of human adiponectin multimers in serum (lane 1) with those secreted by *in vitro*-differentiated human primary adipocytes (lane 2) and stably transfected CHO (lane 3) and HEK (lane 4) cells, after analysis by non-reducing SDS-PAGE on gradient gels. Lane 5 shows C-terminally HA-tagged adiponectin, from stable HEK transfectants, which forms similar multimers to those of the untagged protein (lane 4). B, Adiponectin multimer composition of conditioned serum-free medium from CHO cells transiently expressing WT adiponectin, K2–3R, or K4–5R after analysis as in A. C, Adiponectin multimer composition of conditioned medium from CHO stable cell lines expressing WT (W), C36S (C), or K2–5R (K) analyzed as in A. D, Crude fractionation of LMW and HMW multimers by velocity sedimentation analysis of the samples in C. E, Secretion kinetics of HA-tagged WT, C36S, and K2–5R adiponectin from stably expressing HEK cell lines. Cells were allowed to secrete into serum-free medium for various periods of time before harvest of medium, precipitation (using trichloroacetic acid), and analysis by SDS-PAGE and immunoblotting for HA. A representative experiment (of three independent experiments) is shown. F, CHO cells stably expressing WT (W), C36S (C), or K2–5R (K) were cultured in the presence or absence of 5 μg/ml BFA for 5 h. Monomer (M), dimer (D), and hexamer (H) are readily apparent in lysates of WT stables with the HMW species accumulating upon inhibition of secretion by BFA. Trimer failed to accumulate intracellularly under any condition even in C36S lysates. HMW species are lacking in K2–5R lysates even after inhibition of secretion with BFA. The *lower panel* shows longer exposure of the *upper region of the main panel*, demonstrating HMW multimers in cells expressing WT adiponectin but not the two mutant forms. *Asterisks* mark nonspecific bands. Molecular weight markers are indicated. W, WT; C, C36S; K, K2–5R; H, hexamer; T, trimer; D, dimer; M, monomer.

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

from *in vitro*-differentiated primary human adipocytes (lane 2) and with human serum (lane 1) revealed a similar profile and distribution of multimers, although the hexamer species consistently appeared as a doublet in serum (Fig. 3A). These stable lines were therefore employed for our subsequent investigations.

HMW Multimer Formation Is Dependent on Conserved, Hydroxylated, and Glycosylated Lysine Residues in the Collagenous Domain

Although the structural role of the disulfide-forming cysteine residue in the variable region of adiponectin is now well established (2, 11, 15), the role of other PTMs in multimer formation has not been addressed. Conserved proline and lysine residues in the variable and collagenous domains of the protein are known to undergo hydroxylation, which, in the case of lysines, is followed by addition of a glucosylgalactosyl moiety (17, 18). Four of the five conserved and modified lysines occur in the collagenous domain of the protein, and conservative substitution of these in murine adiponectin severely compromised activity of the protein in primary hepatocytes (17). However, the mechanism behind this loss of function was not examined. We therefore investigated the role of these four modified lysines, and the fifth modified lysine in the variable domain of the protein, in multimerization of human adiponectin. Mutants containing K-to-R substitution of the first (K1R), second and third (K2–3R), fourth and fifth (K4–5R), second to fifth (K2–5R), or first to fifth (K1–5R) residues (see Fig. 1C) were generated by site-directed mutagenesis and expressed transiently or stably in CHO cells. Transfected cells were cultured in serum-free medium, and secreted adiponectin was analyzed for multimer composition on gradient gels. Conservative substitution of either the first two (K2–3R) or last two (K4–5R) lysines in the collagenous domain resulted in impaired HMW multimer production and a greater proportion of trimer than was seen in WT adiponectin from transient transfections (Fig. 3B). The phenotype was more severe in K2–3R, suggesting that these two lysines may serve a more critical function than the fourth and fifth lysines. Mutation of all four lysines in the collagenous domain (K2–5R) had an additive effect, resulting in a complete loss of HMW multimers, and this was confirmed by velocity sedimentation on sucrose gradients (Fig. 3, C and D). The disulfide-incompetent mutant, C36S, formed only trimers as has been reported for the corresponding mutant of murine adiponectin (C39S) (2, 11, 15). Comparison of the secretion rates of WT and mutant adiponectin also revealed striking differences, the K2–5R being secreted with approximately 50% reduced efficiency, whereas the C36S was secreted at 1.5–2 times the rate of WT (Fig. 3E). It was therefore possible that the loss of HMW multimers in secreted K2–5R was due to a failure in secretion of these species rather than a failure in their intracellular formation. To address this, lysates from stable cell lines were analyzed for the presence of HMW multimers after 5-h culture in the presence of brefeldin A (BFA) to inhibit secretion and accumulate the protein in the secretory pathway (23–25). Although an accumulation of HMW multimers was apparent after BFA treatment of WT-expressing cells, K2–5R cell lysates were devoid of HMW multimers in both BFA-treated and untreated cells (Fig. 3F), indicating a defect in formation of these larger multimers by the mutant protein. A decrease in mobility of all species in lysates was routinely observed after culture in BFA and may result from altered processing due to redistribution of Golgi enzymes into the endoplasmic reticulum (ER) with BFA-induced fusion of the two organelles (24, 25). Intriguingly, no intracellular accumulation of trimer was observed under any condition in the stable cell lines or in primary human adipocytes (data not shown) but did occur after overexpression by transient transfection (data not shown). Analysis of the KR1 mutant containing substitution of the single lysine in the variable domain revealed no apparent effect on multimerization compared with WT (Fig. 4). Similarly, no exacerbation of the quadruple mutant (K2–5R) phenotype was observed upon mutation of all five lysines (K1–5R) (Fig. 4). These observations demonstrate an important functional role for the conserved hydroxylated and glycosylated lysine residues, selectively in the collagenous domain of adiponectin, in the intracellular assembly and secretion of HMW multimers.

Inhibition of Proline and Lysine Hydroxylation by 2,2′-Dipyridyl Inhibits Multimerization

In a complementary approach to the mutagenesis studies described above, we employed a pharmaco-

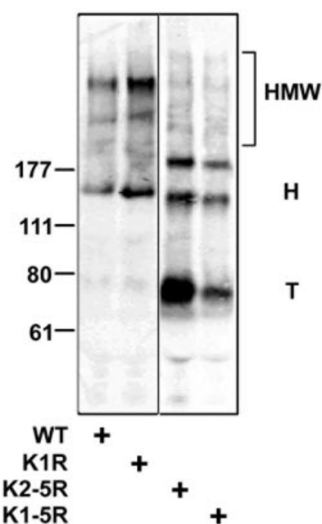


Fig. 4. Substitution of a Hydroxylated and Glycosylated Lysine Residue in the Variable Domain Has No Effect on Multimerization

Adiponectin multimer composition of conditioned, serum-free medium from CHO cells transiently expressing WT adiponectin, K1R, K2–5R, or K1–5R as analyzed by SDS-PAGE on gradient gels. H, Hexamer; T, trimer.

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

logical inhibitor of prolyl- and lysyl-hydroxylases, 2,2′-dipyridyl. This agent has been used previously to demonstrate hydroxylation and subsequent glycosylation of lysine residues in the CSL (21). 2,2′-Dipyridyl treatment of primary rat hepatocytes was shown to inhibit secretion of CSL, and this was thought to occur as a consequence of failed proline and lysine hydroxylation and, thus, subsequent glycosylation of hydroxylysines (26). CHO cells stably expressing WT adiponectin were depleted of intracellular adiponectin by treatment with cycloheximide for 20 h. The ribosomal inhibitor was then removed, and protein synthesis was allowed to resume for 3 h in the presence or absence of BFA or 2,2′-dipyridyl. Analysis of lysates revealed a complete absence of hexamer and HMW species in 2,2′-dipyridyl-treated cells, although these multimers were seen in WT samples and accumulated upon inhibition of secretion with BFA (Fig. 5A). Similar results were obtained in primary human adipocytes (data not shown). Analysis of medium samples from the same experiment, after reduction and heat denaturation, revealed a partial inhibition of adiponectin secretion by 2,2′-dipyridyl (Fig. 5A), in agreement with the previously observed effects of the drug on CSL secretion (26). By comparison, BFA completely inhibited secretion (Fig. 5A). Multimer analysis of medium after a longer (24 h) period of secretion revealed that adiponectin secreted from 2,2′-dipyridyl-treated cells is predominantly in the form of trimer (Fig. 5B). Note that multimers in 2,2′-dipyridyl-treated cells migrate more rapidly than those from untreated and BFA-treated cells, consistent with a loss of PTMs with 2,2′-dipyridyl (Fig. 5B). Thus, inhibition of proline hydroxylation in addition to lysine hydroxylation resulted in a more severe impairment of multimer formation, with loss of both hexamer and HMW species. The observed inhibition of adiponectin secretion by 2,2′-dipyridyl mirrors that observed for the K2–5R mutant and is likely due to the loss of hydroxyproline and glucosylgalactosyl hydroxy-lysine residues. However, we cannot exclude the possibility that nonspecific/indirect effects of the drug may also compromise adiponectin multimerization and secretion. Indeed, fragmentation of the Golgi apparatus was observed by immunofluorescence labeling for the *cis*/medial Golgi marker, GM130, and may well be accompanied by a widespread disruption to ER-to-Golgi transport and/or exocytic vesicle traffic (Fig. 5C). However, dispersion of the Golgi marker was also apparent in BFA-treated cells (Fig. 5C), where formation of HMW adiponectin multimers was uncompromised.

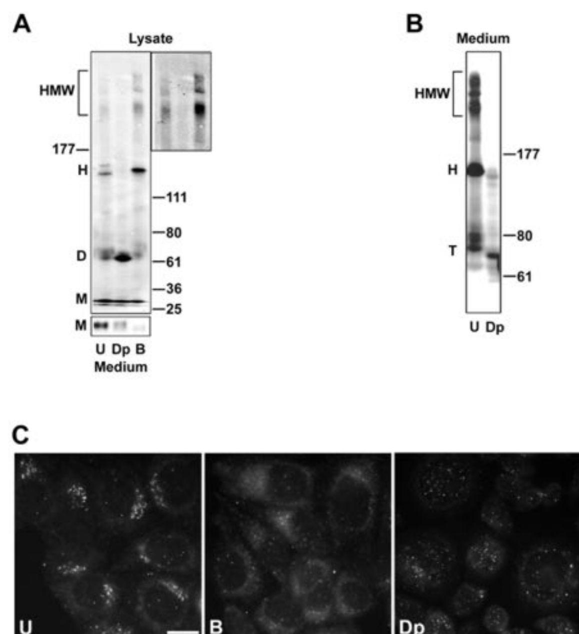


Fig. 5. 2,2′-Dipyridyl-Mediated Inhibition of Prolyl- and Lysyl-Hydroxylases Impairs Adiponectin Multimerization and Secretion

A, CHO cells stably expressing WT human adiponectin were cultured in cycloheximide for 20 h to deplete intracellular adiponectin before washing to remove cycloheximide. Cells were then either left untreated (U) or treated with 3.5 mM 2,2′-dipyridyl (Dp) or 5 μg/ml BFA (B) and allowed to secrete newly synthesized adiponectin into serum-free medium containing the same dose of drug. Lysates and medium were harvested after 3 h for analysis under non-reducing, non-heat-denaturing or reducing, denaturing conditions, respectively. Panel to the *right* shows a longer exposure of the corresponding region of the main blot. B, Multimer composition of conditioned medium from cells treated as in A but allowed to secrete for 24 h. C, Cells treated as in A were immunofluorescently labeled for the *cis*/medial Golgi marker, GM130. U, Untreated; Dp, 2,2′-dipyridyl-treated; B, BFA-treated; H, hexamer; T, trimer; D, dimer; M, monomer. *Scale bar*, 10 μm.

Adiponectin Expressed in Bacteria Lacks PTMs and Fails to Form Trimers, Hexamers, and HMW Multimers

To further examine the role of PTMs in multimer formation of human adiponectin, we expressed the protein in bacteria, which lack the capacity to perform many of the PTMs of eukaryotic cells. Human adiponectin lacking the 18-amino acid mammalian signal peptide sequence was expressed in BL21(DE3)pLys-S cells after induction with isopropyl-β-D-thiogalactopyranoside (IPTG). Cell extracts were fractionated into soluble and particulate components, and the soluble fraction was analyzed under various conditions alongside CHO-produced WT adiponectin (CHO-Ad) (Fig. 6). In all analyses, the bacterially produced protein exhibited significantly greater mobility than CHO-Ad, consistent with a lack of eukaryotic PTMs (Fig. 6A, compare lanes 4 and 6). When samples were boiled to disassemble trimers and larger multimers to their constituent monomers and dimers (2), CHO-Ad was present almost exclusively as dimer. In contrast, bacterially produced adiponectin was largely monomeric with only a trace of dimer (Fig. 6A). Analysis of the same samples after both reduction and boiling revealed complete conversion of the CHO-Ad dimer to

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

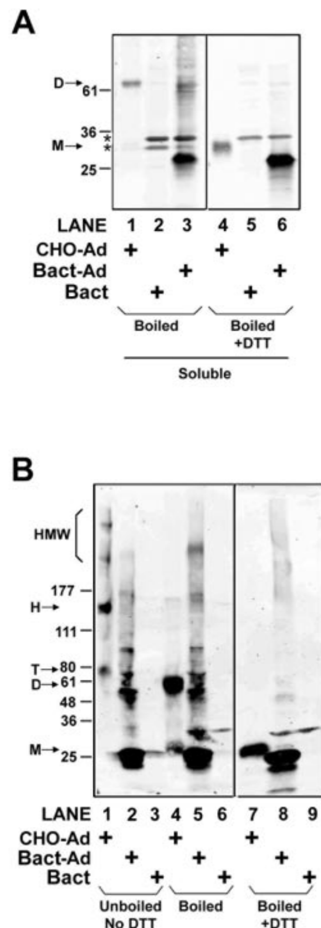


Fig. 6. Bacterially Expressed Human Adiponectin Fails to Form Multimers Despite Formation of Disulfide Bonds

The soluble fraction of lysate from IPTG-induced bacterial cells expressing human adiponectin (Bact-Ad) was analyzed alongside the same lysate fraction from nontransformed, induced cells (Bact) and the CHO-expressed protein (CHO-Ad) after boiling alone or boiling and reduction with DTT (A). Whereas the CHO-Ad exists predominantly as dimer after boiling alone, Bact-Ad is predominantly monomer with a small proportion of dimer, which is lost upon treatment with DTT. Bands present in Bact samples were labeled with *asterisks* as nonspecific. B, Multimer analysis of the same samples under non-reducing and non-heat-denaturing conditions on gradient gels reveals much larger species in addition to monomer and dimer in Bact-Ad. Unlike the multimers present in CHO-Ad, the larger species in Bact-Ad were resistant to boiling, despite being similarly sensitive to DTT. Multimers of CHO-Ad are indicated. H, Hexamer; T, trimer; D, dimer; M, monomer.

monomer as well as loss of the bacterial adiponectin dimer species (Fig. 6A). Thus, disulfide bond formation in the bacterially expressed protein appears to be inefficient, at least in the absence of periplasmic targeting. Furthermore, upon analysis of multimer composition on gradient gels, trimer, hexamer, and HMW species were obviously lacking in the bacterially expressed protein, despite the presence of some minor bands in the 60- to 250-kDa size range (Fig. 6B). These species, although disulfide dependent, failed to display the biochemical characteristics of adiponectin multimers from mammalian cells (CHO-Ad), because they were insensitive to boiling (Fig. 6B). Instead, they may represent chaperone-bound intermediates or aggregates. The failure of bacterially expressed adiponectin to multimerize is consistent with the importance of PTMs in this process and has notable implications for functional studies using recombinant adiponectin expressed in prokaryotic systems.

Isoform Composition of WT, C36S, and K2–5R CHO-Ad

Two-dimensional electrophoresis (2DE) of 3T3-L1-produced (murine) adiponectin revealed eight differentially modified isoforms of the protein, of which six isoforms were glycosylated (17). Similarly, bovine serum adiponectin has also been shown to exist as eight to nine isoforms (18). We therefore employed the same technique to determine the isoform composition of WT and mutant human adiponectin secreted from stably expressing CHO cell lines. Western blotting for adiponectin revealed a train of seven isoforms of decreasing pI and mobility in WT CHO-Ad (Fig. 7). At least as many isoforms were apparent in the C36S and K2–5R mutants, as well as in WT CHO-Ad secreted from dipyridyl-treated cells. (Fig. 7). While WT and C36S samples appeared as a single train of spots, a second train of spots was prominent in both K2–5R mutant and WT CHO-Ad upon dipyridyl treatment.

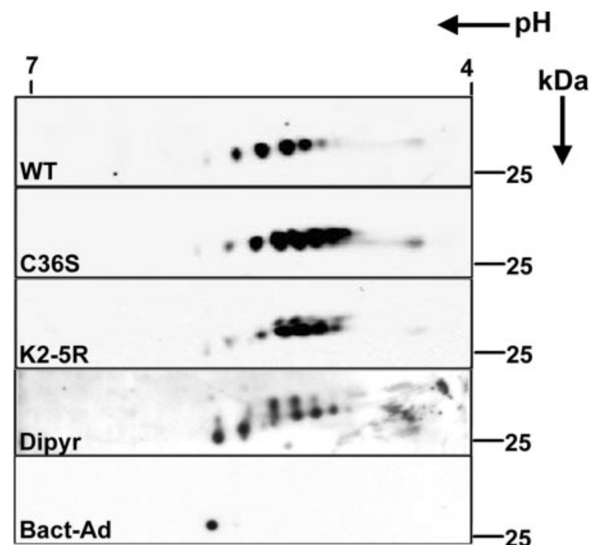


Fig. 7. 2DE Analysis of WT and Mutant Human Adiponectin Produced by CHO Stable Cell Lines and Bacterially Expressed WT Adiponectin

Conditioned serum-free medium from CHO stable cell lines expressing WT adiponectin, C36S, K2–5R, or WT adiponectin after pretreatment with 2,2′-dipyridyl (Dipyr), and bacterially expressed WT adiponectin (Bact-Ad) were analyzed by 2DE and Western blotting to determine the composition of differentially modified isoforms.

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

This was accompanied by an increase in the more basic isoforms after dipyridyl treatment, possibly resulting from the loss of acidic hydroxyproline residues. As expected, the loss of neutral sugars in the K2–5R mutant did not effect a dramatic change in pI of adiponectin isoforms. Moreover, the existence of this mutant as a full complement of pI variants indicates that other modifications contributing to pI can still occur in the absence of these glycosylation events. Human adiponectin expressed in bacteria was present as a single isoform with a pI similar to the most basic of the WT CHO-Ad isoforms, consistent with a lack of PTMs.

Changes in PTMs Are Coincident with Glucose-Induced Increase in HMW Adiponectin

The selective reduction in circulating HMW adiponectin observed in states of insulin resistance (3) and the common incidence of high blood glucose levels under these conditions led us to examine the effects of a high physiological glucose concentration (15 mM) on the multimer composition of adiponectin produced by human adipose tissue explants cultured *in vitro*. Subcutaneous tissue explants, from 15 subjects (4 males, 11 females) undergoing open-abdominal surgery, were cultured in triplicate for 72 h in serum-free medium containing either 5 or 15 mM glucose. Multimer composition of secreted adiponectin in medium samples was subsequently analyzed using gradient gels and by velocity sedimentation. Contrary to our expectations, in no case did high glucose result in a decrease in total adiponectin or the proportion of HMW adiponectin secreted. In fat tissue from 12 individuals, high glucose had no effect (data not shown). However, surprisingly, in tissue from three female individuals, glucose induced an increase in total adiponectin secreted (data not shown) with a predominant increase in the proportion of HMW multimers (Fig. 8, A and B). To determine whether a change in PTM of the protein may be involved in this response to high glucose, we analyzed the samples by 2DE followed by Western blotting. Adiponectin secreted from the glucose-responsive tissue explants displayed a clearly different pattern of isoforms under high glucose conditions compared with low glucose conditions, whereas no such change was apparent in samples from nonresponsive tissue explants also from female individuals (Fig. 8C). These observations indicate a role for differential PTM of adiponectin in the regulation of its multimer composition by environmental factors during production by adipose tissue under physiologically relevant conditions.

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

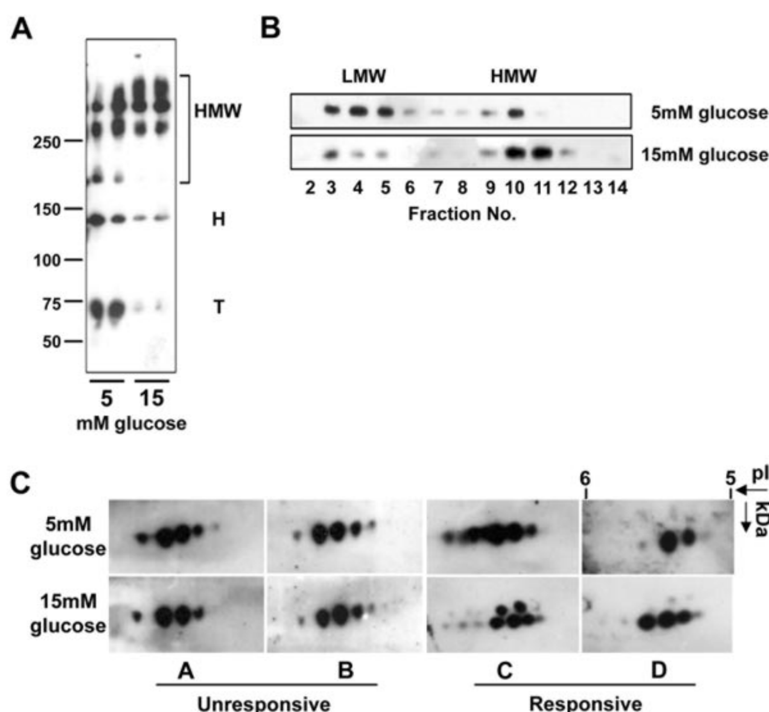


Fig. 8. Glucose-Mediated Changes in Adiponectin Multimer Composition Are Accompanied by Changes in PTMs

A, Adiponectin multimer composition of conditioned serum-free medium after incubation of sc adipose explants in 5 or 15 mM glucose for 72 h. Duplicate samples from one of three different glucose-responsive tissues are shown after SDS-PAGE on a gradient gel and Western blotting. B, Crude fractionation of LMW and HMW multimers by velocity sedimentation (of the samples in A) confirms a selective increase in the proportion of HMW multimers (HMW/total adiponectin) in medium from explants cultured in 15 mM glucose. C, 2DE analysis of explant-conditioned medium samples from two glucose-responsive and two nonresponsive tissue samples. An alteration in the pattern of adiponectin isoforms is evident in samples from the glucose-responsive tissues but not in samples from nonresponsive tissues after culture in 15 mM glucose.

DISCUSSION

In this study, we have investigated the role of conserved lysines and PTMs in multimerization of human adiponectin and, in particular, formation of HMW multimers. Analyses to date reveal two types of interactions necessary for the integrity of adiponectin multimers: noncovalent interactions of the triple helical collagenous domains within trimers, and intertrimer disulfide bonds via a cysteine residue in the variable domain. We have now identified an additional level of control in assembly of HMW multimers involving four hydroxylated and glycosylated lysine residues in the collagenous domain. Our demonstration that conservative amino acid substitution of different combinations of these lysines has a more- or less-severe effect on the efficiency of HMW formation reveals one potential mechanism whereby HMW production may be regulated: by variation of the number of modified lysines. Indeed, mass spectrometric analysis of tryptic peptides revealed differential modification of these lysine residues in HEK-produced recombinant human adiponectin.

We have also attempted to address the function of hydroxyprolines in the collagenous domain of human adiponectin with the use of an inhibitor of both lysine and proline hydroxylation, 2′,2-dipyridyl. Although we observed an additive effect of this drug over that of mutating the five modified lysine residues, interpretation is made difficult by the clear disruption of the Golgi compartment in treated cells. It is notable, however, that BFA treatment, which completely inhibits secretion and causes fusion of the Golgi compartment with the ER, did not inhibit multimerization of adiponectin.

As a third approach, we expressed human adiponectin in bacteria to produce a minimally modified protein lacking proline and lysine hydroxylation as well as any other eukaryotic PTMs. 2DE analysis revealed a single isoform for the bacterially expressed protein, confirming the loss of differential PTMs. By comparison, adiponectin produced by CHO cells consisted of seven or more pI variants of increasing acidity and molecular weight. The lack of multimerization of the bacterially expressed protein was not immediately apparent, however, because disulfide-dependent HMW species were observed by non-reducing, non-heat-denaturing SDS-PAGE. However, the resistance of these species to heat denaturation, while CHO-expressed adiponectin multimers were readily susceptible, is indicative of aberrant intermolecular interactions unlike those that characterize *bona fide* multimers (2). Thus, we conclude that these larger species occur as artifacts of bacterial expression. Early characterizations of bacterially expressed adiponectin demonstrated apparent trimer and hexamer (15, 27) and even HMW species (28), either by gel filtration (15, 27) or SDS-PAGE after chemical cross-linking (27–29). Tsao *et al.* (15) also determined that the apparent hexamer species was disulfide dependent by reduction with dithiothreitol (DTT). However, more extensive ultrastructural or biochemical characterization of these species was not performed. The failure of bacterially expressed adiponectin to form even *bona fide* trimers has important implications for functional studies that have employed bacterially produced recombinant adiponectin and provides an explanation for the discrepancies in outcomes from these studies compared with those employing mammalian expression systems. Our observations that the K2–5R mutant, and bacterially expressed adiponectin, fail to form HMW multimers provides mechanistic insight into the inability of this mutant to regulate hepatic glucose output (17), because various lines of evidence indicate that the HMW multimers mediate adiponectin's effects in the liver (3–6).

Previous MS analyses of adiponectin have been restricted to the mouse and bovine proteins. To confirm the existence of the same modifications in human adiponectin, we have analyzed tryptic digests of recombinant HEK-produced human adiponectin using this technique. Our data show that human adiponectin is hydroxylated or glycosylated on the same Pro and Lys residues shown to be modified in murine and bovine adiponectin. In addition, we have now identified three novel sites of Pro hydroxylation (on residues 71, 76, and 95) and demonstrate the differential hydroxylation of these in human adiponectin. Amino acid sequence alignments revealed that Pro76 and Pro95 are completely conserved in mammals, whereas Pro71 is restricted to primate and chicken sequences (Fig. 9). Because the laser ionization energy used in the MALDI analysis is far lower than that required for removal of a hydroxyl group from a modified lysine or proline residue, it may be concluded that the variable modification of these residues is not an artifact of mass spectrometric analysis and hence may contribute to the multiple pI isoforms observed by 2DE. The presence of additional differential modifications, such as sialylation (16), may also contribute to the multiple pI isoforms and warrants further investigation.

Finally, our proposed hypothesis that multimer composition of adiponectin may be regulated by changes in PTMs was supported by our observations in human

		HxPro71		HxPro76				HxPro95		
Human	GD	P	GLIG	P	K	GDIGETGVPGAEGPR	GF	P	GIQGR	100
Rat	GD	A	GVLG	P	K	GDPGDAGMTGAEGPR	GF	P	GTPGR	100
Mouse	GD	A	GLLG	P	K	GETGDVGMTGAEGPR	GF	P	GTPGR	103
Cow	GD	A	GLLG	P	K	GETGDVGMTGAEGPR	GF	P	GTPGR	95
Pig	GD	T	GLTG	P	K	GDTGESGVTGVEGPR	GF	P	GIPGR	99
Dog	GD	A	GLVG	P	K	GDTGETGVTGVEGPR	GF	P	GTPCR	100
Monkey	GD	P	GLIG	P	K	GDTGETGVTGAEGPR	GF	P	GIQGR	99
Chicken	GE	P	GLQG	V	K	GDTGEKGATGAEGPR	GF	P	GHMGM	98

Fig. 9. Sequence Conservation of the Novel Hydroxylated Prolines

Pro76 and Pro95 are almost completely conserved between species, whereas Pro71 is conserved in only three of the eight sequences compared.

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

adipose tissue explants cultured *in vitro*. A dramatic increase in the proportion of HMW multimers in secreted adiponectin occurred in response to high physiological glucose, and was accompanied by a corresponding alteration in the isoform composition of the secreted protein by 2DE analysis. Interestingly this response was seen only in tissue from a small subset of the subjects studied. Reproduction of the experiment in CHO-WT cells and 3T3-L1 adipocytes or using *in vitro*-differentiated primary human adipocytes isolated from one of the glucose responsive tissue samples failed to demonstrate the same response observed in tissue explants (data not shown). Thus, the response appears not to be a result of genetic factors, but may be dependent on the paracrine input of nonadipocytes in adipose tissue (*i.e.* stromovascular cells, immune cells) and may be influenced by the *in vivo* metabolic environment from which the tissue was extracted. No correlation between the adiponectin response to high glucose and tissue insulin sensitivity (as measured by insulin-stimulated uptake of [^{3}H]deoxyglucose) was apparent, neither did any other subject characteristics [*e.g.* age, body mass index (BMI), waist:hip ratio] cosegregate with the response to glucose (data not shown). The increase in HMW adiponectin production in response to high glucose was unexpected given that HMW adiponectin is reduced in conditions of insulin resistance characterized by chronically elevated blood glucose. However, the observed response may constitute an acute compensatory mechanism for relieving transient hyperglycemia, given the action of HMW adiponectin in reducing hepatic glucose output. Indeed, early studies in mice demonstrated that a 2-fold augmentation of circulating adiponectin, by injection of the full-length recombinant protein expressed in mammalian cells, was able to transiently lower blood glucose levels by inhibition of hepatic glucose production (30, 31). Globular adiponectin produced in bacteria was unable to elicit the same effect (31).

Regulation of adiponectin multimer composition is likely to occur at multiple levels through multiple mechanisms. The molecular mechanisms behind the increase in HMW adiponectin production in response to high glucose remain to be elucidated and will involve identification of the modifications that contribute to the observed changes in pI and mobility. Whether these modifications affect efficiency of HMW formation within the secretory pathway or efficiency of HMW secretion will also be of interest. Xu *et al*. (10) recently demonstrated selective inhibition of HMW multimer secretion (but not formation) by testosterone in 3T3-L1 adipocytes. This indicates the existence of mechanisms in adipocytes for the selective regulation of HMW multimer secretion.

Our parallel analyses of multimer composition by separation on gradient gels as well as by velocity sedimentation on sucrose gradients (see Figs. 3, C and D, and 8) have provided valuable insights into the relative merits of the two methods. SDS-PAGE under non-reducing and non-heat-denaturing conditions allowed resolution of individual multimers for qualitative analyses, whereas sucrose density gradients permitted only crude fractionation into low-molecular-weight (LMW) (trimer and hexamer) and HMW peaks. This was clearly illustrated by analysis, using both methods, of the K2–5R mutant (Fig. 3), which forms trimers and hexamers but no HMW multimers. Quantitation of the absolute amounts of adiponectin present as LMW and HMW species, on the other hand, requires that multimers be first fractionated according to size (either on sucrose gradients or by size-exclusion chromatography) before reduction and denaturation of fractions to constituent monomers for quantitation by SDS-PAGE and Western blotting.

The 2DE pattern of human adiponectin isoforms secreted from CHO cells bears close resemblance to that described nearly two decades ago for the CSL, a 26-kDa plasma protein secreted by hepatocytes (32). CSL is an important component of innate immunity, binding to the surface carbohydrates of pathogens and mediating complement activation (33). A member of the C1q superfamily of structurally homologous proteins, CSL also undergoes hydroxylation of prolines and lysines and subsequent glycosylation of hydroxylysines (21) and forms intramolecular and intermolecular disulfide bonds (26). Also, like adiponectin, CSL is produced as LMW and HMW multimers that fractionate similarly on 5–20% sucrose gradients (32). The functional significance of CSL multimers has recently been demonstrated by Larsen *et al.* (22), who analyzed naturally occurring mutants of the protein expressed in CHO cells. Patients having these mutant alleles have reduced circulating CSL and demonstrate immune deficiencies and a greater susceptibility to develop infections (reviewed in Ref. 22). Mechanistic insights were provided by characterization of the secreted mutant proteins, which revealed a deficiency in HMW multimers of CSL that was accompanied by a loss of ligand-binding activity and ability to activate complement. Thus, a similar relationship between HMW multimer assembly and functional activity appears to exist in this adiponectin-related protein.

In conclusion, the present study elucidates a role for lysine hydroxylation and glycosylation in adiponectin assembly into HMW multimers and, furthermore, demonstrates that the glucose-stimulated increase in HMW multimer production by adipose tissue is associated with changes in PTM of adiponectin.

MATERIALS AND METHODS

Reagents

Chemicals and pharmacologicals were obtained from Sigma-Aldrich (St. Louis, MO). Cell culture and transfection reagents were from Invitrogen (Carlsbad, CA). Antihuman adiponectin polyclonal antibody (AB3784) used for Western blotting and immunofluorescence microscopy was obtained from Chemicon International (Temecula, CA). Secondary antibodies used

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

were IRDye 800-conjugated antirabbit IgG from Rocklands Immunochemicals (Gilbertsville, PA), horseradish peroxidase-conjugated antirabbit IgG from Pierce (Rockford, IL), and Alexa Fluor 488-conjugated antirabbit IgG (Molecular Probes, Eugene, OR). Monoclonal anti-GM130 was obtained from Transduction Laboratories (Lexington, KY). Purified, FLAG-tagged recombinant human adiponectin used for mass spectroscopy analysis was obtained from BioVendor Laboratory Medicine (Candler, NC). Enzymes for molecular biology were obtained from Promega (Madison, WI) or New England Biolabs (Ipswich, MA). Electrophoresis equipment was from Bio-Rad Laboratories (Hercules, CA) unless indicated otherwise.

Molecular Cloning and Site-Directed Mutagenesis

WT adiponectin cDNA was generated from human sc adipose tissue mRNA and amplified by reverse transcription and PCR using the following primers: forward, 5′-ATGCTGTTGCTGGGAGC-3′; reverse, 5′- TCAGTTGGTGTCATGG-3′. The amplicon was cloned into the pGEM-T-Easy shuttle vector (Promega), and a 5′ Kozak sequence was added (forward primer, 5′-GCCACCATGCTGTTGCTGGGAGC-3′) before sequencing and subcloning into the pcDNA5/FRT expression plasmid. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) using complementary primers designed according to the manufacturer's instructions. The following forward primers were used: C36S (5′-cccaagggggccagcacaggttggatgg-3′); K1R (5′-cccctgcccagggggcctgcacagg-3′); K2–3R (5′-cctggtgagcggggtgagcgaggagatcc-3′); K4R (5′-cttattggtcctcggggagacatcgg-3′); K5R (5′-ccaaggcaggcgaggagaacctgg-3′). Reverse primers were exactly complementary to forward primers. The K2–5R and K1–5R mutants were produced by multiple rounds of mutagenesis, and all mutants were confirmed by sequencing.

CHO Cell Culture, Transfection, and Generation of Stable Cell Lines

CHO cells were cultured in HAM's F12 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine and transfected using Lipofectamine Plus reagent according to the manufacturer's instructions. CHO stable cell lines expressing WT or mutant human adiponectin constructs were generated using the Flp-In system from Invitrogen according to the manufacturer's protocols. Briefly, WT and mutant adiponectin cDNA in the expression plasmid, pcDNA5/FRT, were cotransfected with the Flp recombinase-encoding plasmid (pOG44) into the Flp-In CHO host cell line (Invitrogen). Forty-eight hours after transfection, cells were reseeded and cultured in Hygromycin (600 μg/ml)-containing medium for 2 wk to select adiponectin-expressing cells. Immunofluorescence microscopy revealed that approximately 50% of cells in the selected population were expressing adiponectin after this time. Note that, although these were not a single clonal population of stables, all stable transfectants contain one copy of the cDNA integrated into a single FRT site at a defined genomic locus of the Flp-In CHO host cell line.

Adipose Tissue Explant Experiments

Subcutaneous adipose tissue samples were obtained from four male [average age, 33.5 (range, 24–51); average BMI, 55 (range, 46.4–67.9)] and 11 female [average age, 41.4 (range, 21–66); average BMI, 45.5 (range, 26–58)] patients undergoing elective open-abdominal surgical procedures. None of the patients had diabetes or severe systemic illness, and none was taking medications known to affect adipose tissue mass or metabolism. The protocol was approved by the research ethics committees of the University of Queensland, Princess Alexandra Hospital, and Wesley Hospital. All patients gave their informed written consent. The tissue was cut into 10- to 30-mg explants, washed, and cultured in 24-well plates (one explant per well) in 300 μl of DMEM (containing 5 mM glucose) overnight. The next morning, the culture medium was removed, and the explants were cultured in fresh DMEM containing 5 or 15 mM glucose for a further 72 h. At harvest, medium samples were cleared to remove cell/tissue debris before analysis on gradient gels, sucrose density gradients, or by 2DE and Western blotting.

Primary Human Adipocyte Culture

Primary human adipocytes, isolated from sc adipose tissue and differentiated *in vitro* as previously described (34), were kindly provided by Dr. Louise Hutley and Ms. Felicity Newell (Centre for Diabetes and Endocrine Research, Brisbane, Australia). The cells were cultured in DMEM supplemented with 33 μM biotin, 17 μM pantothenate, 10 μg/ml transferrin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin during the course of experiments, which were performed on d 21 of differentiation.

Treatment of Cultured Cells/Tissue Explants

For experiments assessing the effects of 5 or 15 mM glucose, cells were cultured in low-glucose DMEM (containing 1000 mg/liter glucose) or a 1:1 mixture of low-glucose DMEM and high-glucose DMEM (containing 4500 mg/liter glucose), respectively. Cycloheximide treatment of adipocytes and stable CHO cell lines was for 20 h using 10 and 50 μg/ml concentrations of the drug for the two cell types, respectively. BFA and 2,2′-dipyridyl were used on both cell types at 5 μg/ml and 3.5 mM, respectively.

Harvesting and Preparation of Lysate and Medium Samples for Analysis

CHO cells were harvested on ice into lysis buffer [50 mM HEPES, 150 mM NaCl, 1% Triton-X-100, 1 mM Na_3VO_4, 30 mM NaF, 10 mM $Na_4P_2O_7$, 10 mM EDTA, 1 μg/ml antipain, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 250 μM benzamidine, 400 μM 4-(2-aminoethyl)benzenesulfonyl fluoride] and cleared (16,000 × *g* for 20 min at 4 C). Protein concentration of lysates was determined using a bicinchoninic acid protein assay (Pierce) and equal protein loaded for analysis by SDS-PAGE. Medium samples were cleared at 16,000 × *g* for 10 min to remove cell debris and then concentrated by precipitation with trichloroacetic acid or acetone for analysis under reducing and heat-denaturing conditions. For multimer analysis on gradient gels or by velocity sedimentation, samples were concentrated using centricons of 30,000 MW cutoff (Millipore, Billerica, MA) or by ammonium sulfate precipitation (40% wt/vol).

Bacterial Expression of Adiponectin

Human adiponectin cDNA lacking the 18-amino acid mammalian signal sequence was generated by PCR (forward primer, 5′-catatggaaaccacgactcaagggcccgg-3′; reverse primer, 5′-ggatcctcagttggtgtcatggtagagaagaaagcc-3′) and cloned into the pET 20b expression plasmid (using *Nde*I and *Bam*HI restriction sites incorporated into the primers). The insert was sequenced and transformed into BL21(DE3)pLys-S cells for inducible expression. Expression cultures (10 ml) were inoculated from an overnight starter culture and allowed to reach an A_{600} OD of 0.6 AU before induction with 1 mM IPTG for 3 h at 37 C. Cells were extracted with BugBuster Master Mix lysis buffer (Novagen, Madison, WI) and fractionated into soluble and particulate fractions by centrifugation of lysates at 16,000 × *g* for

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

20 min at 4 C. Samples were analyzed by SDS-PAGE and Western blotting.

Velocity Sedimentation on Sucrose Gradients

Fractionation of adiponectin multimers into LMW and HMW multimers was achieved by separation on sucrose gradients essentially as described by Pajvani *et al.* (3). Briefly, 2-ml gradients of 5–20% sucrose were poured stepwise into thin-walled ultracentrifuge tubes and allowed to equilibrate overnight. A 200-μl volume of sample [diluted as required into sucrose gradient buffer; 10 mM HEPES (pH 8), 125 mM NaCl] was applied to the top of the gradient before centrifugation at 55,000 rpm in a Sorvall S-55 rotor for 4 h at 4 C. Gradients were then fractionated from the top into 15 $\times$ 150-μl fractions, which were precipitated with 2 vol of acetone. Fractions were analyzed by SDS-PAGE and Western blotting of equal volumes after reduction and boiling.

SDS-PAGE and Western Blotting

Samples were prepared for SDS-PAGE as described by Waki *et al.* (2). For analysis under non-reducing, non-heat-denaturing conditions, 5$\times$ SDS-PAGE sample buffer lacking reducing agents was added to a final concentration of 1$\times$ [3% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 6.8), 10% glycerol], and samples were incubated for 30 min at room temperature (RT) before analysis on 4–12% or 3–10% gradient gels. For analysis of fully denatured, reduced protein, samples were prepared using 5$\times$ SDS-PAGE sample buffer containing 500 mM DTT and boiled for 10 min before electrophoresis on 12% acrylamide gels. After electrophoretic separation, proteins were blotted onto polyvinylidene difluoride membranes. The membranes were blocked with 1% fish skin gelatin in PBS for 30 min and probed with a 1:5000 dilution of polyclonal antibody to human adiponectin (Chemicon). Membranes were then washed in PBS containing 0.1% Tween and probed with a fluorescent antirabbit secondary (Rocklands Immunochemicals) before scanning of membranes and quantitation of Westerns using the Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NE). Alternatively, membranes were probed with horseradish peroxidase-conjugated secondary antibody and visualized by chemiluminescence using the Supersignal West Pico Chemiluminescent substrate system from Pierce.

2DE

Samples were analyzed by 2DE using the Zoom 2DE system and associated reagents from Invitrogen according to the manufacturer's protocols. In brief, samples of conditioned medium were precipitated with acetone, and the pellets were resuspended in lysis buffer containing ZOOM 2DE Protein Solubilizer 2 and 20 mM DTT. After a 15-min incubation at RT, samples were alkylated by addition of 0.5% *N*,*N*-dimethylacrylamide and incubation for 30 min. Excess DMA was neutralized by further addition of DTT to 40 mM followed by a clearing spin (16,000 $\times$ *g*, 20 min, 4 C) and acetone precipitation. Protein pellets were resuspended in rehydration buffer containing ZOOM 2DE Protein Solubilizer 2, 10 mM DTT, and 0.5% carrier ampholytes (pH 4–7), and applied to ZOOM strips (pH 4–7) for overnight rehydration. Isoelectric focusing was performed using a ZOOM IPG Runner System followed by reduction and alkylation of the strips according to the manufacturer's instructions. Second-phase separation was carried out using 12% minigels and followed by Western blotting.

MALDI-TOF, MALDI/TOF/TOF, and MS/MS analyses

Adiponectin (2 μg) was subject to overnight digestion with trypsin (100 ng) by coprecipitation in methanol at −20 C, resuspension in 10 μl of 40 mM ammonium bicarbonate/10% acetonitrile, and overnight incubation at 37 C. A trypsin-only digest (without adiponectin) was performed as negative control. A virtual trypsin digest of adiponectin (to obtain predicted peptide masses) was performed using MS-Digest software on the Protein Prospector proteomics server.

MALDI-TOF analysis was performed on an Applied Biosystems (ABI) Voyager-DE STR Biospectrometry Workstation. A total of 0.5 μl of the digest was mixed with an equal volume of α-cyano-4-hydroxy-cinnamic acid matrix solution (5 mg/ml in EtOH), spotted, and allowed to dry on the sample plate. All MALDI-TOF analysis was performed in positive reflector mode using a pulsed laser and 20,000-V acceleration. Spectra were mass calibrated using an internal standard mix of angiotensin II (MH+ 1046.5), human ACTH (18–39 clip MH+ 2465.2), and oxidized insulin B chain (MH+ 3494.6). Spectra were analyzed for expected peptide masses using the ABI Data Explorer software. Peptide candidates with probable carbohydrate and hydroxyl group modifications were identified by comparing the experimental and theoretical mass fingerprints. These candidate peptides were submitted for MS/MS and MALDI-TOF-TOF tandem MS sequencing to confirm the amino acid sequence and identify modified residues.

LC/MS/MS sequencing experiments were performed on an ABI 4000 Q-TRAP LC/MS/MS System. Before LC/MS/MS analysis, the tryptic peptide sample was diluted in 40 μl of a 1% trifluoroacetic acid/1% formic acid (AQ) solution and separated on a 0.5-mm (inner diameter) by 150-mm (length) Luna 5μ C18 RP-HPLC (Phenomenex, Torrance, CA) column using a flow rate of 30 μl/min. The eluted peptides were directly applied to the 4000 Q-TRAP MS/MS workstation. Peptide fragmentation data were collected and analyzed using the ABI Analyst QS software package, and sequences were scored using the MASCOT search database.

MALDI-TOF-TOF sequencing experiments were performed on an ABI 4700 Proteomics Discovery System. A total of 0.4 μl of the digest was mixed with an equal volume of α-cyano-4-hydroxy-cinnamic acid matrix solution (5 mg/ml in 60/40 methyl cyanide /0.1% formic acid in 5 mM ammonium phosphate), spotted, and allowed to dry on the sample plate. MS and MS/MS experiments were carried out using 1-kV MS and MS/MS operating modes and 200-Hz laser repetition rate. MS data was externally calibrated using the ABI calibration mix plus Angiotensin (MH+ 1296.685). MS/MS mass calibration was carried out using the fragments from the collision-induced dissociation of the external standard Angiotensin. Precursor ions with predicted modifications from MALDI-TOF MASS fingerprints were collision cell fragmented and sequenced. Sequence analysis was performed using the ABI GPS and DeNovo Explorer software and ranked using the MASCOT search database.

Immunofluorescence Microscopy

Cells were grown on 12-mm round glass coverslips and processed as per Richards *et al.* (35). Briefly, cells were fixed for 20 min in 4% paraformaldehyde and permeabilized in ice-cold methanol for 7 min at −20 C. A 10-min incubation in 50 mM NH_4Cl at RT was followed by a 20-min blocking step in a solution of 0.2% fish skin gelatin and 0.2% BSA in PBS. Cells were incubated in primary antibody solution for 30 min at RT before four 5-min PBS washes and a 20-min incubation in fluorophore-conjugated secondary antibody. After another four 5-min PBS washes, cells were rinsed in MilliQ water and mounted in Moviol mounting medium (Merck Biosciences, San Diego, CA). Specimens were analyzed on an epifluorescence microscope, and images were prepared using Photoshop 7.

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

Acknowledgments

We thank Dr. Louise Hutley and Ms. Felicity Newell for providing *in vitro*-differentiated human adipocytes.

Received September 22, 2005. Accepted February 10, 2006.

Address all correspondence and requests for reprints to: Jonathan P. Whitehead, Centre for Diabetes and Endocrine Research, University of Queensland, Ground Floor, Princess Alexandra Hospital, Ipswich Road, Woolloongabba, Queensland 4102, Australia. E-mail: jwhitehead@cder.soms.uq.edu.au.

This work was supported by a grant from the National Health and Medical Research Council of Australia (to G.A.M., J.P.W., and J.B.P.) and a Diabetes Australia Research Trust grant (to A.A.R). J.P.W was supported by a Lions Senior Medical Research Fellowship.

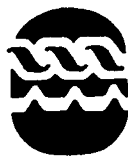
A.A.R., T.S., H.K.C., A.J., G.A.M., J.B.P., and J.P.W. have nothing to declare.

REFERENCES

1. Trujillo ME, Scherer PE 2005 Adiponectin—journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. J Intern Med 257:167–175
2. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, Vasseur F, Froguel P, Kimura S, Nagai R, Kadowaki T 2003 Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin. J Biol Chem 278:40352–40363
3. Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, Scherer PE 2004 Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. J Biol Chem 279:12152–12162
4. Fisher FF, Trujillo ME, Hanif W, Barnett AH, McTernan PG, Scherer PE, Kumar S 2005 Serum high molecular weight complex of adiponectin correlates better with glucose tolerance than total serum adiponectin in Indo-Asian males. Diabetologia 48:1084–1087
5. Hammarstedt A, Sopasakis VR, Gogg S, Jansson PA, Smith U 2005 Improved insulin sensitivity and adipose tissue dysregulation after short-term treatment with pioglitazone in non-diabetic, insulin-resistant subjects. Diabetologia 48:96–104
6. Tonelli J, Li W, Kishore P, Pajvani UB, Kwon E, Weaver C, Scherer PE, Hawkins M 2004 Mechanisms of early insulin-sensitizing effects of thiazolidinediones in type 2 diabetes. Diabetes 53:1621–1629
7. Kishida K, Nagaretani H, Kondo H, Kobayashi H, Tanaka S, Maeda N, Nagasawa A, Hibuse T, Ohashi K, Kumada M, Nishizawa H, Okamoto Y, Ouchi N, Maeda K, Kihara S, Funahashi T, Matsuzawa Y 2003 Disturbed secretion of mutant adiponectin associated with the metabolic syndrome. Biochem Biophys Res Commun 306:286–292
8. Kobayashi H, Ouchi N, Kihara S, Walsh K, Kumada M, Abe Y, Funahashi T, Matsuzawa Y 2004 Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin. Circ Res 94:e27–e31
9. Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, Makishima M, Shimomura I 2003 Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes 52:1655–1663
10. Xu A, Chan KW, Hoo RL, Wang Y, Tan KC, Zhang J, Chen B, Lam MC, Tse C, Cooper GJ, Lam KS 2005 Testosterone selectively reduces the high molecular weight form of adiponectin by inhibiting its secretion from adipocytes. J Biol Chem 280:18073–18080
11. Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M, Scherer PE 2003 Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications for metabolic regulation and bioactivity. J Biol Chem 278:9073–9085
12. Combs TP, Berg AH, Rajala MW, Klebanov S, Iyengar P, Jimenez-Chillaron JC, Patti ME, Klein SL, Weinstein RS, Scherer PE 2003 Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. Diabetes 52:268–276
13. Berg AH, Combs TP, Scherer PE 2002 ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. Trends Endocrinol Metab 13:84–89
14. Shapiro L, Scherer PE 1998 The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. Curr Biol 8:335–338
15. Tsao TS, Tomas E, Murrey HE, Hug C, Lee DH, Ruderman NB, Heuser JE, Lodish HF 2003 Role of disulfide bonds in Acrp30/adiponectin structure and signaling specificity. Different oligomers activate different signal transduction pathways. J Biol Chem 278:50810–50817
16. Sato C, Yasukawa Z, Honda N, Matsuda T, Kitajima K 2001 Identification and adipocyte differentiation-dependent expression of the unique disialic acid residue in an adipose tissue-specific glycoprotein, adipo Q. J Biol Chem 276:28849–28856
17. Wang Y, Xu A, Knight C, Xu LY, Cooper GJ 2002 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. J Biol Chem 277:19521–19529
18. Wang Y, Lu G, Wong WP, Vliegenthart JF, Gerwig GJ, Lam KS, Cooper GJ, Xu A 2004 Proteomic and functional characterization of endogenous adiponectin purified from fetal bovine serum. Proteomics 4:3933–3942
19. Uitto J, Lichtenstein JR 1976 Defects in the biochemistry of collagen in diseases of connective tissue. J Invest Dermatol 66:59–79
20. Wu GY, Pereyra B, Seifter S 1981 Specificity of trypsin and carboxypeptidase B for hydroxylysine residues in denatured collagens. Biochemistry 20:4321–4324
21. Colley KJ, Baenziger JU 1987 Identification of the post-translational modifications of the core-specific lectin. The core-specific lectin contains hydroxyproline, hydroxyl-ysine, and glucosylgalactosylhydroxylysine residues. J Biol Chem 262:10290–10295
22. Larsen F, Madsen HO, Sim RB, Koch C, Garred P 2004 Disease-associated mutations in human mannose-binding lectin compromise oligomerization and activity of the final protein. J Biol Chem 279:21302–21311
23. Misumi Y, Miki K, Takatsuki A, Tamura G, Ikehara Y 1986 Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. J Biol Chem 261:11398–11403
24. Lippincott-Schwartz J, Yuan LC, Bonifacino JS, Klausner RD 1989 Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell 56:801–813
25. Scheel J, Pepperkok R, Lowe M, Griffiths G, Kreis TE 1997 Dissociation of coatomer from membranes is required for brefeldin A-induced transfer of Golgi enzymes to the endoplasmic reticulum. J Cell Biol 137:319–333
26. Colley KJ, Baenziger JU 1987 Post-translational modifications of the core-specific lectin. Relationship to assembly, ligand binding, and secretion. J Biol Chem 262: 10296–10303
27. Tsao TS, Murrey HE, Hug C, Lee DH, Lodish HF 2002 Oligomerization state-dependent activation of NF-κB

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022

signaling pathway by adipocyte complement-related protein of 30 kDa (Acrp30). J Biol Chem 277: 29359–29362
28. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T 2003 Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423: 762–769
29. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T 2002 Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 8:1288–1295
30. Combs TP, Berg AH, Obici S, Scherer PE, Rossetti L 2001 Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. J Clin Invest 108: 1875–1881
31. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE 2001 The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nat Med 7:947–953
32. Colley KJ, Baenziger JU 1987 Biosynthesis and secretion of the rat core-specific lectin. Relationship of post-translational modification and assembly to attainment of carbohydrate binding activity. J Biol Chem 262:3415–3421
33. Turner MW, Hamvas RM 2000 Mannose-binding lectin: structure, function, genetics and disease associations. Rev Immunogenet 2:305–322
34. Hutley L, Shurety W, Newell F, McGeary R, Pelton N, Grant J, Herington A, Cameron D, Whitehead J, Prins J 2004 Fibroblast growth factor 1: a key regulator of human adipogenesis. Diabetes 53:3097–3106
35. Richards AA, Stang E, Pepperkok R, Parton RG 2002 Inhibitors of COP-mediated transport and cholera toxin action inhibit simian virus 40 infection. Mol Biol Cell 13:1750–1764



***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Downloaded from https://academic.oup.com/mend/article/20/7/1673/2738433 by guest on 16 August 2022