Oligomerization and Regulated Proteolytic Processing of Angiopoietin-like Protein 4*

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Angiopoietin-like protein 4 (Angptl4) is a recently identified circulating protein expressed primarily in adipose tissue and liver. Also known as peroxisome proliferator-activated receptor (PPAR)-γ angiopoietin-related, fasting induced adipose factor, and hepatic fibrinogen/angiopoietin-related protein, recombinant Angptl4 causes increase of plasma very low density lipoprotein levels by inhibition of lipoprotein lipase activity. Similar to angiopoietins and other angiopoietin-like proteins, Angptl4 contains an amino-terminal coiled-coil domain and a carboxyl-terminal fibrinogen-like domain. We report here that Angptl4 is evolutionarily conserved among several mammalian species and that full-length Angptl4 protein is an oligomer containing intermolecular disulfide bonds. Oligomerized Angptl4 undergoes proteolytic processing to release its carboxyl fibrinogen-like domain, which circulates as a monomer. Angptl4’s N-terminal coiled-coil domain mediates its oligomerization, which by itself is sufficient to form higher order oligomeric structure. Adenovirus-mediated overexpression of Angptl4 in 293 cells shows that conversion of full-length, oligomerized Angptl4 is mediated by a cell-associated protease activity induced by serum. These findings demonstrate a novel property of angiopoietin-like proteins and suggest that oligomerization and proteolytic processing of Angptl4 may regulate its biological activities in vivo.

Until recently, adipose tissue has been thought of as largely a passive energy storage depot for periods of nutritional excess. It releases stored energy in the form of free fatty acids and glycerol upon hydrolysis of esterified lipids by lipases that are under hormonal control (1). The cloning of leptin from adipose tissue and the subsequent discoveries that adipose tissue is also an active endocrine organ, and the expression patterns of these secreted factors are under both nutritional and hormonal controls (2–4).

One of the recently identified secreted factors from adipose tissue is angiopoietin-like protein 4 (Angptl4)‡, also named PPARγ angiopoietin-related, fasting induced adipose factor, or hepatic fibrinogen/angiopoietin-related protein. It is a secreted protein selectively expressed in adipose tissue, liver, and placenta (5–7). 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 carboxyl terminus. Such domain organization is preserved in both angiopoietins and angiopoietin-like proteins.

Transcriptional regulation of Angptl4 is rather complex. Several transcription factors have been identified as having a role in Angptl4 transcription, including two members of the peroxisome proliferator-activated receptor family of transcription factors, PPARγ and PPARα (6, 7), as well as hypoxia-inducible factor 1α (8). As a transcriptional target of PPARγ, Angptl4 has been hypothesized to play a role in the modulation of adipogenesis, insulin sensitivity, or energy metabolism (7). Mice heterozygous for PPARγ knockout have reduced Angptl4 mRNA expression in adipose tissue, where PPARγ is normally abundantly expressed. Mice with PPARα deficiency also exhibit undetectable Angptl4 mRNA levels in liver, where PPARα is normally abundantly expressed. Conversely, when mice are treated with a potent agonist of PPARγ, WY14643, Angptl4 transcription is increased (6). In vitro, treatment of NIH 3T3 cells stably expressing PPARγ with its agonist, pioglitazone, caused an increase of at least 10-fold in Angptl4 mRNA levels in the absence of protein synthesis (7). PPARα and hypoxia-inducible factor 1α also cause the synergistic activation of Angptl4 transcription in cardiomyocytes (8). In a recent report, Angptl4 was found to be one of nine genes silenced by methylation in human gastric cancers (9). In aggregate, these data suggest that Angptl4 may play a variety of roles in vivo, ranging from adipogenesis and angiogenesis to carcinogenesis (10).

Because both ischemic tissue and solid tumor tissue are in a hypoxic state, Angptl4 could be playing a shared role of modulating angiogenesis under both conditions.

The expression of angptl4 is also under nutritional and hormonal control. Under fasting conditions, transcription of Angptl4 in both liver and adipose tissue is induced independently of the presence of PPARγ (6). Circulating levels of Angptl4 are also increased in genetically obese mice (ob/ob or db/db) that lack leptin signaling (7). However, in rodents fed a high-fat diet, circulating levels of Angptl4 are reduced, provid-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY393999.

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‡‡ The abbreviations used are: Angpt, angiopoietin-like protein; PPAR, peroxisome proliferator-activated receptor; PBS, fetal bovine serum; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; GFP, green fluorescent protein; PNGase, peptide N-glycosidase F.
ing further evidence that Angptl4 may play a role in response to the availability of nutrients (6).

The function of Angptl4 is not completely understood. Recently, it was reported that Angptl4 is a potent hyperlipidemia-inducing factor and an inhibitor of lipoprotein lipase (11). To obtain better insight on the structural basis of Angptl4 function, we have now determined that full-length Angptl4 is an oligomer, similar to angiopoietin 1 (12). In contrast to angiopoietin 1, however, Angptl4 undergoes regulated proteolysis to release its carboxyl-terminal fibrinogen-like domain both in vitro and in vivo, the first example of proteolysis-mediated processing of a member of this family of proteins.

**MATERIALS AND METHODS**

**Reagents**—All PCR primers were synthesized by Sigma-Genosys or IDT Technologies. All restriction endonucleases as well as PNGase F were from New England Biolabs, Inc. (Beverly, MA). Taq DNA polymerase and protease inhibitor mixture tablets were from Roche (Indianapolis, IN). ECL-Western blotting reagents and Superdex 200 column (10/30) were from Amersham Biosciences. TALON Metal Affinity resin was from BD Biosciences Clontech. Polyclonal antibodies against Angptl4 were generated in rabbits by Covance Research Products, Inc. (Denver, PA). Fetal bovine serum (FBS) was from Gemini Bio-Products (Woodland, CA). SuperScript II RNase H1 reverse transcriptase was from Invitrogen.

**Cloning of Rat Angptl4 and Construction of Adenoviral Expression Vectors**—To amplify cDNA encoding full-length Angptl4 from rat adipose tissue, first-strand cDNA was generated by reverse transcription of total RNA using SuperScript II RNase H1 reverse transcriptase. Both full-length Angptl4 and the amino-terminal coiled-coil domain of Angptl4 were amplified using the same 5’ primer, CL307. It contains an XhoI site (underlined) for in-frame subcloning into the mammalian expression vector pcDNA3.1 (+)/Myc-HisA and has the following sequence: 5’-CCGCTCGAGAATCATGCGCTGCGCTCCG-3’; the 3’ ends of the full-length and the amino-terminal coiled-coil domain of Angptl4 were amplified by primers CL379 and CL380, each containing a HindIII site (underlined) for in-frame subcloning into the same vector. The sequences are: CL379, 5’-CCC AAG CTT AGA GGC TGC TGT AGC CTC CAT G-3’; CL380, 5’-CCC AAG CTT CTG GGT CAT CTT GGG AAG-3’. Clones containing inserts from primer pairs CL307-CL379 and CL307-CL380 were verified by sequencing analysis and transient transfection.

To generate adenoviruses encoding the N-terminal coiled-coil domain (Ad-Angptl4(S)-myc) or full-length (Ad-Angptl4(L)-myc) rat Angptl4, the AdEasy system was used, with a slight modification (13). Briefly, to facilitate detection of virally produced Angptl4 proteins, inserts in the pcDNA3.1 (+)/Myc-His vector were removed along with the carboxyl-terminal c-myc tag as a PmeI fragment and ligated to the EcoRV site of pShuttle-CMV. The resulting plasmid was then linearized with PmeI, 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 KanR colonies. PacI digestion was then performed to confirm the presence of inserts. Typically, this procedure yields ~50% recombinants. Viral DNA was transformed into DH5α cells and propagated before being used for transfection into low passage 293 cells as described previously (14).

**Purification of Angptl4 Protein**—Conditioned media with or without serum from 293 cells infected with Ad-Angptl4(L)-myc virus were collected and filtered through a 0.22-μm filter. Buffering capacity and salt concentrations were adjusted to 20 mM Tris-HCl, pH 8.0, and 300 mM NaCl by diluting conditioned media with 1/10 volume of 200 mM Tris-HCl, pH 8.0, and 3 M NaCl. 5 μL of TALON Metal Affinity resin was pre-equilibrated with 50 ml of wash buffer (20 mM Tris-HCl, pH 8.0, and 300 mM NaCl), before 1000 ml of conditioned medium was applied to the resin. Resin and medium were rocked gently at 4°C overnight. Resin
Fusional protein was run on preparative SDS-PAGE and cut out after staining with Coomassie blue. Gel slice was homogenized and injected into female New Zealand White rabbits for antigen production. In all cases, specificity of the antibodies was determined out after staining with Coomassie brilliant blue. Gel slice was homogenized and injected into female New Zealand White rabbits for antibody production. Antigen 2 was generated using a glutathione fusion system (Novagen). A 512-bp cDNA sequence encoding the carboxyl terminus of Angptl4 was prepared from mammalian cells (antigen 1) or bacteria (antigens 2 and 3) for generation of rabbit polyclonal antibodies using different expression systems. Antigen 1 was purified from processed carboxyl terminus of Angptl4 from conditioned medium containing 5% FBS of Ad-Angptl4(L)-myc virus-infected 293 cells as described above. Partially purified Angptl4 protein was separated on preparative 12% SDS-PAGE, and the Angptl4 was band cut out after staining with Coomassie brilliant blue. Gel slice was homogenized and injected into female New Zealand White rabbits for antibody production. Antigen 2 was generated using a pET expression system (Novagen). A 512-bp cDNA sequence encoding the carboxyl fibrinogen-like domain of Angptl4 was PCR-amplified and cloned into the SmaI site of pET-44b vector (Novagen). Fusion protein was expressed in bacteria and purified according to the manufacturer’s instructions. The purified protein was also used for injection into rabbit. Antigen 3 was generated using a glutathione S-transferase fusion protein of Angptl4 as follows. Full-length Angptl4 cDNA (minus the signal peptide) was fused to the carboxyl terminus of glutathione S-transferase and expressed in bacteria, following standard methods. Fusion protein was run on preparative SDS-PAGE and cut out after staining with Coomassie blue. Gel slice was homogenized and injected into female New Zealand White rabbits for antibody production. In all cases, specificity of the antibodies was determined by serial dilution of antigen, by comparison with the signal recognized by the c-myc monoclonal antibody, and by comparison with pre-immune serum. All blots shown are recombinant Angptl4 expressed after transient transfection or adenoviral infection.

Expression and Detection of Angptl4 in 293 Cells—293 cells were cultured in 6-well plates until 80–90% confluent. Culture medium was replaced with fresh Dulbecco’s modified Eagle’s medium with or without 5% FBS before adenoviruses encoding Angptl4 were added at a multiplicity of infection of 100. Multiplicity of infection was determined based on the virus titer, obtained by serial dilution of viruses and infections of individual wells of 96-well plates. Typically, titer of purified viruses ranges from $10^6$ to $10^7$ plaque-forming units per milliliter.

Forty-eight hours after virus infection, conditioned medium was collected and centrifuged to obtain supernatant. 200 μl of conditioned medium was diluted 20-fold in PBS to remove possible protease inhibitors. Concentration of β-mercaptoethanol is 0.1% in lane labeled “reduced” in B. Both experiments were repeated for three times with similar results.

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RESULTS

Angptl4 Is Evolutionarily Conserved between Rodents and Humans—Mouse and human Angptl4 share 75% nucleotide identity and 77% amino acid identity (7). However, mouse Angptl4 contains 410 amino acids, whereas that of humans contains only 405 amino acids. To determine how rat Angptl4 shares evolutionary conservation with mouse and human Angptl4, we performed reverse transcription PCR on the first-strand cDNA derived from total RNA of rat adipose tissue. The full-length coding sequence of rat Angptl4 was obtained. Sequence comparisons revealed that, similar to human Angptl4, rat Angptl4 also encodes a protein of 405 amino acids. However, it shares only 73% identity with the human protein. On the other hand, even though rat Angptl4 has a 5-amino acid internal deletion compared with mouse Angptl4, their amino acid sequences share 97% identity, with the conserved coiled-coil domain and fibrinogen-like domain being essentially identical between mouse and rat Angptl4 proteins. In all experiments described in this study, rat Angptl4 cDNA was used. The complete nucleotide sequence of rat Angptl4 has been submitted to GenBank under accession number AY393999. The alignment of the amino acid sequences of mouse, rat, and human Angptl4 is shown in Fig. 1.

Native Angptl4 Is a Variably Sized Oligomer Containing Intermolecular Disulfide Bonds—Angiopoietin 1, the founding member of angiopoietin and angiopoietin-like protein family of secreted proteins, consists of distinct modular domains responsible for receptor binding, dimerization, and superclustering and characterization. Animal use was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center at Dallas.

Time Course of Serum-induced Angptl4 Processing—293 cells were cultured in 6-well plates until 80–90% confluent. Cells were then washed once with PBS and medium was replaced with 2 ml of fresh Dulbecco’s modified Eagle’s medium without serum; adenoviruses encoding full-length Angptl4 were added to cells at a multiplicity of infection of 100. Twenty-four hours after virus infection, FBS was added to cells to a final concentration of 5%. FBS was applied to all wells except one, which was used as control. Conditioned medium was collected at different time points from 0 to 48 h after addition of FBS and used directly for Western blotting to detect recombinant Angptl4. No protease inhibitors were added to media at any stages of Angptl4 expression.

To determine whether serum-activated proteolytic activity is released into conditioned media and whether adenovirus infection per se causes release of nonspecific proteolytic activity, supernatant was collected from 293 cells cultured in serum-containing media, with or without infection by the control adenovirus Ad-GFP. About 0.1 μg of Angptl4 protein was then incubated with collected media at 37 °C for 24 h and used for Western blotting with c-myc monoclonal antibody. Molecular mass markers are indicated on the left. Experiments were repeated for more than three times with similar results.

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To determine whether Angptl4 also forms higher order structure, we over-expressed rat Angptl4 in 293 cells via infection of adenoviruses encoding full-length Angptl4 (Ad-Angptl4(L)-myc). When secreted recombinant Angptl4 was detected by Western blotting, three bands were recognized by the C-terminal tag antibody (Fig. 2A, Total). The three bands consist of an upper band of ~74 kDa and a doublet of ~50 kDa. Further analysis demonstrated that the 74-kDa band represents full-length Angptl4 protein, whereas the 50-kDa doublet is the proteolytic product of Angptl4, corresponding to its carboxyl fibrinogen-like domain. The amino acid sequence boundaries of the 50-kDa doublets are not known because the N termini of both bands are blocked when subjected to sequencing analysis. One explanation for the appearance of the doublet band is the additional cleavage of primary proteolysis product, because the doublet is detected by the same carboxyl-terminal antibodies recognizing fibrinogen-like domain or c-myc tag of Angptl4.

The same supernatant containing recombinant Angptl4 was loaded onto a Superdex 200 column, and fractions were collected and analyzed by Western blotting to determine the elution profile of Angptl4 protein. Fig. 2A shows that Angptl4 first appears in an early fraction, near the void volume of the column, which excludes globular proteins of 600 kDa and larger. It continues to elute over a broad range to a position that corresponds to a protein of ~100 kDa. Its broad range of mobility in a gel filtration column suggests that Angptl4, similar to angiopoietin-1 (12), also forms variable-sized multimers. We also performed sucrose density gradient sedimentation experiments, with results similar to those obtained by gel filtration (data not shown).

Interestingly, the doublet band of recombinant Angptl4 from total starting supernatant, a proteolytic product of full-length Angptl4, eluted as an apparent monomer from the same Superdex 200 column. This doublet is recognized by both a c-myc monoclonal antibody, which recognizes the c-myc tag fused to the carboxyl terminus of Angptl4, as well as a polyclonal antibody generated in rabbit using the carboxyl fibrinogen-like domain of Angptl4 as antigen (data not shown). These results demonstrate that the monomeric carboxyl fibrinogen-like domain is converted from oligomerized, full-length Angptl4. Consequently, the carboxyl fibrinogen-like domain of Angptl4 loses the ability to form higher order structure.

Formation of high molecular weight protein complexes frequently involves disulfide bond formation between protein monomers, such as adiponectin/ACRP30 (16). We asked whether the oligomerization of full-length Angptl4 also requires disulfide bond formation between individual Angptl4 molecules. Fig. 2B shows that when denatured full-length Angptl4 was treated with SDS-PAGE sample buffer containing β-mercaptoethanol or not, the presence of β-mercaptoethanol caused Angptl4 protein to migrate at a size predicted of a monomer; in the absence of β-mercaptoethanol, bands that migrate at the size of a dimer and higher molecular weight complexes also form. Because the sizes of large proteins in a nonreducing gel are difficult to estimate, it is impossible to accurately determine the number of Angptl4 monomers that participate in disulfide bond formation. The results from this experiment are consistent with the broad elution profile of full-length Angptl4 protein during size exclusion chromatography (Fig. 2A).

**Coiled-coil Domain of Angptl4 Is Sufficient to Form Higher Order Oligomers**—The elution profile of the carboxyl fibrinogen-like domain of Angptl4 in FPLC suggests that this domain is insufficient to form an oligomer. To determine whether the N-terminal coiled-coil domain, in the absence of fibrinogen-like domain, is sufficient to mediate Angptl4 oligomerization, we generated adenoviral vectors expressing only the N-terminal coiled-coil domain (Fig. 3A). Both the full-length and the coiled-coil domain of Angptl4 were tagged with c-myc and polyhistidine epitopes at the carboxyl terminus to facilitate detection of recombinant proteins. When supernatant of 293 cells infected with viruses encoding full-length Angptl4 (Ad-Angptl4(L)-myc) or N-terminal coiled-coil domain of Angptl4 (Ad-Angptl4(S)-myc) was run on a Superdex 200 column and detected with the c-myc monoclonal antibody, both proteins eluted at similar positions as very large high molecular weight complexes (Fig. 3B). These results demonstrate that the N-terminal coiled-coil domain is sufficient to form high-order oligomeric structures. This conclusion is also supported by the observation that under nonreducing conditions, both full-length and N-terminal coiled-coil domain of Angptl4 migrates as larger molecular weight species (Fig. 3C).

**Serum-Dependent Processing of Full-length Angptl4**—We proceeded to determine how processing of full-length, oligomerized Angptl4 is regulated. Ad-Angptl4(L)-myc was used to infect 293 cells again and cells were maintained in the presence...
Ad-Angptl4(L)-myc. Cells were washed with PBS and replaced with either serum-free (−) or serum-containing (+) media. Cells were treated either with protease inhibitor mixture (mixture; +) or left untreated (−). Incubation was continued for 24 h (day 1) or 48 h (day 2) before supernatant was removed for Western blotting analysis. In the presence of protease inhibitor mixture, processing of full-length Angptl4 protein was completely inhibited in the presence of serum. A, proteolytic processing of Angptl4 is not related to a protease released in response to serum or adenovirus infection. Full-length Angptl4 protein was loaded onto SDS-PAGE directly (lane 1), incubated with fresh media (lane 2) or conditioned media from cells not infected with virus (lane 3), or infected with Ad-GFP virus (lane 4). Samples from lanes 2 to 4 were incubated at 37 °C for 24 h. No proteolysis of Angptl4 was observed in any lanes. Incubation with trypsin caused the complete disappearance of Angptl4 signal (data not shown).

C. To gain a better understanding of the cleavage process of full-length Angptl4, we expressed Angptl4 in the presence of protease inhibitors. Recombinant Angptl4 secreted into the media under either serum-free or serum-containing conditions remained mostly intact in the presence of complete protease inhibitor mixture for up to 2 days (Fig. 5A). We then determined whether the conversion of full-length, oligomerized Angptl4 to monomeric form is mediated by a protease activity present in serum or that induced in the presence of serum. Incubation of full-length Angptl4 protein with conditioned media from cells cultured in the presence of serum, with or without infection by control Ad-GFP virus shows that the protease is not released into media (Fig. 5B, lane 3), nor was processing a nonspecific effect caused by adenovirus infection (Fig. 5B, lane 4). These results demonstrate that proteolytic processing of full-length, oligomerized Angptl4 requires the presence of serum as well as molecules from cells. The time course of protease induction is shown in Fig. 5C. Angptl4 was first allowed to express in serum-free media for 24 h before serum was added. 24 to 48 h after serum addition, the appearance of the ~50-kDa doublet reaches peak levels, suggesting full induction of proteolytic activity. In aggregate, these results demonstrate that the proteolytic processing of Angptl4 occurs before its release into media, mediated by a cell-associated protease activity that is activated by serum.

Both the Full-length and the Fibrinogen-like Domain of Angptl4 Are Glycosylated—Because full-length Angptl4 ob-

| Time (hr) | No addition | 5% FBS |
|----------|-------------|--------|
| 0        | 24 Hrs. serum-free |
| 48       |             |        |
| 1        |             |        |
| 2        |             |        |
| 4        |             |        |
| 8        |             |        |
| 24       |             |        |
| 48       |             |        |

or absence of serum to determine whether the presence of serum is necessary for Angptl4 processing. Fig. 4A shows that when 293 cells were infected with adenoviruses encoding Angptl4 or GFP and maintained in media without serum, Angptl4 is secreted predominantly as a full-length protein of ~74 kDa (Fig. 4A, long arrow). In the presence of 5% serum, Angptl4 protein detected in the supernatant exists as either a full-length protein as that secreted into serum-free media or as a doublet of ~50 kDa (Fig. 4A, short arrow). Supernatant or pellet samples of cells infected with Ad-GFP showed no signal. Both the full-length and the carboxyl fibrinogen-like domain of Angptl4 were detected by the c-myc antibody, which recognizes the carboxyl terminus of Angptl4 (Fig. 4A) and polyclonal antibodies against Angptl4 (data not shown). No processed Angptl4 fragment was detected in total cell lysate when cells were cultured in either serum-free or serum-containing media, suggesting that cleavage of full-length did not occur during the transport of Angptl4 to plasma membrane. The amino acid boundaries at the N terminus of the doublet are not known because sequencing analysis revealed that the N termini of both bands are blocked (data not shown).

We tested whether full-length Angptl4 expressed in vivo also undergo similar proteolytic processing as in vitro. The same adenovirus construct, Ad-Angptl4(L)-myc, which encodes full-length Angptl4, was infused into mice intravenously and recombinant Angptl4 was detected by Western blotting from both liver lysate and plasma. Fig. 4B shows that in plasma, only the 50-kDa doublet band of Angptl4 was observed, which is indistinguishable in size from that present in serum-containing tissue culture media (Fig. 4B, lanes 3 and 4), suggesting the relative instability of full-length Angptl4 protein in vivo. Lysates from virus-infected cells or liver contain only full-length Angptl4 protein (Fig. 4B, lanes 7 and 8). Taken together, these studies demonstrate that proteolytic processing of Angptl4 is induced in the presence of serum both in vitro and in vivo.

To gain a better understanding of the cleavage process of full-length Angptl4, we expressed Angptl4 in the presence of protease inhibitors. Recombinant Angptl4 secreted into the media under either serum-free or serum-containing conditions remained mostly intact in the presence of complete protease inhibitor mixture for up to 2 days (Fig. 5A). We then determined whether the conversion of full-length, oligomerized Angptl4 to monomeric form is mediated by a protease activity present in serum or that induced in the presence of serum. Incubation of full-length Angptl4 protein with conditioned media from cells cultured in the presence of serum, with or without infection by control Ad-GFP virus shows that the protease is not released into media (Fig. 5B, lane 3), nor was processing a nonspecific effect caused by adenovirus infection (Fig. 5B, lane 4). These results demonstrate that proteolytic processing of full-length, oligomerized Angptl4 requires the presence of serum as well as molecules from cells. The time course of protease induction is shown in Fig. 5C. Angptl4 was first allowed to express in serum-free media for 24 h before serum was added. 24 to 48 h after serum addition, the appearance of the ~50-kDa doublet reaches peak levels, suggesting full induction of proteolytic activity. In aggregate, these results demonstrate that the proteolytic processing of Angptl4 occurs before its release into media, mediated by a cell-associated protease activity that is activated by serum.

Both the Full-length and the Fibrinogen-like Domain of Angptl4 Are Glycosylated—Because full-length Angptl4 ob-

FIG. 5. Proteolytic processing of Angptl4 is serum-dependent and can be inhibited by protease inhibitors. A, proteolytic processing of Angptl4 may be inhibited by protease inhibitors. 293 cells grown in Dulbecco’s modified Eagle’s medium containing 5% FBS were infected with Ad-Angptl4(L)-myc. Cells were washed with PBS and replaced with either serum-free (−) or serum-containing (+) media. Cells were treated either with protease inhibitor mixture (mixture; +) or left untreated (−). Incubation was continued for 24 h (day 1) or 48 h (day 2) before supernatant was removed for Western blotting analysis. In the presence of protease inhibitor mixture, processing of full-length Angptl4 protein was completely inhibited in the presence of serum.

B. Proteolytic processing of Angptl4 is not related to a protease released in response to serum or adenovirus infection. Full-length Angptl4 protein was loaded onto SDS-PAGE directly (lane 1), incubated with fresh media (lane 2) or conditioned media from cells not infected with virus (lane 3), or infected with Ad-GFP virus (lane 4). Samples from lanes 2 to 4 were incubated at 37 °C for 24 h. No proteolysis of Angptl4 was observed in any lanes. Incubation with trypsin caused the complete disappearance of Angptl4 signal (data not shown).
The fibrinogen-like domain of Angptl4 is glycosylated. 293 cells were infected with adenoviruses encoding GFP, full-length Angptl4 (Lmyc), or its coiled-coil domain (Smyc). Secreted recombinant Angptl4 protein was obtained from either serum-free supernatant (−) or serum-containing supernatant (+). Samples were treated with buffer alone or buffer plus with PNGase F as indicated. All samples were run on reducing SDS-PAGE and blotted with the c-myc monoclonal antibody, which recognizes the c-myc epitope fused to the carboxyl terminus of Angptl4. Each lane contains ~50 of ng Lmyc (with or without serum) or ~10 ng of Smyc (with or without serum). The mobility of both full-length Angptl4 (lane 6, top band) and its processed fibrinogen-like domain (lane 7, lower band) is increased, whereas that of the coiled-coil domain was not changed by PNGase F treatment (lanes 4 and 8). The experiment was repeated three times.

DISCUSSION

In this report, we demonstrated that a recently identified secreted protein, Angptl4, which is selectively expressed in adipose tissue, liver, and placenta, exists both as a full-length oligomerized protein as well as proteolytically processed forms. Oligomerized Angptl4 is processed by a serum-induced proteolytic activity to release its carboxyl fibrinogen-like domain, which circulates as a monomer. This unique property has not been reported in any other angiopoietins or angiopoietin-like proteins. The N-terminal coiled-coil domain of Angptl4 is sufficient to form high molecular weight structure. The monomeric fibrinogen-like domain is glycosylated and its function has not been elucidated.

The processing of oligomerized, full-length Angptl4 is reminiscent of that of adiponectin/ACRP30. In both cases, proteolytic conversion is associated with changes of the properties of full-length proteins. In the case of adiponectin/ACRP30, processing of full-length adiponectin/ACRP30 causes enhanced effect on muscle fat oxidation and glucose transport (17, 18). The formation of high molecular weight complex of adiponectin/ACRP30 is regulated by insulin and dependent on Cys-39 of adiponectin/ACRP30. Its plasma levels are also under complex hormonal control and may play a key role in determining systemic insulin sensitivity under the respective conditions (19, 20). Whereas cysteine is required for dimer formation of Angptl4, the key residue involved in disulfide formation and the regulation of Angptl4 processing from high molecular weight complexes remain to be determined.

An earlier report indicates that Angptl4 causes hyperlipidemia by inhibiting lipoprotein lipase activity (11). Another circulating protein structurally related to Angptl4, angiopoietin-like protein 3 (Angptl3), also causes elevation of plasma very low density lipoprotein elevation by inhibition of LPL (21). Analogous to Angptl3, Angptl4 may thus be another circulating regulator 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 (22, 23), whereas Angptl4 is a target of PPARα and PPARγ. Because both PPARs and liver X receptor dimerize with retinoid X receptor for full biological activity, it is conceivable that both Angptl3 and -4 are also regulated by the same factor(s) in vivo. In addition, Angptl3 has also been reported to activate lipolysis by directly binding to adipocytes to stimulate the release of FFA and glycerol (24). However, Angptl3 is expressed exclusively by liver, whereas Angptl4 is expressed selectively by adipose tissue, liver, and placenta, and potentially other tissues. In addition, the levels of Angptl3 expression are not regulated by nutritional status. The differences in sites and regulation of expression could thus confer tissue specific actions of Angptl3 and -4.

In summary, we show that Angptl4, a circulating protein primarily expressed by adipose tissue and liver, resembles angiopoietin 1 in forming a higher order structure consisting of variable-sized multimers (12). In contrast to angiopoietin 1, however, oligomerized full-length Angptl4 undergoes regulated proteolysis into an amino-terminal coiled-coil domain and a carboxyl fibrinogen-like domain, the first example among all angiopoietins and angiopoietin-like proteins. An exciting possibility is that proteolytic processing of full-length, oligomerized Angptl4 could serve as a mechanism of activation, with each released domain having distinct roles. One such consequence is the hyperlipidemic effect of Angptl4 (11), which could be mediated by either domain. It is intriguing to speculate that inappropriate proteolytic conversion of Angptl4 in vivo could be associated with certain disorders of altered plasma lipid homeostasis as well as possibly other pathological conditions.

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Note Added in Proof—While this manuscript was under review, Ono et al. (Ono, M., Shimizugawa, T., Shimamura, M., Yoshida, K., Noj-Sakikawa, C., Ando, Y., Koishi, R., and Furukawa, H. (2003) J. Biol. Chem. 278, 41804–41809) also reported the proteolytic cleavage of angiopoietin-like 3, a protein sharing homology with Angptl4.

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