Proteolytic Processing of Angiopoietin-like Protein 4 by Proprotein Convertases Modulates Its Inhibitory Effects on Lipoprotein Lipase Activity*

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Angiopoietin-like protein 4 (ANGPTL4) has been associated with a variety of diseases. It is known as an endogenous inhibitor of lipoprotein lipase (LPL), and it modulates lipid deposition and energy homeostasis. ANGPTL4 is cleaved by unidentified protease(s), and the biological importance of this cleavage event is not fully understood with respect to its inhibitory effect on LPL activity. Here, we show that ANGPTL4 appears on the cell surface as the full-length form, where it can be released by hepatic fibrinogen/angiopoietin-related protein, fasting-inhibitory protein (FIP), or adiponectin. ANGPTL4 is proteolytically cleaved into several forms by proprotein convertases (PCs). Several PCs, including furin, PC5/6, paired basic amino acid-cleaving enzyme 4, and PC7, are able to cleave human ANGPTL4 at a consensus site. PC-specific inhibitors block the processing of ANGPTL4. Blockage of ANGPTL4 cleavage reduces its inhibitory effects on LPL activity and decreases its ability to raise plasma triglyceride levels. In summary, the cleavage of ANGPTL4 by these PCs modulates its inhibitory effect on LPL activity.

Angiopoietin-like protein 4 (ANGPTL4) is also known as hepatic fibrinogen/angiopoietin-related protein, fasting-induced adipose factor, peroxisome proliferator-activated receptor-α, angiotensinogen, and γ-angiopoietin-related protein. It is one of the seven members of the ANGPTL family (ANGPTL1–7). Mainly produced in hepatocytes in humans and adipocytes in mice, ANGPTL4 exerts its biological effects by means of autocrine/paracrine and endocrine processes. ANGPTL4 has been implicated in a variety of diseases, including cardiovascular disease (1, 2), cancer metastasis (3), obesity (4), diabetes (5), wound repair (6, 7), inflammation (8), and arthritis (9). ANGPTL4 is a fusion protein consisting of an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain.

These two domains have been shown to have distinct biological functions (10). The N-terminal domain is responsible for the inhibitory effects on LPL, converting the active form of LPL into an inactive form (11), and the C terminus mediates its antiangiogenic functions (12). Interestingly, these two domains are separated by a short linker that can be cleaved after secretion. The cleavage phenomenon has been shown to occur in humans as well as in rodents (13, 14). Cleavage of ANGPTL4 appears to be tissue-dependent in humans; liver secretes cleaved ANGPTL4, whereas adipose tissue secretes the full-length form (14).

The physiological relevance of the proteolytic processing of ANGPTL4 is largely unknown. In a human study, treatment with fenofibrate, a potent peroxisome proliferator-activated receptor-α agonist, markedly increased plasma levels of cleaved ANGPTL4. On this basis, it was proposed that the cleaved form of ANGPTL4 may have specific functions (14). There is evidence that the antiangiogenic activity of ANGPTL4 is regulated by an interaction of its coiled-coil domain with heparan sulfate proteoglycans (HSPGs) (18). This may be due to an ability of the N-terminal domain to facilitate the interaction between the C-terminal domain and the cell surface. Regarding its effects on lipid metabolism, the cleavage of ANGPTL4 is not absolutely required for its LPL inhibitory effects (15). However, whether cleavage influences the metabolic effects of ANGPTL4 is unclear.

Early studies in endothelial and human embryonic kidney 293 (HEK293) cells showed that ANGPTL4 is cleaved after a conserved basic sequence (-RXKR-), and mutation of all four of these amino acids abolishes the cleavage of ANGPTL4 in vitro and in vivo (15, 16). α-1-Antitrypsin Portland variant (α1-PDX), which was generated by mutating the reactive-site loop of α1-antitrypsin to contain the minimal consensus sequence for proprotein convertase (PC) cleavage (-RIPR-), acts as a competitive inhibitor of several PCs (16) and can partially block the processing of ANGPTL4 in human umbilical vein endothelial cells (HUVECs). Thus, PCs are suspected to act as the enzymes that cleave ANGPTL4. However, ANGPTL4 can be processed in furin-deficient cell lines, and overexpression of furin has only modest effects on ANGPTL4 cleavage in HUVECs and HEK293

*This work was supported, in whole or in part, by National Institutes of Health Grants HL081861, HL55323, and HL08186104S1 from the NHLBI. This work was also supported by research grants from the Canadian Institutes of Health Research, Fonds de la Recherche en Santé du Québec, and the Ministère du Développement Économique, de l’Innovation et de l’Exportation du Québec (to R. D.).
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The abbreviations used are: LPL, lipoprotein lipase; PC, proprotein convertase; TG, triglyceride; BisTris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)propane-1,3-diol; HSPG, heparan sulfate proteoglycan; HUVEC, human umbilical vein endothelial cell; EGFP, enhanced GFP.
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cells, suggesting that furin is not the only protease responsible for ANGPTL4 cleavage (15, 16). There are nine members of the PC family (seven are basic amino acid-cleaving enzymes), but little is known about the relative effectiveness of each of them in the normal processing of ANGPTL4. Here, we examined the influence of PCs on the biological processing of ANGPTL4 in hepatocytes and its effect on metabolism.

EXPERIMENTAL PROCEDURES

Materials—We obtained polyclonal anti-Myc (IgG fraction) from Biovision (Mountain View, CA), horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG (affinity-purified) from Jackson ImmunoResearch (West Grove, PA), rabbit anti-human ANGPTL4 polyclonal antibody from Biovendor (RD181073100, Modrice, Czech Republic), the human hepatoma cell line Huh7 from American Type Culture Collection (Manassas, VA), Lipofectamine 2000 transfection reagent, and BisTris NuPAGE gels (10% resolving gel; 4% stacking gel) from Invitrogen. Purified PCs were produced by transfecting S2 insect cells with full-length human cDNAs of each PC and purified as described previously (17).

Plasmids—Human ANGPTL4 and the N terminus of ANGPTL4 (amino acids 1–160) with a Myc-His tag was cloned into pcDNA3.1-Myc-His vector. Site mutagenesis of ANGPTL4 was achieved using the QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. PCR primer sequences used for cloning are available upon request. All plasmids were verified by DNA sequencing. The profurin, furin, PC5, PC7, PC9, and PACE4 bicistronic constructs also contained an EGFP expression cassette, which is controlled by an internal ribosome entry site.

Cell Culture and Transfection—Monolayers of Huh7 cells were cultured in 5% CO₂ at 37 °C in DMEM containing 100 units/ml penicillin and 100 μg/ml streptomycin supplemented with 10% (v/v) FBS from Invitrogen. The cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For one well of a 6-well plate, 2 μg of DNA and 5 μl of Lipofectamine 2000 were used. The culture media were changed to serum-free DMEM with 10 units/ml heparin at 24 h following transfection. The medium was collected at 48 h post-transfection. The media collected with 10 units/ml heparin are referred to as conditioned media. The cells were lysed using the RIPA buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris, pH 7.5) for 10 min at 4 °C, and the cell lysate was then collected after centrifugation at 14,000 × g for 10 min. The EGFP expression in the cell lysate was measured (excitation/emission, 482/515 nm) using a SpectraMax M2 plate reader (MDS Analytical Technology). It was considered to be positive when it was 5-fold higher than the signal from the untreated cell lysate. The LPL-myc stably expressing cells were used as described previously (18). Murine primary hepatocytes were isolated as described previously (19).

Cell Surface Protein Labeling by Biotinylation—The cells were washed with cold PBS and incubated with 2 mg/ml sulfo-NHS-biotin (Pierce) in biotinylation buffer (2 mM CaCl₂, 150 mM NaCl, and 10 mM triethanolamine, pH 7.5) for 40 min at 4 °C. The reaction was quenched by incubation with 100 mM glycine in PBS for 20 min. The cells were washed with PBS and then incubated with DMEM for the indicated time. The biotin-labeled proteins in the media were captured using streptavidin-coated agarose beads for 1 h at 4 °C. The beads were then washed three times with cold PBS. The bound proteins were eluted from beads using 4× sample loading buffer for 10 min at 70 °C. The elute was separated from the beads by centrifugation at 10,000 × g for 10 min at room temperature and readied for protein analysis.

Western Blotting and Protein Analysis—SDS-PAGE and immunoblot analysis were carried out as described previously (20). The ANGPTL4 cleavage analysis was performed using the anti-Myc or anti-ANGPTL4 antibodies. Blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibody followed by detection with chemiluminescent horseradish peroxidase substrate (Pierce). A modified ELISA was used to measure any Myc tag-containing protein as described previously (19).

Reverse Transcription-PCR and Real Time PCR—Primers for PC1–9 were confirmed to be specific for the targeted genes by bioinformatics analysis. Total RNA was isolated using the RNeasy kit (Qiagen) according to manufacturer’s instructions. One microgram of total RNA was converted into cDNA using the SuperScript™ first-strand synthesis system (Invitrogen). PCR primer sequences used for cloning are available upon request. Real time PCR was carried out using the ABI 7900 real time PCR system. All of data were normalized to β-actin expression.

Lipase Activity Assay and Lipid Analysis—Triglyceride lipase activity was assayed as described previously (21). Triglyceride levels were measured using the triglyceride kit from Wako Chemicals Inc. (Richmond, VA).

Construction of Adenoviral Vectors—Recombinant adenoviruses encoding human ANGPTL4 (AdANGPTL4) and its mutant (AdR161A) were generated using the Viralpower Expression kit from Invitrogen. Both constructs expressed a C-terminal Myc-His in-frame with ANGPTL4.

Animal Studies—Female 8-week-old C57BL/6 mice obtained from Taconic Farms, Inc. (Germantown, NY) were used for the experiments. All procedures were conducted in conformity with the United States Public Health Service Policy on Human Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the State University of New York Downstate Medical Center. Adenovirus was administered at the indicated dose via the tail vein on day 0 of the study. For blood sampling, mice were fasted for 4 h and bled from the saphenous vein at days 0, 3, and 6 after virus injection. Mice were sacrificed under anesthesia, and livers were removed and snap-frozen at day 6. Liver protein extracts were prepared as reported previously (19). Post-heparin samples were collected at 5-min intervals after heparin injection (i.v., 250 units/kg).

Statistical Analyses—Values for LPL activity are expressed as the mean ± S.D. of triplicate determinations. Comparisons between the two groups were analyzed by the unpaired Student’s t test. Comparisons among multiple mouse groups were analyzed by analysis of variance followed by unpaired Student’s t tests if necessary to determine significant differences.
RESULTS

Extracellular Processing of ANGPTL4—Because human ANGPTL4 is expressed and cleaved in the liver, a human hepa
toma cell line, Huh7, was used to study the expression and processing of human ANGPTL4 protein in cell culture. Endog
genous ANGPTL4 mRNA expression in Huh7 cells could be detected by PCR, but ANGPTL4 protein could not be detected
with a commercial antibody to ANGPTL4 (data not shown). Therefore, a plasmid encoding a C-terminal Myc-His-tagged
version of human ANGPTL4 was constructed for detection and purification of ANGPTL4 protein (ANGPTL4-myc, Fig. 1A).
The same tag has been used by other groups and was reported to not interfere with ANGPTL4 function (22). ANGPTL4 pro
tein was secreted in conditioned medium as three forms under reducing conditions. The 52-kDa band corresponded to the
full-length protein (with a Myc-His tag); the 37-kDa band corresponded to the C-terminal fragment (with a Myc-His tag, Fig.
1B), and the 15-kDa band corresponded to the N-terminal fragment (Fig. 1C). Therefore, ANGPTL4 in the conditioned
medium is also cleaved in Huh7 cells, similar to what is seen in HEK293 and HUVEC cells (15, 16). Thus, the enzyme(s)
responsible for cleaving ANGPTL4 are also present in hepatocytes.

To trace the turnover of cell surface ANGPTL4 protein, cell surface proteins were labeled with biotin, and the appearance of
ANGPTL4 in the culture medium was monitored in the presence or absence of heparin over 24 h (10 units/ml). The biotin
labeled ANGPTL4 proteins were captured using streptavidin beads and detected by immunoblot analysis. As shown in Fig.
2A, the full-length ANGPTL4 appeared in the medium first, and then cleavage products increased during incubation. In the
presence of heparin, more cell surface ANGPTL4 was rapidly released into culture media in the full-length form, indicating
ANGPTL4 is tethered to the cell surface via HSPGs. Similar data were obtained using murine primary hepatocytes (Fig. 2B).
These results are consistent with previous data on endogenous ANGPTL4 secretion in HUVECs (16). Thus, the full-length
ANGPTL4 bound to the cell surface is likely the precursor of ANGPTL4 in the culture medium.

![Diagram](https://via.placeholder.com/150)

**FIGURE 1. Different forms of ANGPTL4.** A, scheme of human ANGPTL4. Human ANGPTL4 protein was detected in the conditioned medium after transduction using the anti-Myc antibody (B) or the anti-ANGPTL4 antibody (C). SP, signal peptide; F, C, and N denote the full-length form and the C- and N-terminal fragments of ANGPTL4, respectively.

| Incubation time (hrs) | 24 | 0 | 2 | 4 | 8 | 24 | 0 | 2 | 4 | 8 | 24 |
|-----------------------|----|---|---|---|---|----|---|---|---|---|----|
| Heparin               | -  | - | - | - | - | +  | + | + | + | +  | +  |
| ANGPTL4-myc           | -  | + | + | + | + | +  | + | + | + | +  | +  |
| Lane                  | 1  | 2 | 3 | 4 | 5 | 6  | 7 | 8 | 9 | 10|11 |

**FIGURE 2. Turnover of the cell surface ANGPTL4 in Huh7 cells (A) and murine primary hepatocytes (B).** The conditioned media were collected at indicated times after cell surface protein biotinylation as described under “Experimental Procedures.” The biotin-labeled proteins in the media were captured using streptavidin beads. ANGPTL4 was resolved using an SDS-polyacrylamide gel and detected by immunoblot analysis with an anti-Myc antibody. F and C denote the full-length form and the cleaved C-terminal fragment of ANGPTL4, respectively. This experiment was repeated three times, and similar results were obtained.
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Processing of ANGPTL4 Requires a PC Consensus Sequence—To identify the precise cleavage site, we expressed ANGPTL4 protein in Huh7 cells by transient transfection, using a plasmid encoding ANGPTL4-myc. Proteins containing a His epitope from the cell culture medium were purified using a nickel-nitrilotriacetic acid column, separated by SDS-PAGE, and transferred to membranes. The C terminus of ANGPTL4-myc was confirmed by Western blotting. Edman degradation-based amino acid sequencing analysis of the major cleavage products revealed that the cleavage of ANGPTL4 occurs immediately after residue 164 (data not shown), consistent with data on ANGPTL4 cleavage from HEK293 cells (23).

The angptl4 gene and its translated protein are present in at least 40 different species from mammals to fish based on information available through Ensembl. The protein homology among these species ranges from 37 to 76%. However, the basic residues surrounding the cleavage site are only conserved in mammals. The residues of ANGPTL4 adjacent to the cleavage site for representative species are shown in the Fig. 3A, upper panel. In each case, the conserved PC consensus recognition sequence, RXKR, was present before the cleavage site. To test whether the basic amino acids in the conserved PC consensus motif are important for the ANGPTL4 cleavage, the arginines at positions 161 or 164 were mutated to alanines, and the mutants ANGPTL4 constructs were expressed in Huh7 cells. Both mutants R161A and R164A dramatically reduced the cleavage of ANGPTL4 (Fig. 3A, lower panel). Thus, both arginines at site 161 and 164 are critical for ANGPTL4 cleavage.

Hereafter, we designate the R161A mutant as the cleavage-resistant mutant.

PCs Are Responsible for ANGPTL4 Cleavage—Because the sequence RXKR is a typical PC consensus motif, PC inhibitors were used to study their effects on the proteolytic processing of ANGPTL4. The proteoforms of furin and PC5/6 are potent autoinhibitors of PC activity by noncovalently attaching to PCs. Profurin and pro-PC5/6 inhibit both furin and PC5/6 (18). Both are also highly potent inhibitors of several other PCs (with $K_i$ in the low nanomolar range) (17). The ability of overexpressed profurin and pro-PC5/6 to inhibit the processing of ANGPTL4 was examined. As seen in Fig. 3B, comparing the ratio between cleaved C terminus to full-length ANGPTL4, overexpression of profurin or pro-PC5/6 resulted in a significant inhibition of ANGPTL4 cleavage. Also, profurin had a dose-dependent effect on ANGPTL4 processing (data not shown). Therefore, the members of the PC family are likely responsible for the cleavage of ANGPTL4. These data are consistent with an early observation using another specific PCs inhibitor, α-1 PDX (16).

Only Some PCs Have the Ability to Cleave ANGPTL4—To determine which member(s) of the PC family cleave ANGPTL4, ANGPTL4-myc was co-expressed with each of the PCs individually in the Huh7 cell line. The different forms of ANGPTL4 protein were detected using a polyclonal antibody against ANGPTL4. Profurin, PC5/6, furin, and PC7 were all found to significantly reduce the ratio of full-length to C-terminal ANGPTL4 (Fig. 4A), except PCSK9. Similar changes in the ratio of full-length to N-terminal fragments were also observed (data not shown). The presence of each PC was confirmed by measuring the EGFP signal in the cell lysates (Fig. 4A, lower panel). To test whether cleavage by furin, PC5/6, PACE4, or PC7 required the intact RXKR consensus sequence of ANGPTL4, the cleavage-resistant mutant of ANGPTL4 (R161A) was co-expressed with furin, PC5/6, PACE4, or PC7 in Huh7 cells. Cleavage of R161A was slightly increased after co-expression of PC5/6 and PACE4 but not furin and PC7 (Fig. 4B); therefore, the cleavage-resistant mutant is indeed resistant to cleavage by different PCs (Fig. 4, B versus A).

To directly prove that PCs can cleave ANGPTL4, purified individual PCs and ANGPTL4 were incubated at 37 °C overnight. The processing of ANGPTL4 was determined by immunoblotting. In order of potency, PC5/6, PACE4, and PC7 but not soluble furin were able to cleave ANGPTL4 (Fig. 4C).

The mRNA expression of endogenous PCs in Huh7 cells was assessed by reverse transcription-PCR. PC-specific primers covering at least two exons were designed for all members of the PC family. Furin, PC5/6, PACE4, and PC7 were found to be expressed in the Huh7 cells but not PC1/3 and PC2 (data not shown). In order of abundance, as determined by real time PCR, PACE4 was most abundant, followed by PC7, furin, PC5/6, and PC1 (data not shown). Thus, more than one of the PCs that were able to cleave ANGPTL4 was in fact normally expressed in hepatocytes.

ANGPTL4 Cleavage and LPL Activity in Vitro—To understand the functional effects of ANGPTL4 cleavage on LPL activity, two cell-based assays were used to mimic the endocrine effects of ANGPTL4. The N terminus of ANGPTL4 pro-
Protein was generated by transfecting cells with a plasmid coding amino acids 1–160 of human ANGPTL4 protein, and the full-length ANGPTL4 was generated by transfecting cells with the ANGPTL4R161A mutant or by co-transfection of wild-type ANGPTL4 with profurin (which equally inhibits all the relevant convertases). The concentration of these proteins was determined by using a modified ELISA (data not shown). The LPL-myc stably expressing cells were incubated with similar amounts (based on molarity) of ANGPTL4 proteins in the conditioned media for 24 h. As shown in Fig. 5A, the N terminus of ANGPTL4 was more effective in inhibiting LPL activity than the cleavage-resistant mutant R161A ($p < 0.05$). Also, blockage of ANGPTL4 cleavage by co-expression of profurin reduced its inhibitory effects on LPL activity ($p < 0.05$, Fig. 5B). Thus,
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A

Cleavage of ANGPTL4 and LPL in a co-culture system. Huh7 cells were cultured on the top chamber, which is separated from the lower chamber by a membrane with pore size of 0.4 μm. HEK293 cells stably expressing human LPL-myc were maintained in the lower chamber. Huh7 were transfected with either EGFP or wild-type ANGPTL4 or the cleavage-resistant ANGPTL4 (R161A). The conditioned medium after 24 h of incubation in the lower chamber was collected to be analyzed for LPL activity (Fig. 5).

B

ANGPTL4 Cleavage and LPL Activity in a Co-culture System—To mimic the effects of ANGPTL4 on LPL function in vivo, a co-culture assay was developed. Huh7 cells were cultured on the porous membrane of the top well, and the stably LPL-myc-expressing cells were maintained in the lower chamber. As shown in Fig. 6A, both wild-type and cleavage-resistant forms of ANGPTL4 inhibited LPL activity, but the cleavage-resistant form had significantly less inhibitory effect (p < 0.05), despite the fact that similar expressions of wild-type and mutant of ANGPTL4 proteins were detected in the media of Huh7 cells (Fig. 6B). Interestingly, the cleavage-resistant ANGPTL4 and wild-type ANGPTL4 also enhanced, although to a similar extent, the N terminus of ANGPTL4. As shown in Fig. 6B, ANGPTL4 can enhance its own processing. We incubated ANGPTL4-myc- or LPL-myc-expressing cells with the purified N-ANGPTL4 for 24 h. The conditioned media were collected to be analyzed for the expression of LPL, ANGPTL4, and N-ANGPTL4 by immunoblot analysis. Importantly, there was a linear increase of plasma TG levels with respect to plasma ANGPTL4 expression (Fig. 7C), and there was a linear decrease of plasma LPL activity (Fig. 7D). The N terminus of ANGPTL4 could only be detected at a high dose (>2·10^10 viral particles per mouse) after extended exposure times (Fig. 7E).

Because both ANGPTL4 and LPL can be cleaved by PCs and ANGPTL4 enhances LPL cleavage, we examined whether ANGPTL4 can enhance its own processing. We incubated ANGPTL4-myc- or LPL-myc-expressing cells with the purified N terminus of ANGPTL4. As shown in Fig. 6C, the N terminus of ANGPTL4 enhanced the cleavage of LPL but not of ANGPTL4.

Blockage of ANGPTL4 Cleavage Reduces Its Effects on Raising Plasma TG Levels in Vivo—To test the effects of ANGPTL4 cleavage on plasma TG metabolism, the adenoviral vector system was used to express ANGPTL4-myc protein or its cleavage-resistant R161A-myc mutant in mice. We normalized the titer of AdANGPTL4 (WT) and AdR161A vectors in vitro by examining the expression of ANGPTL4 protein after infecting cells using different doses of vectors (Fig. 7A). When wild-type ANGPTL4 was expressed at the dose range of 10^10 to 20·10^10 viral particles per mouse (Fig. 7B), very little ANGPTL4 protein was detected in the pre-heparin samples; however, abundant ANGPTL4 proteins were found in the post-heparin samples at day 3 after transduction. This is consistent with the observation that most of ANGPTL4 binds to the cell surface via HSPGs. Importantly, there was a linear increase of plasma TG levels with respect to plasma ANGPTL4 expression (Fig. 7C), and there was a linear decrease of plasma LPL activity (Fig. 7D). The N terminus of ANGPTL4 could only be detected at a high dose (>2·10^10 viral particles per mouse) after extended exposure times (Fig. 7E).

We tested whether cleavage of ANGPTL4 affected its ability to increase plasma TG levels. Pre-matched (both TG and total cholesterol) female wild-type mice were given either Adempty, WT, or R161A, at the dose of 10·10^10 viral particles per mouse. As expected, overexpression of ANGPTL4 resulted in a significant increase of plasma TG levels, up to 15-fold change on day 3 post-injection compared with mice injected with the control virus. However, the cleavage-resistant mutant form, R161A, raised plasma TG levels significantly less than did the wild-type ANGPTL4 (Fig. 8A). Consistent with these data, the cleavage-resistant mutant form of ANGPTL4 inhibited less LPL activity than the wild type (Fig. 8B), despite the fact that both wild-type and mutant forms of ANGPTL4 proteins were expressed at similar levels in the mouse livers (Fig. 8C, upper panel), and similar concentrations of the full-length ANGPTL4 protein...
were detected in the post-heparin samples (Fig. 8D). Very little cleavage product of the cleavage-resistant R161A mutant (about 6% of the wild-type ANGPTL4) was detected in mouse plasma using the anti-Myc antibody (data not shown). Therefore, the less ANGPTL4 is cleaved, the less it can increase plasma TG levels.

To understand why the R161A mutant is much less effective than wild-type ANGPTL4 in vivo, two sets of samples from either the AdANGPTL4 or AdR161A group with similar hepatic expression of ANGPTL4 proteins were chosen for the comparison (Fig. 9). Lower amounts of full-length (Fig. 9, upper panel), C terminus (upper panel), and N terminus (lower panel) of the ANGPTL4 protein were detected in the pre-heparin plasma from mice expressing the R161A mutant than from mice expressing the WT protein (lanes 9 and 10 versus lanes 5 and 6). Therefore, the cleavage of ANGPTL4 modulates the secretion of ANGPTL4 from the cell surface into the plasma. In other words, cleavage enhances its endocrine function. Also, heparin treatment released the cell surface ANGPTL4 in vivo (Fig. 9, lanes 5 and 6 versus lanes 7 and 8 and lanes 9 and 10 versus lanes 11 and 12).

**DISCUSSION**

The major findings of our study are that ANGPTL4 appears on the cell surface as the full-length form, which can be released by heparin treatment in culture and in vivo. ANGPTL4 protein is then proteolytically cleaved into several forms by PCs. Cleavage of ANGPTL4 by PCs was directly demonstrated in vitro and...
Interestingly, some PCs, including furin, PC5/6, PACE4, and PC7, are able to cleave ANGPTL4 but not PCSK9. Among these PCs, PACE4 and PC5/6 are the most potent. The proteolytic cleavage releases the ANGPTL4 from the cell surface to allow it to exert its endocrine functions. Also, it generates the more potent inhibitor of LPL, the N terminus of ANGPTL4. Moreover, ANGPTL4 can promote the cleavage of LPL. Finally, the cleavage of ANGPTL4 by PCs influences its inhibitory effects on LPL activity and controls its ability to raise plasma TG levels.

The organization of ANGPTL4 protein appears to show gain of function during evolution. Several truncated forms of ANGPTL4 exist in many other species. In *Oryctolagus cuniculus*, only the C-terminal form but not the N-terminal form of ANGPTL4 is present. In *Macaca mulatta*, only the N-terminal form but not the C-terminal form of ANGPTL4 exists. ANGPTL4 from *Ornithorhynchus anatinus* has the C terminus without the consensus cleavage sequence. The presence of these very different forms supports the notion that different domains of ANGPTL4 have distinct physiological functions in humans.

PCs are key players in lipid and lipoprotein metabolism. In our earlier studies, we showed that endothelial lipase and LPL are targets of a subset of PCs, including furin, PACE4, and PC5/6, and cleavage of endothelial lipase is an inactivation process (19). In this study, ANGPTL4 is observed to be activated by these PCs, increasing its LPL inhibitory effects. Although ANGPTL4, endothelial lipase, and LPL are cleaved by these same PCs, LPL is the least sensitive to these proteases. This subset of PCs modulates plasma lipid and lipoprotein metabolism through their catalytic activity that is quite different from PCSK9, which mainly targets the LDL receptor through its noncatalytic activity (24). Moreover, many known PC substrates typically require activation at the late trans-Golgi network by cleavage. However, although cleavage of ANGPTL4 is not absolutely required for inhibiting LPL activity, it is important in regulating its LPL inhibitory function. Thus, cleavage of

![FIGURE 8. Cleavage of ANGPTL4 and plasma TG levels. A, fasting plasma TG levels in mice at day 3 after transduction. B, LPL activity in post-heparin plasma. Four mice were included for each group. Error bars represent S.D. *, p < 0.05. C, ANGPTL4 protein was detected by Western blotting in mouse liver lysates (50 μg of total protein/lane, upper panel), and actin proteins in liver were detected as loading control (lower panel) at day 3 after transduction. D, human ANGPTL4 protein was detected in post-heparin plasma (0.2 μl per lane) at day 3 after transduction. Each lane represents a different animal. F and C denote the full-length form and the cleaved C-terminal fragment of ANGPTL4, respectively.](image)

![FIGURE 9. Plasma forms of ANGPTL4. Human ANGPTL4 and mutant protein were detected in matched pre- and post-heparin plasma (0.2 μl per lane) at day 3 after transduction. Each lane represents a different animal. F, N, and C denote the full-length form, the N-terminal fragment, and the C-terminal fragment of ANGPTL4, respectively.](image)
ANGPTL4 by the PCs provides another example of how proteases regulate protein function. The PCs modulate lipase activities through two distinct mechanisms, by directly inactivating a lipase through proteolytic cleavage and by indirectly inactivating lipases through activation of lipase inhibitors in plasma. LPL is active on the endothelial surface. Furin is a type I transmembrane protein. ANGPTL4, PACE4, and PCSK6 have been reported to be tethered on the cell surface via HSPG (25). All these lines of evidence indicate that these proteins can interact with each other on the endothelial surface. Thus, the interplay between PCs, ANGPTL4, and lipases may play significant roles in lipid homeostasis and endothelial biology.

ANGPTL4 and ANGPTL3 are similar in that both have the ability to enhance LPL cleavage by PCs and that this property is not influenced by the extent to which they themselves are cleaved (26) (Fig. 5). This effect also seems LPL-specific (26). ANGPTL3 cleavage is reported to be affected by the O-glycosylation state of threonine at amino acid position 226 by polypeptide N-acetylgalactosaminyltransferase 2, adjacent to the proprotein convertase-processing site (27). However, this O-glycosylation site is not conserved among ANGPTL4 proteins. Thus, this modification is expected to be absent in ANGPTL4. Of note, ANGPTL4 is N-glycosylated at amino acid position 177 (Fig. 1), but this does not affect the cleavage of ANGPTL4 by PCs (data not shown). Also, ANGPTL3 and ANGPTL4 inhibit LPL activity by different mechanisms in vitro (28), have unique tissue distributions, and are differentially regulated (29). Thus, ANGPTL3 and ANGPTL4 play distinct roles in lipid metabolism.

The most dramatic metabolic effect we noted was that the reduced inhibition of LPL after cleavage of ANGPTL4 was blocked correlated with a reduced increase in plasma TG levels. Because only the fragments containing the N terminus of ANGPTL4 are active inhibitors of LPL, we postulate that four possible mechanisms may contribute to this observation. First, cleavage of ANGPTL4 generates more potent inhibitors of LPL, as supported by our in vitro studies (Fig. 4, A and B). Limited cleavage of ANGPTL4 combined with intermolecular interaction of ANGPTL4 molecules may generate several intermediates that may have different properties with respect to their ability to inhibit LPL activity. Future experiments are needed to identify whether there are some specific intermediates responsible for these effects. Second, cleavage of ANGPTL4 releases the N terminus of ANGPTL4 from the cell surface, which then can target LPL via its endocrine function. ANGPTL4 protein produced in liver has to be released to exert its inhibitory effects on lipases that are endothelium-bound. Also, different forms of ANGPTL4 have been shown to be associated with different lipoproteins (30). It is possible that ANGPTL4 carried by these lipoproteins inhibits lipase activity, while the lipoproteins interact with LPL. Third, ANGPTL4 promotes PC-mediated LPL cleavage as does ANGPTL3 (21). Fourth, cleavage of ANGPTL4 may be involved in the turnover of ANGPTL4 protein, thereby regulating ANGPTL4 protein concentration in the circulation. Yin et al. (15) observed that the cleavage-defective mutant ANGPTL4-GSGS accumulated in the circulation to much higher levels than did the wild-type mice. These data should be carefully interpreted because this mutant was also shown to be expressed at much higher levels in the mouse liver, indicating more efficient transduction or other possibilities. We did not observe the accumulation of our R161A mutant in the circulation. This discrepancy needs to be elucidated in the future.

Compared with the wild-type ANGPTL4, our cleavage-resistant mutant (R161A) yielded similar in vitro results but opposite in vivo data from that of the cleavage-defective mutant (GSGS) used by Yin et al. (15). Because the human PC recognition sequence contains four basic residues (RRKR), it is attempting to speculate that this region is critical for HSPG binding. Thus, mutations of all four residues could cause this GSGS mutant to be less effective in binding to the cell surface. This may create an artificial phenomenon, in which this GSGS mutant protein is more effectively released into the bloodstream and can inhibit LPL activity and increases plasma TG levels to a greater extent than wild-type ANGPTL4. Also, it could explain the conflicting results from their in vivo data using this GSGS mutant (15). Further head-to-head comparisons between these two mutants are required to test this idea.

The development of ANGPTL4 inhibitors for the treatment of hypertriglyceridemia, an independent factor for atherosclerosis, is hindered by observations from neutralizing antibody studies in mice. Inhibition of ANGPTL4 caused lymphatic vessel malformation, even in the adult mouse (31). ANGPTL4 knockout mice are also susceptible to saturated fatty acid induced inflammation (8). Here, we provide an alternative approach involving inhibition of ANGPTL4 cleavage to increase LPL activity. Because cleavage of ANGPTL4 is not absolutely required for its inhibitory effects on LPL activity, a complete inhibition of ANGPTL4 cleavage would be expected to reduce its inhibitory effect on LPL activity, providing a safer route to lowering plasma TG. This approach may also be suitable for treating certain types of dyslipidemia by increasing LPL activity. Alternatively, PC inhibitors that target PACE4 and PCSK6 simultaneously and do not penetrate cell membranes could be very effective in treating hypertriglyceridemia. Nevertheless, the importance of cleavage of ANGPTL4 for other biological processes, including endothelial function, apoptosis, angiogenesis, and cancer metastasis, needs to be further studied in vivo before developing any approach to inhibiting ANGPTL4 cleavage.

Acknowledgments—We thank Dr. Daniel J. Rader, Dr. Frank Scalia, and Dr. John S. Millar for helpful discussions. We are indebted to Megan Donovan and Sandra Gagnon for excellent technical assistance.

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