Oligomerization state-dependent hyperlipidemic effect of angiopoietin-like protein 4

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Abstract Angiopoietin-like protein 4 (Angptl4) is the second member of the angiopoietin-like family of proteins previously shown to increase plasma triglyceride (TG) levels in vivo. We recently reported that Angptl4 is a variable-sized oligomer formed by intermolecular disulfide bonds and undergoes regulated proteolytic processing upon secretion. We now show that adenoviral overexpression of Angptl4 potently increases plasma TG levels by a mechanism independent of food intake or hepatic VLDL secretion. We determined that cysteine residues at positions 76 and 80 of Angptl4, conserved among mouse, rat, and human, are required to form higher order structures. By generating adenoviral expression vectors of Angptl4 containing different epitope tags at both N and C termini, we show that loss of oligomerization results in decreased stability of the N-terminal coiled-coil domain of Angptl4 as well as decreased ability to increase plasma TG levels, suggesting that intermolecular disulfide bond formation plays important roles in determining the magnitude of the hyperlipidemic effect of Angptl4. **Because Angptl4 is more potent than Angptl3 in increasing plasma TG levels in mice, inappropriate oligomerization of Angptl4 could be associated with disorders of lipid metabolism in vivo.**—Ge, H., G. Yang, X. Yu, T. Pourbahrami, and C. Li. **Oligomerization state-dependent hyperlipidemic effect of angiopoietin-like protein 4.** ***J. Lipid Res.*** **2004.** 45: **2071–2079.**

Supplementary key words lipoprotein lipase • plasma triglyceride • disulfide bond

One of the recently identified secreted factors from adipose tissue is angiopoietin-like protein 4 (Angptl4), also named PGAR [peroxisome proliferator-activated receptor γ (PPARγ) angiopoietin related], FIAF (fasting-induced adipose factor), or HFARP (hepatic fibrinogen/angiopoietin-related protein). Angptl4 is a secreted protein selectively expressed in adipose tissue, placenta, liver, and pituitary (1–4). Mouse Angptl4 is composed of 410 amino acids that include an N-terminal signal sequence, a coiled-coil domain, and a fibrinogen-like motif at the C terminus. This domain structure is preserved in a family of proteins called angiopoietins and angiopoietin-like proteins. In contrast to angiopoietins, angiopoietin-like proteins do not bind to Tie2, the receptor for angiopoietins (5). At least six angiopoietin-like proteins are now known, with two of these, Angptl3 and Angptl4, shown to be involved in the regulation of lipid metabolism (6–8).

The expression of Angpt4 is under complex nutritional and hormonal control. Its expression is induced by fasting in adipose tissue, liver, and pituitary (2–4); its mRNA levels are also increased by severalfold in liver of mice lacking stearoyl-CoA desaturase-1, the rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids (9); treatment of primary cultured keratinocytes with a PPARβ/δ agonist, GW501516, increases Angptl4 mRNA levels by more than 12-fold (10); conjugated linoleic acid, a PPARα ligand, also potently induces Angptl4 expression (11); in cardiomyocytes, Angptl4 is induced by hypoxia mediated by hypoxia-inducible factor 1α (12, 13). Conversely, mRNA levels of Angptl4 are reduced in mice hemizygous for either PPARα or PPARγ. However, under fasting conditions, transcription of Angptl4 in both liver and adipose tissue can be induced even in the absence of PPARα (2). Circulating levels of Angptl4 are increased in genetically obese mice (*ob/ob* or *db/db*) that lack leptin signaling (3).

The liver is the most important organ of energy distribution at times of both nutritional excess and food restriction. There are two essentially opposite patterns of metabolism in liver during the fed and fasted states. In the fed state, it converts ingested nutrients to glycogen or triglyceride (TG). Newly synthesized TG is packaged into lipoproteins, mainly in the form of VLDL, and released for uptake by other tissues. In the fasted state, liver takes up fatty acids released by adipose tissue and oxidizes them into ketone bodies. Free fatty acids are derived primarily from the adipose tissue by the action of hormone-sensitive lipase (14). Increased oxidation of fatty acids during fasting also generates precursors of gluconeogenesis, causing

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the increased release of glucose for use by other tissues, such as brain and red blood cells.

The function of Angptl4 is not well understood. Angptl4 is a potent hyperlipidemia-inducing factor and an inhibitor of lipoprotein lipase (8). It has been hypothesized to play a role in the modulation of adipogenesis, insulin sensitivity, and/or energy metabolism. Recently, we reported that full-length Angptl4 is an oligomer, a characteristic also observed for angiopoietin 1 (15). In contrast to angiopoietin 1, Angptl4 undergoes regulated proteolysis to generate a truncated C-terminal fibrinogen-like domain in vitro and in vivo. However, it is not known if oligomerization affects the ability of Angptl4 to increase plasma lipid levels. In this report, we transiently overexpressed full-length Angptl4 via adenovirus-mediated gene transfer and studied its effect on plasma TG levels in vivo. We show that consistent with an earlier report using bacterially produced Angptl4, overexpression of Angptl4 adenovirally increases plasma TG levels, which we show to be caused without increasing hepatic VLDL secretion. We also demonstrate that cysteine residues at positions 76 and 80 are required for Angptl4 oligomerization. Furthermore, when both cysteine residues are mutated to render Angptl4 oligomerization-defective, the hyperlipidemic effect of Angptl4 is reduced. These results raise the possibility that changes in the oligomerization state of Angptl4 in vivo may underlie certain disorders of lipid metabolism.

MATERIALS AND METHODS

Reagents

All PCR primers were synthesized by Sigma-Genosys or IDT Technologies. All restriction endonucleases and the random primer labeling kit were from New England Biolabs, Inc. (Beverly, MA). Taq DNA polymerase and anti-*c-myc* peroxidase were from Roche (Indianapolis, IN). Trizol reagent and Superscript II RNase H^- reverse transcriptase were from Invitrogen (Carlsbad, CA). Anti-FLAG-peroxidase, tyloxapol (Triton WR-1339), and the Infinity Cholesterol Reagent and Triglyceride (Serum Triglyceride Determination) Kit were from Sigma (St. Louis, MO). [9.10-^{3}H]palmitic acid (57 Ci/mmol, 5 mCi/ml) was from Perkin-Elmer (Boston, MA). ECL-Western blotting reagents and Superose 6 columns (10/30) were from Amersham Biosciences (Piscataway, NJ).

Construction of adenoviral expression vectors

To generate adenoviruses encoding the full-length rat Angptl4 with both FLAG and *c-myc* tags (Ad-FLAG-Angptl4-myc), full-length rat Angptl4 cDNA was amplified from pcDNA-Angptl4 using primers CL561 (5′-CCCAAGCTTCAAGGGCGCCCTGCACAGCCG-3′) and CL562 (5′-GCTCTAGAGGCTGTAGCCTCCATG-3′). They contain *Hind*III and *Xba*I sites (underlined) for in-frame subcloning into the same sites of p3×FLAG-myc-CMV-23 vector. The AdEasy system, with a slight modification, was used to generate adenoviruses (16). Briefly, to facilitate the detection of virally produced Angptl4 proteins, the insert in the p3×FLAG-myc-CMV-23 vector was removed along with the FLAG and *c-myc* tags as a blunt-end fragment using Pfu-derived PCR amplification and ligated to the *Pme*I site of pAdenoVator-CMV5. The primers used here were CL495 (5′-ACCATGTCTGCACTTATCC-3′) and CL444 (5′-GGGTCACAGATCCTCTTCTG-3′). The resulting plasmid was then linearized with *Eco*RI, purified using the MinElute Kit (Qiagen), and co-electroporated with pAdEasy1 into BJ5183 cells to generate recombinant viral DNA. Recombinants were screened by running an aliquot of undigested miniprep DNA of 2 ml cultures from kanamycin-resistant colonies. *Pac*I digestion was then performed to confirm the presence of inserts. Viral DNA was transformed into DH5α cells and propagated before being used for transfection into low-passage HEK293 cells as described (17, 18).

Construction of Angptl4 mutants

Substitutions of C76A, C80A, and C76A/C80A of p3×FLAG-Angptl4-myc were carried out by site-directed mutagenesis using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) and synthetic oligonucleotides as shown. For C76A, CL564 (5′-CCGCATGGCTGCCGCCGGTAACGCTTGTC-3′) and CL565 (5′-GACAAGCGTTACCGGCGGCAGCCATGCGG-3′); for C80A, CL566 (5′-CTGCGGTAACGCTGCTCAGGGGCCCAAG-3′) and CL567 (5′-CTTGGGCCCCTGAGCAGCGTTACCGCAG-3′); for C76A/C80A, CL568 (5′-CCGCATGGCTGCCGCCGGTAACGCTGCTCAGGGGCCCAAG-3′) and CL569 (5′-CTTGGGCCCCTGAGCAGCGTTACCGGCGGCAGCCATGCGG-3′).

Adenovirus infusion

ICR or C57BL/6 mice were purchased from Jackson Laboratories and housed in a pathogen-free facility in the Animal Resources Center at University of Texas Southwestern on a 12 h light/dark cycle. Before virus administration, each mouse was anesthetized by injection of a cocktail containing xylazine and ketamine in PBS. Each mouse received 2×10^{11} virus particles in 0.1 ml of PBS via the tail vein or jugular vein, as described (17, 19).

Plasma preparation and lipoprotein analysis

Blood was sampled from the tail vein into EDTA-containing Eppendorf tubes (final concentration, ~2 mM). Plasma was prepared by low-speed centrifugation (5,000 *g*) for 10 min at room temperature. It was then diluted 1:1 (v/v) with PBS and filtered through a 0.22 μm syringe filter (Millipore). One hundred microliters of diluted sample was injected into a Superose 6 HR 10/30 gel filtration column for the analysis of plasma lipoproteins, performed using a fast-protein liquid chromatography (FPLC) system (Pharmacia ÄKTA) at 4°C, as described (20). Samples were eluted at a flow rate of 0.4 ml/min in buffer containing 0.05 M phosphate, pH 7.0, and 0.15 M NaCl. Fractions of 0.3 ml were collected for analysis. Total plasma cholesterol and TG levels as well as those in each fraction were measured enzymatically using the Infinity Cholesterol Reagent and Triglyceride (GPO-Trinder) Kit. One hundred microliters of each fraction was used for cholesterol or TG assay.

Treatment of mice with Triton WR-1339

Mice were fasted for 2 h before a dose of 20 mg of tyloxapol (Triton WR-1339) was injected into the jugular vein in a volume of 100 μl. Blood was sampled from the tail vein (~30 μl) just before and at 30, 60, 120, and 180 min after Triton injection. Mice were then killed by cervical dislocation. Plasma TG levels were assayed as described above.

In vivo synthesis of ^{3}H-labeled VLDL and turnover

[^{3}H]palmitic acid in ethanol was evaporated under nitrogen and redissolved in 0.95% NaCl containing 2 mg/ml BSA to a final concentration of 1 mCi/ml. One hundred microcuries of [^{3}H]palmitic acid solution was injected intravenously via jugular vein into each mouse, followed by 100 μl of 20% Triton WR-1339 to inhibit the clearance of lipoproteins. Mice were anesthetized and bled from the abdominal aorta 25 min after injection. One hundred microliters of plasma was loaded on a Superose 6 HR 10/30 column to isolate ^{3}H-labeled VLDL. Fractions (300 μl) were collected using a 96-well plate, and radioactivity in each well

was measured to determine the position of eluted VLDL. To determine the turnover rate of VLDL in Angptl4-overexpressing mice, 7 week old male C57BL/6J mice were jugular vein-injected with 10^{11} particles of Ad-Angptl4-myc or Ad-green fluorescent protein (GFP) viruses. Two days after virus injection, mice were injected, also via the jugular vein, with 60,000 cpm of ^{3}H-labeled VLDL in a volume of 150 μl. Approximately 40 μl of blood was collected at different times after tracer injection. The clearance rate of ^{3}H-labeled VLDL was determined by measuring the radioactivity of 10 μl of plasma using a Beckman LS 5000TD β-counter.

Northern blotting and other molecular biology techniques

Ten micrograms of total RNA from the livers of treated mice was loaded onto a 1% formaldehyde gel and transferred to a positively charged Hybond-N^{+} nylon membrane. Probes corresponding to sterol-regulatory element binding protein-1c (SREBP-1c), acetyl-CoA carboxylase 1 (ACC1), FAS, peroxisome proliferators-activated receptor gamma coactivator-1α (PGC-1α), phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase, apolipoprotein C-III, apolipoprotein A-V, GAPDH, and Angptl4 were synthesized by PCR and labeled with a random primer labeling kit using [α-^{32}P]dCTP (3,000 Ci/mM, 10 mCi/ml; ICN Radiochemicals). Labeled probe was purified from free radionucleotides with a spin column, Probe Quant G-50 (Amersham Biosciences). Filters were hybridized in RapidHyb buffer in a 65°C oven (Robbins Scientific). Washed filters were exposed to X-ray film (Kodak) at −70°C and developed with an automatic developer.

RESULTS

Adenovirus-mediated overexpression of Angptl4 causes an increase of plasma TG levels associated with VLDL particles

Because Angptl4 is a glycoprotein (21), we asked if glycosylation plays a role in the hyperlipidemic effect of Angptl4, using an adenoviral expression system to selectively overexpress Angptl4 in livers of mice, a protocol routinely used in our laboratory for in vivo studies to express proteins containing posttranslational modifications (17–19). cDNA encoding full-length Angptl4 was tagged with the *c-myc* epitope at the C terminus to facilitate the detection of expressed protein (**Fig. 1A**). To test the effect of full-length Angptl4 on plasma TG levels, we infused Ad-Angptl4-myc or a control virus, Ad-GFP, into mice and analyzed the plasma lipid composition of these mice. In agreement with the earlier study that had delivered bacterially derived recombinant Angptl4 protein by injection, adenoviral overexpression of full-length Angptl4 caused a 10- to 30-fold increase in circulating TG (Fig. 1B), a 3- to 4-fold increase in total cholesterol, and a 2-fold increase in free fatty acids (data not shown). These data demonstrate that glycosylation is not required for the hyperlipidemic effects

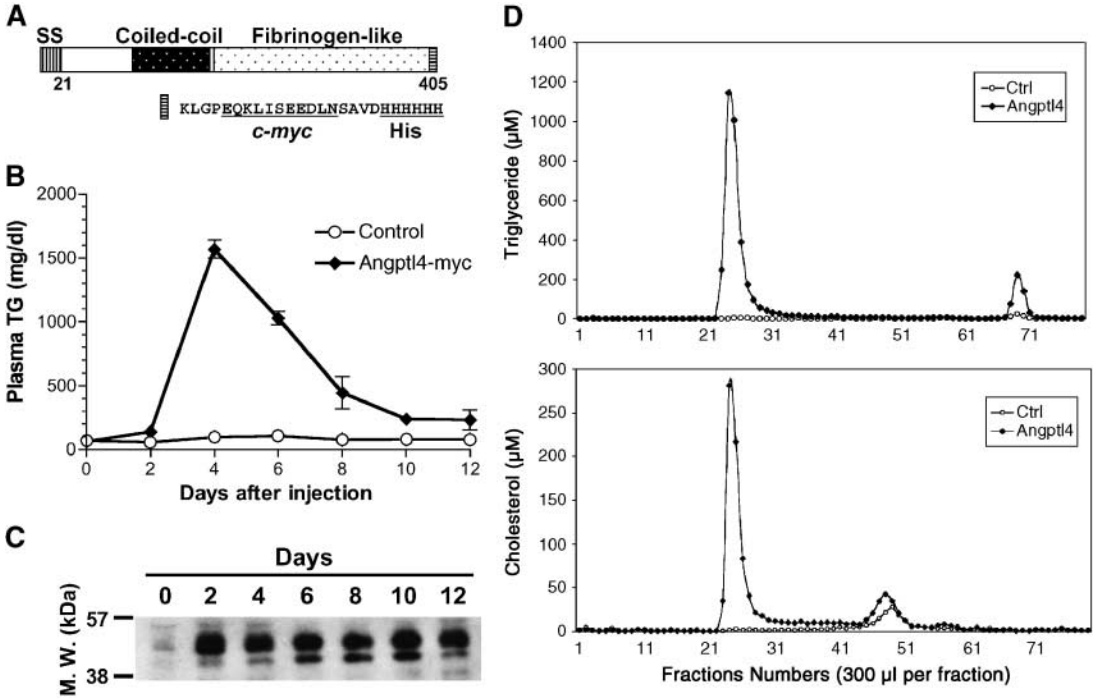

Fig. 1. Adenoviral constructs of Angptl4 (A), its hyperlipidemic effects in vivo (B), expression (C), and determination of lipoprotein species in plasma by FPLC (D). In (A), a schematic diagram is shown illustrating the domain structure as well as the boundaries of each domain within Angptl4 (top). Numbers below the diagram indicate the positions of the last amino acid of Angptl4 as well as the position at the end of signal sequence (SS). The amino acid sequences of epitope tag are indicated below. In (B), adenoviruses encoding full-length Angptl4 (Angptl4-myc) or control adenovirus encoding GFP (control) were infused into mice. Levels of plasma triglyceride were determined at regular intervals as indicated (n = 5 mice/group). In (C), plasma samples (1 μl/lane) were separated by SDS-PAGE and blotted with c-myc-HRP antibody. In (D), plasma from day 4 after virus expression was fractioned by gel filtration and levels of TG and cholesterol determined. Plasma samples were collected from mice infused with Ad-GFP (Ctrl) or Ad-Angptl4-myc (Angptl4) virus, loaded onto Superose 6 column, and eluted as 300 μl fractions. The triglyceride and cholesterol content in each fraction was determined enzymatically. The peak position of triglyceride and cholesterol indicates that VLDL is the lipoprotein that is elevated after Angptl4 over-expression. Error bars represent standard deviation of the mean of five mice/group.

of Angptl4 but could still be involved in other aspects of Angptl4 function. When analyzed by Western blot analysis (Fig. 1C), only a protein the size of the C-terminal fibrinogen-like domain of Angptl4 is recognized by the *c-myc* antibody, in agreement with our earlier finding that full-length Angptl4 is rapidly processed, generating a stable fragment containing its fibrinogen-like domain (21).

Interestingly, the time course of the increase of circulating TGs does not coincide with the expression profile of virally expressed Angptl4. Even though adenovirus-mediated expression of Angptl4, detected by Western blotting using an anti-*c-myc* monoclonal antibody, remains robust for more than 2 weeks (Fig. 1C), plasma levels of TG show a different kinetics. They increase to peak levels at ~4 days in wild-type mice and then return to near-normal levels within 2 weeks. The mechanism responsible for the discrepancy between the viral expression of Angptl4 and plasma TG levels remains to be determined.

To determine the nature of lipoprotein species that may account for the observed increase of plasma TG and cholesterol, we performed gel filtration analysis on plasma samples of mice treated with control virus or Ad-Angptl4. Plasma components were separated using FPLC. Measurement of TG and cholesterol levels in all eluted fractions revealed that the increase of circulating TG and cholesterol is attributable to the increase of circulating VLDL (Fig. 1D). Interestingly, a second, smaller peak of TG is also observed from plasma of Angptl4-treated mice that is not accompanied by a similar increase of cholesterol levels, suggesting that the composition of other lipoproteins also occurred as a consequence of Angptl4 overexpression.

Adenovirus-mediated overexpression of Angptl4 does not increase VLDL secretion rate

An increase in plasma VLDL levels could be attributable to three possible mechanisms: an increase of diet-derived chylomicron, an increase of hepatic VLDL synthesis, or an inhibition of clearance of circulating VLDL. We performed food restriction, Triton WR-1339 injection, and injection of ^{3}H-labeled VLDL into mice treated with control virus or Ad-Angptl4-myc to test each of these possibilities.

To test if intestinally derived chylomicron contributes to the observed increase of VLDL in plasma induced by adenovirus-mediated overexpression of truncated Angptl4, we determined the lipoprotein profile of plasma of mice fed ad libitum and food-restricted mice for 8 h. In the mouse, chylomicron production does not occur when food is restricted for more than 4 h. Both groups of mice had received the same dose of adenoviruses before blood sampling.

We compared the plasma TG contents and lipoprotein profiles of virus-treated mice with or without food restriction (**Fig. 2A** and data not shown). Fasting for 8 h had a negligible effect on plasma TG level of Angptl4-treated mice. This result suggests that the increased plasma VLDL level after Ad-Angptl4-myc treatment did not result from increased intestinal output of lipoproteins in the form of chylomicron.

We next asked if Ad-Angptl4 causes an increase of VLDL release from the liver or a decrease of VLDL clearance through the inhibition of lipoprotein lipase. To distinguish between these two scenarios, we tested if overexpression of full-length Angptl4 can alter VLDL accumulation in plasma when Triton WR-1339, which inhibits plasma hydrolysis, is infused intravenously into mice fed ad libitum. Two days before Triton injection, two groups of mice were administered Ad-Angptl4 or Ad-GFP. As shown in Fig. 2B, Ad-Angptl4 overexpression did not cause a further increase of plasma TG levels over a 6-h test period, indicating that hepatic VLDL release is not altered by overexpression of full-length Angptl4.

Finally, we determined the rate of VLDR hydrolysis in Ad-Angptl4-treated mice using ^{3}H-labeled VLDL as a tracer. We performed the experiment 2 days after mice were given Ad-Angptl4-myc or Ad-GFP. At this time point, Angptl4 expression is already high but plasma TG levels have not increased substantially above control levels (Fig. 1B, C). Figure 2C shows that upon [^{3}H]VLDL injection into the jugular vein of mice treated with Ad-Angptl4-myc or Ad-GFP, the clearance rate of [^{3}H]VLDL from plasma is delayed in mice that received Ad-Angptl4-myc virus. This result confirms that Angptl4 causes delayed clearance of VLDL in vivo.

Consistent with the above observations, mRNA levels of the rate-limiting enzymes in the VLDL synthesis pathway were not changed by Ad-Angptl4 infection. Northern blotting was performed on total RNA from livers of mice treated with Ad-Angptl4 or Ad-GFP. The expression levels of key genes in fatty acid and lipoprotein synthesis pathways, including SREBP-1c, ACC1, fatty acid synthase, and apolipoproteins C-III and A-V, were determined (**Fig. 3**). In parallel, we also determined the expression of genes in the gluconeogenesis pathway, including PGC-1α, glucose-6-phosphatase, and PEPCK. Compared with Ad-β-Gal-treated mice, no significant changes in mRNA expression were detected in liver of mice treated with Ad-Angptl4 (Fig. 3). In aggregate, these results suggest that Angptl4 induces hyperlipidemia without major effects on VLDL secretion or gene expression profiles in the liver.

Both cysteine residues within the coiled-coil domain of rat Angptl4 are required for oligomerization

To determine the role of oligomerization in the function of Angptl4, we performed site-directed mutagenesis to determine the key cysteine residues that control Angptl4 oligomer assembly. Examination of the amino acid sequences within the coiled-coil domain of Angptl4 revealed the presence of two cysteine residues, at positions 76 and 80. Both of these residues are conserved among mouse, rat, and human (**Fig. 4A**). Because we already reported that the coiled-coil domain is sufficient for oligomerization of the Angptl4 protein (21), we mutated these two residues individually or in combination to alanine residues. Three mutants are thus generated: C76A, C80A, and C76A/C80A. All three mutants, along with wild-type Angptl4 cDNA expression constructs, were transiently transfected into HEK293 cells for protein expression. The levels of recombinant protein expression are similar, suggesting that substitution of cysteine residues with alanine does not al-

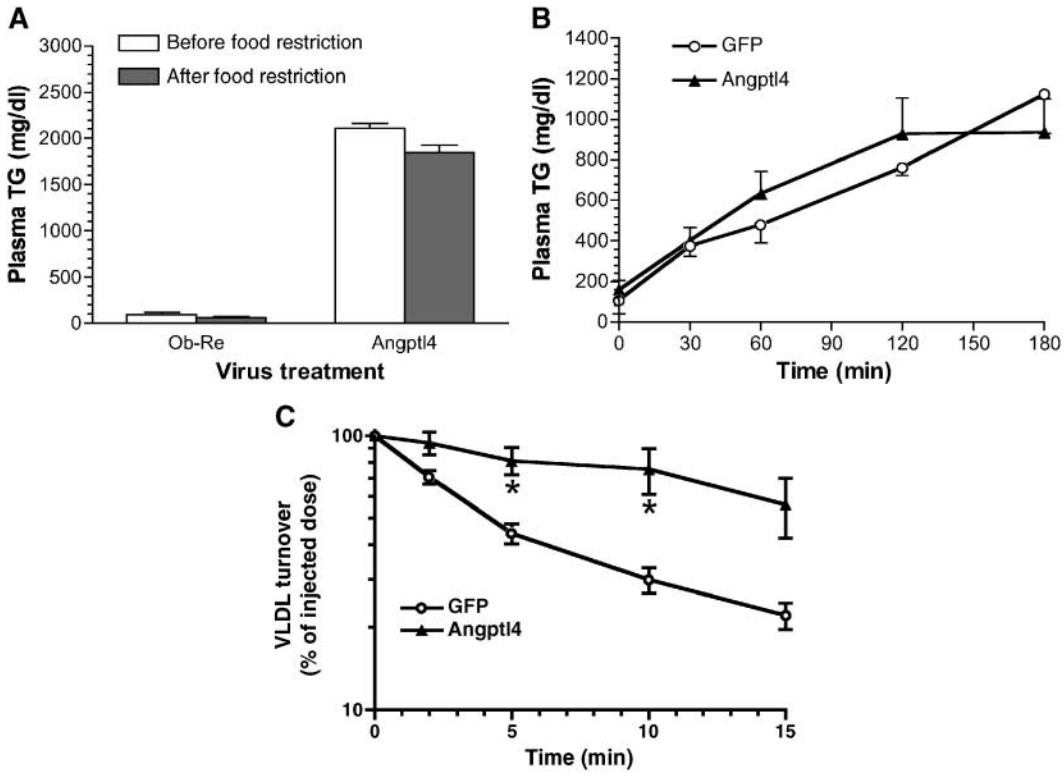

Fig. 2. Origin of accumulation of circulating VLDL in Angptl4-treated mice. A: Lack of effect of food intake on plasma VLDL levels in mice overexpressing Ad-Angptl4-myc (Angptl4). Adenoviruses overexpressing soluble leptin receptor (Ob-Re) were used as a control. Four days after adenovirus injection, food (normal chow, 4% fat) was removed from mice at 9 a.m. for 8 h. Plasma levels of TG were determined at the start and end of food removal. B: Secretion rate of VLDL from liver of Ad-Angptl4-myc-treated mice was not increased. Two days after virus injection, Angptl4 expression level was already high but plasma TG had not increased substantially (Fig. 1B). This time point was chosen to avoid the potential interference of high TG levels in Angptl4-treated mice on subsequent TG measurements after Triton treatment. Mice overexpressing adenovirally encoded GFP or Angptl4 were injected with Triton-1339 for the indicated times, and plasma samples were obtained to determine the accumulation of circulating TG. C: Delayed clearance of [^{3}H]VLDL tracer in mice overexpressing Angptl4. Two days after virus injection, equal amounts of [^{3}H]VLDL tracer (60,000 cpm) prepared from [^{3}H]palmitate-injected donor mice were administered intravenously into mice that received control adenovirus (GFP) or adenovirus encoding Angptl4. Plasma samples were collected at the indicated times, and an exponential decay curve of injected tracer was calculated. * $P < 0.05$. n = 5 mice/group. Error bars represent standard deviation of the mean of five mice/group.

ter the stability of the expressed protein (Fig. 4B, left panel). When all proteins were run under nonreducing conditions and blotted for Angptl4, both C76A and C80A retain the ability to form an apparent dimer but not a higher order oligomer (Fig. 4B, right panel, lanes 3 and 4). However, when both cysteine residues are mutated, recombinant Angptl4 runs exclusively as a monomer (Fig. 4B, both panels, lane 5) in the absence or presence of β-mercaptoethanol.

Oligomerization-defective Angptl4 has reduced efficacy in increasing plasma TG levels

To determine the role of oligomerization on the hyperlipidemic effect of Angptl4, we generated an adenovirus vector encoding C76A/C80A-Angptl4, which is incapable of forming a higher order structure. Because of the instability of the N-terminal coiled-coil domain of Angptl4, we first engineered a double-tagged expression vector containing an additional three copies of the FLAG tag immediately after the signal sequence of Angptl4, so that mature full-length Angptl4 will have three copies of FLAG at the N terminus and one copy of *c-myc* at the C terminus (**Fig. 5A**). Detection of the same protein containing both tags indicated that the sensitivity obtained using the FLAG tag antibody is higher than that using the *c-myc* antibody (Fig. 5B).

Double-tagged Angptl4 vectors encoding wild-type Angptl4 or C76A/C80A-Angptl4 were injected into mice. Expression levels of both Angptl4 constructs are similar, as judged by Northern blotting (data not shown) and detection of the C-terminal fibrinogen-like domain of Angptl4 in plasma (**Fig. 6A**, lower panel). Plasma was collected at different time points after virus infection, and the profile of TG levels is shown in Fig. 6B. Compared with wild-type Angptl4, Ad-C76A/C80A-Angptl4 showed significantly reduced capacity to increase plasma TG levels. This reduced capacity is positively correlated with the amount of N-terminal coiled-coil domain present in plasma: although it is possi-

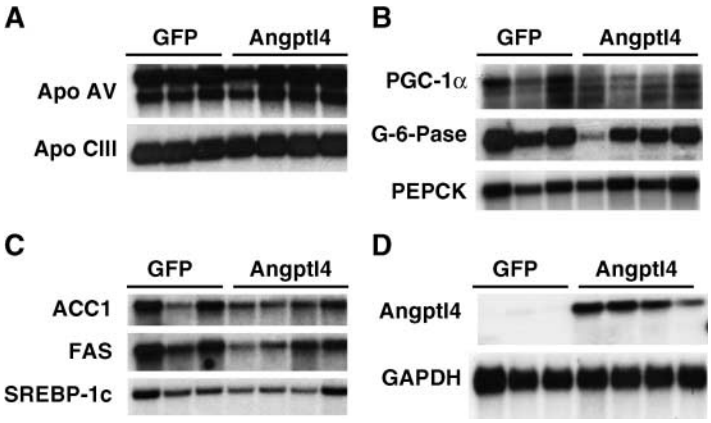

Fig. 3. Angptl4 overexpression does not cause significant changes in the genes encoding apolipoproteins (A), key molecules in TG synthesis (B), or those controlling gluconeogenesis (C). Each lane was loaded with 10 μg of total RNA from the liver of a single mouse treated with Ad-GFP or Ad-Angptl4 for 4 days. The genes examined are labeled on the left of each blot. Viral expression of Angptl4 was confirmed by hybridization with an Angptl4 cDNA probe. Equal loading was confirmed by hybridization with a GAPDH cDNA probe (D). ACC1, acetyl-CoA carboxylase 1; Apo AV and Apo CIII, apolipoproteins A-V and C-III; G-6-Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferators-activated receptor gamma coactivator-1 α; SREBP-1c, sterol-regulatory element binding protein-1c.

ble to detect this domain in mice injected with Ad-Angptl4 using FLAG antibody, signal from mice infected with Ad-C76A/C80A-Angptl4 is essentially nondetectable (Fig. 6A, upper panel). Coupled with the observation that the N-terminal coiled-coil domain of Angptl3 is responsible for its hyperlipidemic effect (7), our results demonstrate that oligomerization of Angptl4 is not necessary for its plasma TG-increasing effect. We suggest that oligomerization of Angptl4 at its N-terminal coiled-coil domain stabilizes it and thus causes an enhanced effect on increasing plasma TG levels.

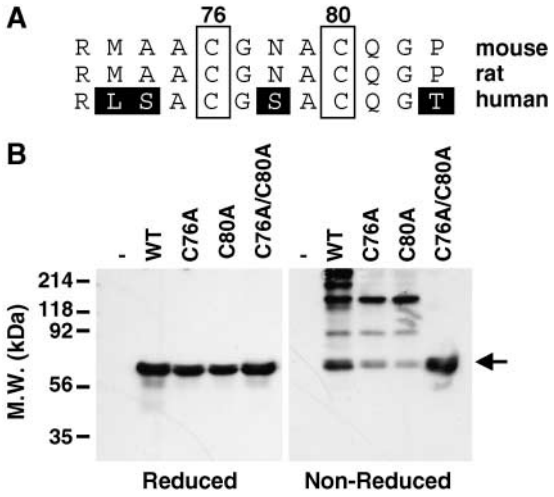

Fig. 4. Determination of cysteine residues required for Angptl4 oligomerization. A: Alignment of amino acid sequences of mouse, rat, and human Angptl4 flanking the two cysteine residues within the coiled-coil domain. The position of each cysteine is boxed, with residue numbers labeled at the top. Residues that differ between species are shaded in black. B: Both Cys-76 and Cys-80 of Angptl4 are required for oligomerization. Full-length cDNA encoding Angptl4 was mutated at Cys-76 and Cys-80 singly (C76A, C80A) or in combination (C76A/C80A) and transfected into HEK293 cells in the presence of protease inhibitor cocktail to prevent proteolysis (21). Supernatant was run in SDS-PAGE under reducing or nonreducing conditions as indicated and blotted with the *c-myc* monoclonal antibody. A single mutation (C76A or C80A) causes Angptl4 to migrate as an apparent dimer under nonreducing conditions, whereas a double mutation (C76A/C80A) causes Angptl4 to be completely monomeric. M.W., molecular mass; WT, wild type.

DISCUSSION

Previously, we reported that a recently identified secreted protein, Angptl4, also known as PGAR/FIAF/HFARP, circulates in plasma in full-length as well as proteolytically processed forms (21). Oligomerized Angptl4 is converted to a C-terminal fibrinogen-like domain in vitro and in vivo, mediated by a serum-induced protease. This is a unique property that has been reported in only two members of the family of angiopoietins or angiopoietin-like proteins (8, 21). The N-terminal coiled-coil domain of Angptl3 is responsible for its hyperlipidemic effect, possibly by directly inhibiting the activity of lipoprotein lipase. The function of the monomeric fibrinogen-like domain is not known.

Earlier, Yoshida et al. (8) reported that injection of recombinant Angptl4 into mice causes hypertriglyceridemia in mice. The same study also reported that Angptl4 is an inhibitor of lipoprotein lipase in vitro. However, these studies used bacteria-derived recombinant Angptl4 protein. As we have recently demonstrated that Angptl4 is a glycoprotein (21), it raised the question of whether this unmodified Angptl4 would reproduce all aspects of Angptl4 activity. Many studies have shown that glycosylation can be essential in maintaining productive protein-protein interactions (22, 23). Our results demonstrate that glycosylation, which occurs within the C-terminal fibrinogen-like domain of Angptl4, is not required for the plasma TG-increasing effect of Angptl4. Because the N-terminal coiled-coil domain of recombinant Angptl4 inhibits LPL action both in vitro and in vivo (8), it is likely that Angptl4 binds LPL directly and inhibits its enzymatic activity. Additional experiments are required to test this hypothesis further.

We injected ^{3}H-labeled VLDL to estimate the clearance rate of plasma VLDL in the presence of overexpressed Angptl4 (Fig. 2C). To increase the yield of labeled VLDL, we injected Triton WR-1339 into donor mice. Although this method has been used frequently to inhibit lipoprotein turnover (24, 25), the presence of Triton WR-1339 is likely to change lipoprotein particle composition because of its intrinsic properties as a detergent. Triton WR-1339 that is copurified with labeled VLDL may change the plasma turnover rate in the absence of Angptl4. Given these limi-

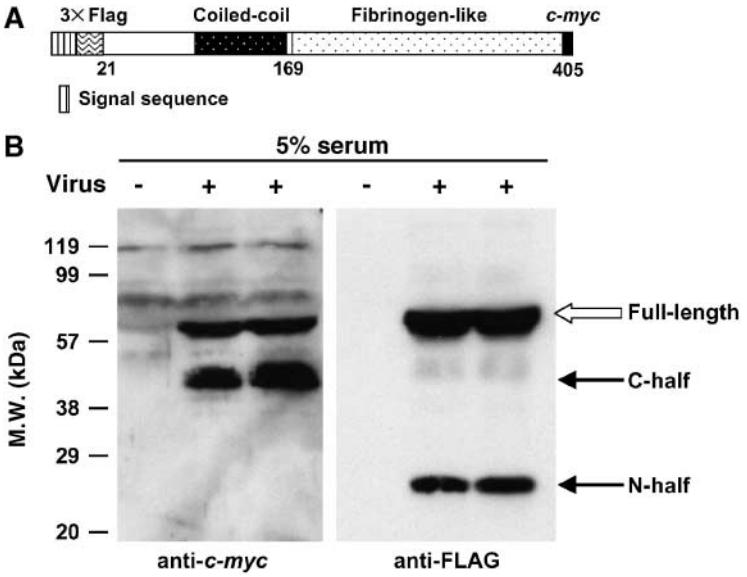

Fig. 5. Generation and expression of the double-tagged Angptl4 expression construct. A: Diagram of the adenovirus construct encoding an N-terminal FLAG tag and a C-terminal *c-myc* tag. The domain structure (top) and the boundaries of each domain (bottom) within Angptl4 are indicated. B: Proteolytic processing of the double-tagged Angptl4 by Western analysis. HEK293 cells were mock-infected (−) or infected with Ad-Angptl4 (+) and allowed to express recombinant Angptl4 in the presence of 5% serum. Supernatant was collected 48 h after infection and blotted for Angptl4 with antibodies against both the FLAG tag, which recognizes the N terminus of Angptl4, and the *c-myc* tag, which recognizes the C terminus of Angptl4. Both antibodies recognize the full-length Angptl4 (open arrow), whereas each also recognizes only the proteolytically processed N-terminal coiled-coil domain or the C-terminal fibrinogen-like domain (arrows) of Angptl4. The antibodies used are indicated at the bottom of each blot. M.W., molecular mass.

tations, the extent of inhibition of VLDL turnover by Angptl4 may be different from that shown in Fig. 2C.

It is not known if Angptl4 also affects the metabolism of chylomicrons. Because LPL can hydrolyze both VLDL and chylomicrons, it is likely that Angptl4 also inhibits the clearance of chylomicron similarly. However, it is difficult to address this question experimentally, such as by feeding of a fat-rich meal to mice already expressing Angptl4. Because of the transient and potent nature of virally produced Angptl4 on plasma VLDL levels (Fig. 2), the increase in plasma TG caused by the accumulation of both VLDL and chylomicron and that caused by VLDL accumulation alone may not be distinguishable.

The processing of oligomerized, full-length Angptl4 is reminiscent of that seen for adiponectin/ACRP30 (26–28). In both cases, proteolytic conversion is associated with changes of the properties of full-length proteins. In the case of adiponectin/ACRP30, processing of the full-length protein causes enhanced effects on muscle fat oxidation and glucose transport, whereas intact adiponectin inhibits hepatic glucose production (29–32). The formation of a high molecular weight complex of adiponectin/ACRP30 is regulated by insulin and dependent on Cys-39. Its plasma levels are also under complex hormonal control and may play a key role in determining systemic insulin sensitivity under specific conditions (26, 27, 33, 34). In Angptl4, cysteine residues are also required for higher order oligomer formation, mediated by both Cys-76 and Cys-80 (Fig. 3). It is not clear if a receptor for Angptl4 exists, and if so, whether full-length Angptl4 and its C-terminal fibrinogen-like domain binds Angptl4 receptor with equal affinity.

Angptl4 does not act directly on hepatocytes to modulate the synthesis and secretion of lipoproteins. Hepatic expression of genes involved in lipogenesis, such as SREBP-1c, ACC1, and FAS, were not affected by Ad-Angptl4 treatment (Fig. 3). This result is consistent with an earlier re-

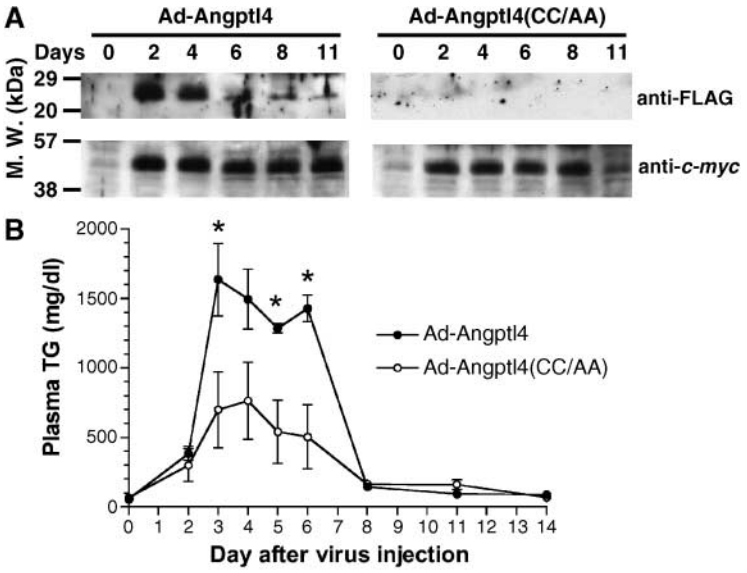

Fig. 6. Reduced hyperlipidemic effect of monomeric Angptl4. A: Determination of the levels of processed, N-terminal, and C-terminal halves of Angptl4 expressed from Ad-Angptl4 or Ad-Angptl4(CC/AA) at the indicated time points (days) after virus administration. Antibodies used were as described in Fig. 5. Although similar amounts of the C-terminal fibrinogen-like domain of Angptl4 were present in plasma of mice receiving either virus (lower panel), the N-terminal coiled-coil domain of monomeric Angptl4, Angptl4(CC/AA), is essentially nondetectable (upper panel), suggesting that oligomerization may serve to stabilize this domain. M.W., molecular mass. B: Adenoviruses encoding wild-type Ad-Angptl4 or monomeric Angptl4 [Ad-Angptl4(CC/AA)] were infused into mice, and plasma TG levels were determined as in Fig. 1. n = 5 mice/group. The experiment was performed three times. * $P < 0.05$. Error bars represent standard deviation of the mean of five mice/group.

port that Angptl4 inhibits lipoprotein lipase activity in vitro (8). The increased VLDL levels in plasma of Ad-Angptl4-overexpressing mice are analogous to those of knockout mice with deletion of lipoprotein lipase (35). Restoration of lipase activity from a muscle-specific LPL transgene or via adenovirus-mediated rescue corrected the increased VLDL in plasma of these mice (35, 36).

Another circulating protein structurally related to Angptl4, Angptl3, also causes increased plasma VLDL by inhibition of LPL (6). In this regard, Angptl4 may constitute another novel component in the regulation of plasma lipid homeostasis. The similarities between Angptl3 and Angptl4 also extend to their regulation by nuclear hormone receptors: Angptl3 is a direct target of liver X receptor (37, 38), whereas Angptl4 is a target of PPARα and PPARγ (3, 11). Because both PPARs and liver X receptor dimerize with retinoid X receptor for biological activity, it is conceivable that both Angptl3 and Angptl4 are also regulated in a similar manner via retinoid X receptor activation. In addition, Angptl3 has also been reported to activate lipolysis by directly binding to adipocytes to stimulate the release of FFA and glycerol (39). On the other hand, Angptl3 is expressed exclusively by liver, whereas Angptl4 is expressed selectively by adipose tissue, liver, and placenta. The differences in sites of expression could confer tissue-specific action of Angptl3 and Angptl4.

The function of the C-terminal fibrinogen-like domain of Angptl4 is not known. Its stability in plasma suggests that it could have an endocrine role to mediate additional biological activities of Angptl4. The same domain of angiopoietin 1 binds its receptor Tie2 to induce angiogenesis, providing the exciting possibility that an Angptl4 receptor may also exist and that the C-terminal fibrinogen-like domain could also perform similar functions (13). Additional experiments are needed to address these possibilities.

In summary, we show that Angptl4/PGAR/FIAF/HFARP, an adipose tissue-derived circulating protein, resembles Angptl3 in increasing plasma TG levels (15). Both proteins also undergo proteolytic processing into two species, which may serve to expose the functional moiety of the N-terminal coiled-coil domain in mediating its hyperlipidemic effect and its C-terminal fibrinogen-like domain in possibly other functions (7, 21). It is intriguing to speculate that Angptl4 could have evolved to have two distinct functions that together serve a common purpose. One possibility is that its fibrinogen-like domain may serve as an angiogenesis factor, which recruits sources of energy by inhibiting VLDL breakdown and utilization at other sites by inhibition of lipoprotein lipase activity. The inappropriate proteolytic conversion could be associated with altered plasma lipid homeostasis and possibly other biological processes.

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