Angiopoietin-like Protein 3 Inhibits Lipoprotein Lipase Activity through Enhancing Its Cleavage by Proprotein Convertases*[S]

Jun Liu[‡§]**, Huq Afroza**[¶]**, Daniel J. Rader**[‡]**, and Weijun Jin**[‡¶1]

From the ‡*Translational Medicine and Therapeutics and Cardiovascular Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, the* §*Department of Cardiology, First Affiliated Hospital of Sun Yat-Sen University, Guang Zhou City, 510080 Guang Dong Province, China, and the* ¶*Department of Cell Biology, State University of New York Downstate Medical Center, Brooklyn, New York 11203*

Lipoprotein lipase (LPL)-mediated lipolysis of triglycerides is the first and rate-limiting step in chylomicron/very low density lipoprotein clearance at the luminal surface of the capillaries. Angiopoietin-like protein 3 (ANGPTL3) is shown to inhibit LPL activity and plays important roles in modulating lipoprotein metabolism *in vivo*. However, the mechanism by which it inhibits LPL activity remains poorly understood. Using cell-based analysis of the interaction between ANGPTL3, furin, proprotein convertase subtilisin/kexin type 5 (PCSK5), paired amino acid converting enzyme-4 (PACE4), and LPL, we demonstrated that the cleavage of LPL by proprotein convertases is an inactivation process, similar to that seen for endothelial lipase cleavage. At physiological concentrations and in the presence of cells, ANGPTL3 is a potent inhibitor of LPL. This action is due to the fact that ANGPTL3 can enhance LPL cleavage by endogenous furin and PACE4 but not by PCSK5. This effect is specific to LPL but not endothelial lipase. Both N- and C-terminal domains of LPL are required for ANGPTL3-enhanced cleavage, and the N-terminal domain of ANGPTL3 is sufficient to exert its effect on LPL cleavage. Moreover, ANGPTL3 enhances LPL cleavage in the presence of either heparan sulfate proteoglycans or glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1). By enhancing LPL cleavage, ANGPTL3 dissociates LPL from the cell surface, inhibiting both the catalytic and noncatalytic functions of LPL. Taken together, our data provide a molecular connection between ANGPTL3, LPL, and proprotein convertases, which may represent a rapid signal communication among different metabolically active tissues to maintain energy homeostasis. These novel findings provide a new paradigm of specific protease-substrate interaction and further improve our knowledge of LPL biology.

ANGPTL3 is a newly discovered modulator of plasma triglyceride levels, an independent risk factor of atherosclerosis. Genome-wide association studies in humans link single nucleotide polymorphisms near *ANGPTL3* to plasma triglyceride levels (1–3). Several loss of function mutations of *ANGPTL3* contribute to variation in plasma triglyceride levels in the Dallas Heart Study population as well as in subjects in the Atherosclerosis Risk in Communities study (4). However, two small studies suggested that plasma ANGPTL3 protein concentration does not correlate with plasma triglyceride levels (5, 6). Data from ANGPTL3 knock-out mice, the mutant KK/san mice, neutralization using antibody, and overexpression studies demonstrate that ANGPTL3 raises plasma triglyceride levels by inhibiting LPL,[2] a rate-limiting enzyme in plasma triglyceride metabolism (7). ANGPTL3 is mainly made in liver, whereas most active LPL protein resides in adipose and muscle, yet the nature of its interaction with LPL is not fully understood. Based on several studies from cell-free systems (8), ANGPTL3 is proposed to directly bind and inhibit the LPL catalytic activity, and the inhibitory constant IC_{50} of ANGPTL3 for LPL activity is around 180 nM, which is at least 30-fold higher than its plasma concentration in humans and mice (9, 10). Furthermore, soluble glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1) has been shown to block the inhibitory effect of ANGPTL3 on LPL activity at least 40-fold in the presence of 100 nM soluble GPIHBP1 (11). Because a large pool of cell surface LPL is believed to be bound to GPIHBP1, it is unlikely that ANGPTL3 can effectively inhibit cell surface LPL activity *in vivo* based on this direct inhibition mechanism. Another intriguing feature of ANGPTL3 is that its triglyceride-raising effect is dominant in the fed state but not the fasting state (12), although in mice, the liver mRNA expression of ANGPTL3 does not differ in these two conditions (13). Therefore, other factors or regulatory steps may be required for ANGPTL3 to effectively inhibit LPL activity *in vivo*.

The change of LPL activity is not always consistent with the change of LPL mass or mRNA. Circumstantial evidence suggests that post-translational regulation of LPL is important to modify LPL activity during physical and pathological conditions (14, 15). We recently reported that certain convertases, including PACE4 (gene *PCSK6*), furin (gene *PCSK3*), and PCSK5 (gene *PCSK5*), are able to cleave LPL at the linker

---

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–9.

[1] To whom correspondence should be addressed: Dept. of Cell Biology, State University of New York Downstate Medical Center, BSB, 2-106, 450 Clarkson Ave., Brooklyn, NY 11203. Tel.: 718-221-5639; Fax: 718-270-3732; E-mail: weijun.jin@downstate.edu.

[2] The abbreviations used are: LPL, lipoprotein lipase; HSPG, heparan sulfate proteoglycan; PC, proprotein convertase; EGFP, enhanced GFP; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; EL, endothelial lipase; LDL, low density lipoprotein; HL, hepatic lipase.

region between its N-terminal catalytic domain and C-terminal lipid binding domain *in vitro* (16). Such a cleavage of LPL by overexpressed PCs is rather inefficient compared with that of endothelial lipase (EL), another member of the lipase family, which contains a similar PC recognition site. This raises questions whether LPL is indeed cleaved by PCs and if other factor(s) is/are also involved. The biological consequence of LPL cleavage is unknown because many intermediates of LPL could be generated through incomplete proteolysis of the LPL homodimer. In the case of EL, active EL forms a head-to-tail dimer like LPL (17) and is inactivated by PCs through proteolytic cleavage (16).

ANGPTL3 protein also contains a consensus PC recognition site like LPL and EL and is cleaved in mice and humans (7). We previously showed that cleavage of ANGPTL3 could be blocked by profurin, a proteinaceous inhibitor of furin, PACE4, and PCSK5 (18). The latter PCs (19, 20), LPL and ANGPTL3, have been shown to reside on the cell surface via HSPG, yet very little is known about the interaction among them. Here, we provide evidence that ANGPTL3 enhances the cleavage of LPL by PACE4 and furin. This process would determine the fate of cell surface LPL by promoting LPL dissociation from the cell surface, thereby eliminating its catalytic and noncatalytic functions. Thus, we provide the first evidence that ANGPTL3 functions as a specific cofactor of PCs for LPL cleavage but not EL.

EXPERIMENTAL PROCEDURES

Materials—We obtained most chemicals from Sigma unless otherwise stated. Polyclonal rabbit anti-Myc (IgG fraction) was from Biovision (Mountain View, CA); horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (affinity-purified) was from Jackson ImmunoResearch; rabbit anti-mouse ANGPTL3 polyclonal antibody was from Biovendor (Modrice, Czech Republic); mouse anti-HL and rabbit anti-scavenger receptor class B, type I, were from Santa Cruz Biotechnology. HEK293 and human Huh7 hepatoma cell lines were from American Type Culture Collection (Manassas, VA), Lipofectamine 2000 transfection reagent and BisTris NuPAGE gels (10% resolving gel; 4% stacking gel) were purchased from Invitrogen. The LPL-Myc stably expressing cells were used as described previously (16). Murine ANGPTL3 ELISA kit was purchased from Enzo Life Sciences.

Plasmids—Mouse ANGPTL3 with a Myc-His tag was cloned into pcDNA3.1-Myc-His vector. Site mutagenesis of ANGPTL3 was achieved using the QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All the primer sequences, including real time PCR, are available upon request. All plasmids were verified by DNA sequencing. EL and LPL chimera, furin, PCSK5, and PACE4 (kindly provided by N. G. Seidah, the Clinical Research Institute of Montreal, Montreal, Quebec, Canada) and shRNA constructs were used as described previously (16). The furin, PCSK5, and PACE4 bicistronic constructs also contained an internal ribosome entry site-controlled EGFP expression cassette.

Cell Culture and Transfection—Monolayers of Huh7 cells were cultured in 5% CO_2 at 37 °C in DMEM containing 100 units/ml penicillin and 100 μg/ml streptomycin supplemented with 10% (v/v) FBS from Invitrogen. In a typical transfection experiment, cells were transfected with Lipofectamine 2000 according to the manufacturer's instructions. The culture media were changed to serum-free DMEM with 10 units/ml heparin at 24 h followed 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 then the cell lysate was collected after centrifugation at $14,000 \times g$ 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.

Cell Surface Protein Labeling by Biotinylation—The cells were washed with cold PBS and incubated with 2 mg/ml sulfo-succinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (Pierce) in biotinylation buffer (2 mM $CaCl_2$, 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 lysed with the lysis buffer containing a protease inhibitor mixture (Roche Applied Science), and the cell lysate was collected after centrifugation at $14,000 \times g$ for 10 min. The biotin-labeled cell surface proteins 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 bead using four times sample loading buffer for 10 min at 70 °C. The elute was separated from the beads by centrifugation at $10,000 \times g$ for 10 min at room temperature and readied for protein analysis.

Real Time PCR—Primers for real time PCR for furin, PCSK5, PACE4, and β-actin were confirmed to be specific for the targeted genes. Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was converted into cDNA using the SuperScript^{TM} first-strand synthesis system (Invitrogen). RT-PCR was performed in an ABI Prism 7300 sequence detector system. All real time PCR samples were run in triplicate. β-Actin mRNA was used as the invariant control for all studies.

Western Blotting—SDS-PAGE and immunoblot analysis were carried out as described previously (21). The LPL cleavage analysis was performed using the anti-Myc antibody, which recognizes the full-length and the C terminus of LPL-Myc.

Triglyceride Lipase Activity Assay—Triglyceride lipase activity was assayed as described previously (21). ^{3}H-Labeled triolein in glycerol-stabilized micelle was used as the substrate. The medium from the untransfected or EGFP-transfected cells yielded similar results and was used as background.

Proprotein Convertase Activity Assay—Proprotein convertase activity was assayed as described previously (18). The assay was performed using the fluorogenic peptide Pyr-RVKR-AMC (Bachem) as the substrate. Conditioned medium from COS-7 cells infected with AdPACE4 or AdPCSK5 was used as the source of these PCs for test tube assay. AdEGFP medium was used as the control. Soluble furin without the transmembrane domain (New England Biolabs) was used as control for the fluorogenic activity.
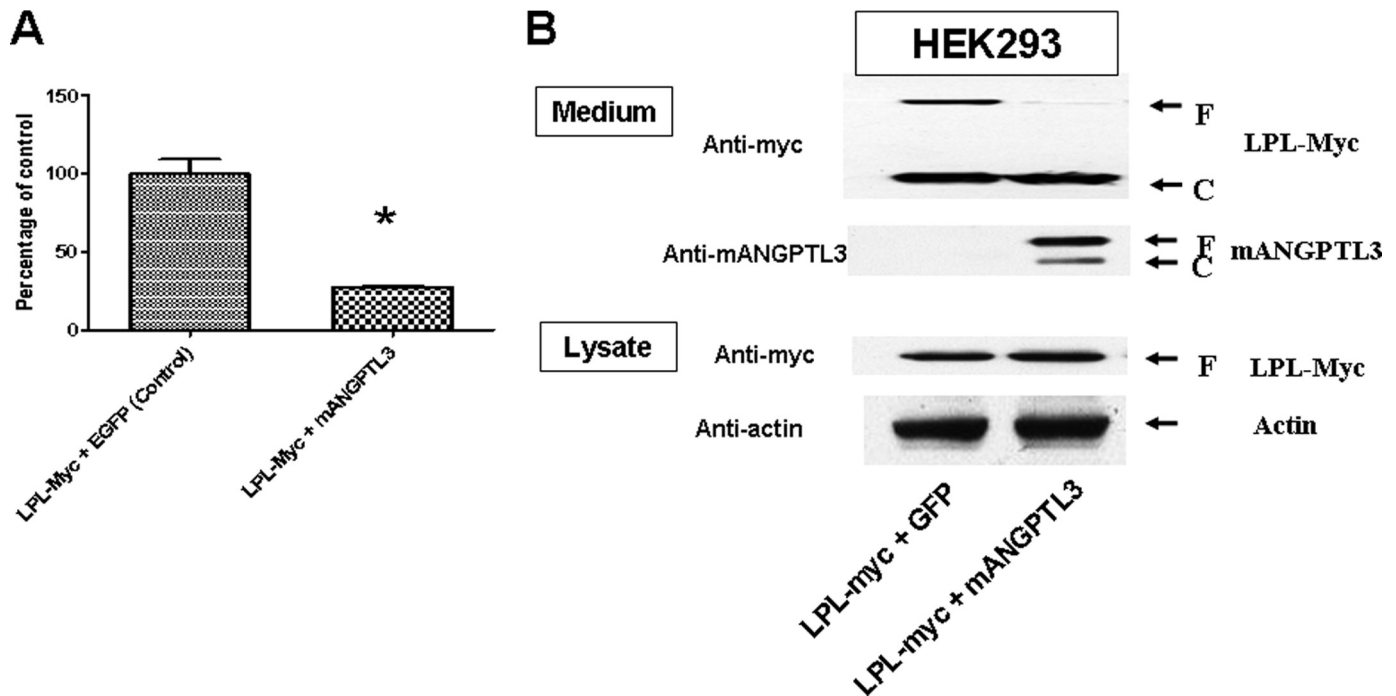
FIGURE 1. **Potent inhibition of LPL activity by mANGPTL3 in cell culture.** HEK293 cells were transfected with constructs expressing LPL-Myc and EGFP or LPL-Myc and mANGPTL3, and conditioned media (with 10 units/ml of heparin) incubated during 24–48 h after transfection and lysates were collected at 48 h followed transfection. *A,* triglyceride lipase activity in the conditioned media. Background was subtracted using the results from GFP-transfected medium, and data were expressed as the percentage change of lipase activity from the LPL-Myc and EGFP treatment. *Error bars* represent S.D. *, $p < 0.05$. *B,* immunoblot analysis of LPL-Myc, ANGPTL3 protein in the conditioned media (*upper panel*), and LPL-Myc and actin in the lysates (*lower panel*). Aliquots of the culture media or lysates were subjected to SDS-PAGE and immunoblot analysis with an anti-Myc or an indicated antibody. *F* and *C* denote the full uncleaved precursor form and the cleaved C-terminal fragment of LPL-Myc or mANGPTL3, respectively. This experiment was repeated three times with similar results.

Lipoprotein Binding Assay—The lipoprotein binding assays were performed as described previously (22). Cells in 12-well plates were incubated with 0.5 ml of serum-free medium supplemented with 0.2% BSA and ^{125}I-labeled LDL for 1 h at 4 °C. A separate set of samples was treated in the above medium with heparin for protein analysis.

Construction of Adenoviral Vectors—Recombinant adenoviruses encoding mouse ANGPTL3 and its mutant were generated using the ViraPower Expression kit from Invitrogen.

Statistical Analyses—Values for LPL activity or binding assay were expressed as the mean ± S.D. of triplicate determinations. Comparisons between two groups were analyzed by the unpaired Student's *t* test.

RESULTS

LPL Activity Is Inhibited by ANGPTL3 in Vitro—We generated a batch of murine ANGPTL (mANGPTL3-)-containing medium from HEK293 cells to examine its inhibitory effect on LPL activity. The mANGPTL3 protein concentration in the medium was ~4-fold higher than that in mouse plasma (1652 ± 126 *versus* 421 ± 26 ng/ml) as determined by ELISA. Adding mANGPTL3 (12 nM) directly into the LPL assay or preincubation of mANGPTL3 with LPL or preincubation of mANGPTL3 with a synthetic triolein substrate had no significant effect on LPL activity (supplemental Fig. 1), which is in sharp contrast to other LPL inhibitors such as ANGPTL4, apoC-III, and apoE (23). We hypothesized that cellular components might be required for ANGPTL3-mediated LPL inhibition, which may explain its inhibitory effects on plasma LPL activity *in vivo* (12).

To test this concept, constructs encoding the cDNA of mANG-PTL3 or EGFP along with another construct expressing LPL-Myc (with C-terminal a Myc-His tag) were cotransfected into HEK293 cells at a ratio of 1:1. Conditioned media containing 10 units/ml heparin were collected during the 24–48-h incubation post-transfection and assayed for LPL activity. Murine ANGPTL3 significantly inhibited LPL activity by 76% compared with the EGFP control (Fig. 1*A*). The expression of mANGPTL3 was confirmed by immunoblotting. Both the full-length and a C-terminal fragment (C-fragment) of LPL-Myc were detected using a rabbit anti-Myc antibody. Interestingly, the presence of mANGPTL3 resulted in the nearly total disappearance from the medium of the full-length LPL but did not affect the release of the C-fragment (Fig. 1*B, upper panel*). In contrast, similar amounts of the intracellular full-length form of LPL were detected whether or not mANGPTL3 was expressed (Fig. 1*B, lower panel*). These results were also confirmed by expressing human ANGPTL3 protein in the LPL-Myc-expressing cells (supplemental Fig. 2). Furthermore, incubation of the mANGPTL3 medium with cells expressing LPL-Myc yielded similar results (Fig. 5). Therefore, ANGPTL3 is a potent inhibitor of LPL only in the presence of cells, which is likely due to its effect on the turnover of the full-length LPL.

ANGPTL3 Enhances LPL Turnover by Increasing Its Cleavage—Because most of the full-length LPL is attached to the cell surface and there was no obvious accumulation of the full-length LPL intracellularly after mANGPTL3 treatment, we tested whether ANGPTL3 promoted the degradation of the full-
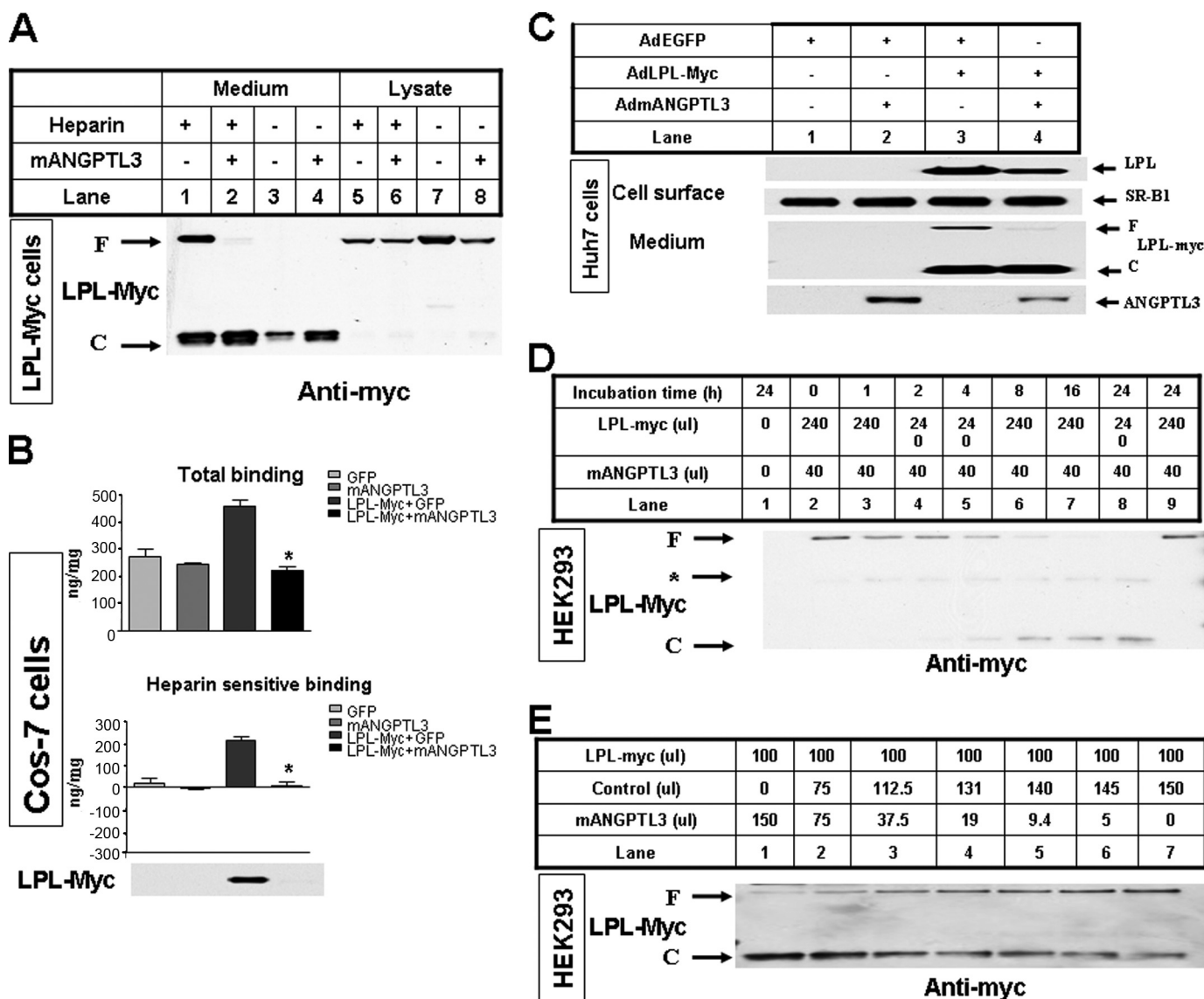
A

	Medium				Lysate			
Heparin	+	+	-	-	+	+	-	-
mANGPTL3	-	+	-	+	-	+	-	+
Lane	1	2	3	4	5	6	7	8

LPL-Myc cells

F →
LPL-Myc
C →

Anti-myc

B

Cos-7 cells

Total binding

□ GFP
▨ mANGPTL3
■ LPL-Myc+GFP
■ LPL-Myc+mANGPTL3

ng/mg

500
400
300
200
100
0

*

Heparin sensitive binding

□ GFP
▨ mANGPTL3
■ LPL-Myc+GFP
■ LPL-Myc+mANGPTL3

ng/mg

300
200
100
0
-100
-200
-300

*

LPL-Myc

C

AdEGFP	+	+	+	-
AdLPL-Myc	-	-	+	+
AdmANGPTL3	-	+	-	+
Lane	1	2	3	4

Huh7 cells

Cell surface

← LPL
← SR-B1

Medium

← F
← C } LPL-myc

← ANGPTL3

D

Incubation time (h)	24	0	1	2	4	8	16	24	24
LPL-myc (ul)	0	240	240	240	240	240	240	240	240
mANGPTL3 (ul)	0	40	40	40	40	40	40	40	40
Lane	1	2	3	4	5	6	7	8	9

HEK293

F →
* →
LPL-Myc
C →

Anti-myc

E

LPL-myc (ul)	100	100	100	100	100	100	100
Control (ul)	0	75	112.5	131	140	145	150
mANGPTL3 (ul)	150	75	37.5	19	9.4	5	0
Lane	1	2	3	4	5	6	7

HEK293

F →
LPL-Myc
C →

Anti-myc

FIGURE 2. **Turnover of the cell surface LPL enhanced by ANGPTL3.** *A,* heparin-independent effect of ANGPTL3 on LPL secretion. The LPL-Myc cells were incubated with and without heparin or ANGPTL3 medium for 6 h. Immunoblot analysis of LPL-Myc was performed in the media and lysates. Both of them are collected in 400 μl per well. *B,* lipoprotein binding assay in COS-7 cells. COS-7 cells infected with the indicated adenoviruses and lipoprotein binding assay were performed as described under "Experimental Procedures." Cell surface LPL-Myc released at 4 °C in the presence of heparin was detected by Western blotting. *Error bars* represent S.D. *, $p < 0.05$. *C,* cell surface protein biotinylation labeling was performed as described under "Experimental Procedures." LPL-Myc and scavenger receptor class B, type I (*SR-B1*), on cell surface (biotinylated) and LPL-Myc and ANGPTL3 in media (nonbiotinylated and collected before labeling) were detected by immunoblotting. *D,* LPL cleavage as a function of the incubation time with ANGPTL3. *, nonspecific band. *E,* LPL cleavage as a function of the concentration of ANGPTL3. *F* and *C* denote the full uncleaved precursor form and the cleaved C-terminal fragment of LPL-Myc or ANGPTL3, respectively. This experiment was repeated three times with similar results.

length LPL, and whether lysosomal protease inhibitors and/or proteasomal inhibitors could block this process. Following coexpression of mANGPTL3 and LPL-Myc in HEK293 cells, conditioned media and cell lysates were collected in the presence of lysosomal inhibitors (25 mM ammonium chloride) or proteasomal inhibitors (10 μM lactacystin and 1 μM MG132). None of these treatments appreciably blocked the effects of ANGPTL3 on the abundance of the full-length LPL in the media, and no cellular accumulation was observed (supplemental Fig. 3). Thus, it is unlikely that ANGPTL3 affects the turnover of the full-length LPL by promoting the intracellular degradation of the full-length LPL.

To understand the effect of ANGPTL3 on LPL secretion, cells expressing LPL-Myc were incubated with and without

heparin and/or the ANGPTL3 medium for 6 h. LPL-Myc proteins were examined in the media and cell lysates. As shown in the Fig. 2*A,* in the presence of heparin, about one-third of total LPL protein in the medium remained as the full-length form (*lane 1*); however, most of LPL protein appeared as the C-fragment after mANGPTL3 treatment (*lane 2*). Similar amounts of the full-length LPL were detected in the cell lysates (Fig. 2*A,* *lanes 5* and *6*). In the absence of heparin, much less LPL was released into the medium, mostly as the C-fragment (Fig. 2*A,* *lane 3 versus lane 1*), but more of the full-length LPL remained in cells (Fig. 2*A,* *lane 7 versus lane 5*). In addition, the presence of ANGPTL3 resulted in a much higher level of LPL C-fragment in the media (Fig. 2*A,* *lane 4 versus lane 3*), and the amount of the full-length LPL retained in cells did not depend
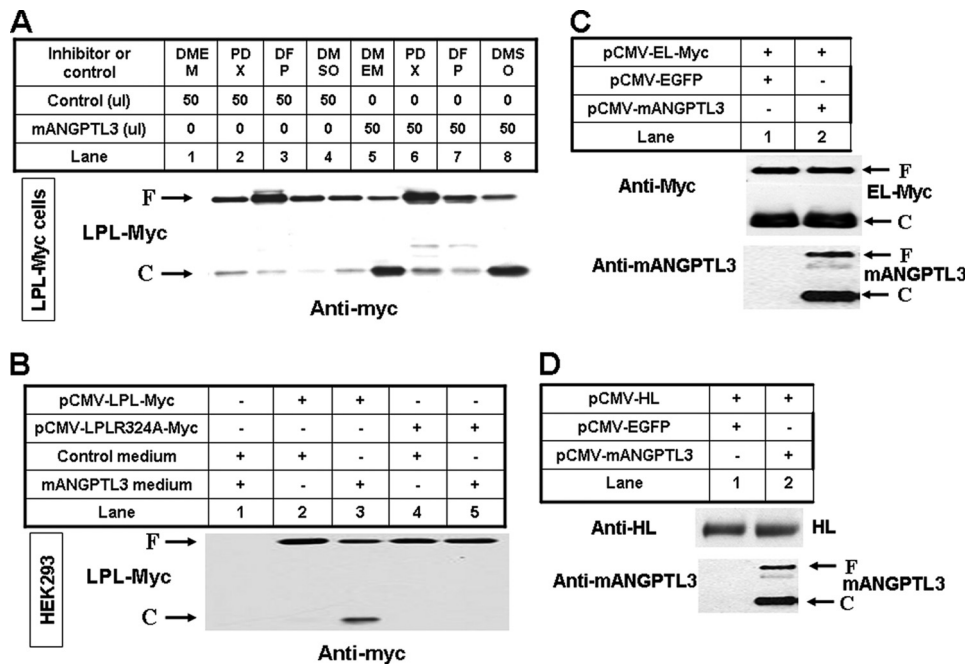
FIGURE 3. **PC-dependent effect of ANGPTL3 on LPL cleavage.** *A,* LPL-Myc protein in the medium was analyzed after incubation with di-isopropyl fluorophosphate (*DFP*) (1 μM) or α_1-PDX (50 μl) in the presence of absence of the mANGPTL3 medium. *B,* mANGPTL3 and LPL-Myc protein or its cleavage-resistant mutant, LPL-R324A-Myc, in the medium was analyzed by immunoblot analysis. *F* and *C* denote the full uncleaved precursor form and the cleaved C-terminal fragment of EL or mANGPTL3, respectively. *C,* EL-Myc or HL protein in the media was detected after transfection by immunoblot analysis using either the anti-Myc or an anti-HL antibody, respectively. This experiment was repeated three times with similar results.

μg/ml) at 4 °C for 30 min. Exogenous EGFP control or mANGPTL3 and LPL-Myc media were incubated with and without HEK293 cells. In the absence of HEK293 cells, ANGPTL3 had minimal effects on the cleavage of the full-length of LPL compared with the control (Fig. 2*D, lane 9*). Therefore, ANGPTL3 is not an enzyme that can directly cleave LPL. In the presence of HEK293 cells, ANGPTL3 exhibited time- and dose-dependent effects on the conversion of full-length LPL-Myc into its C-fragment starting at the 2-h time point (Fig. 2, *D* and *E*). Notably, most concentrations of ANGPTL3 used here were within the physiological range. The absence of any effect on LPL content during incubation further excluded the possibility of intracellular degradation of LPL by ANGPTL3 treatment. Therefore, at close to physiological concentrations, ANGPTL3 enhances the cleavage of LPL, most likely via a protease secreted from the cell. This result was not unique for HEK293 cells but was also true in several other cells lines, including COS-7, Huh7, and CHOK1 cells (Figs. 2 and 7). Therefore, ANGPTL3 enhances LPL cleavage through a general proteolytic mechanism.

ANGPTL3 has been shown to activate several signal pathways (24). To verify whether newly synthesized transcripts or proteins are required for ANGPTL3-enhanced LPL cleavage, RNA transcription or protein translation inhibitors were used to test whether they could block the effect of ANGPTL3 on LPL cleavage. None of these treatments influenced the effects of ANGPTL3 on LPL cleavage (supplemental Fig. 4).

PCs Are Required for ANGPTL3 Enhanced LPL Cleavage— LPL was previously shown to be cleaved by furin, PACE4, and PCSK5 into the C-fragment similar to the one shown here (16). To test whether these PCs were involved in the cleavage of LPL in the presence of ANGPTL3, the LPL-Myc cells were incubated with ANGPTL3 along with different PC inhibitors, including di-isopropyl fluorophosphate and α_1-antitrypsin Portland variant (16). In the presence of these inhibitors, the cleavage of LPL was significantly reduced compared with the controls (Fig. 3*A, lanes 2* and *3 versus lanes 1* and *4*). The LPL cleavage enhanced by ANGPTL3 treatment was also blocked by these treatments (Fig. 3*A, lanes 6* and *7 versus lanes 5* and *8*). These data support the notion that PCs are potentially the proteases cleaving LPL in the presence or absence of ANGPTL3. Furthermore, when another PC-specific inhibitor profurin was used to block the processing of LPL, the secretion of full-length LPL was similar either in the presence or absence of ANGPTL3 (supplemental Fig. 5). Therefore, endogenous PCs are responsible for ANGPTL3-enhanced LPL cleavage. When the cleav-

on heparin treatment (Fig. 2*A,* (*lane 8 versus lane 5*). These data suggest that, similar to heparin, ANGPTL3 promotes the dissociation of the full-length LPL from cells, but different from heparin, ANGPTL3 induces the formation of the C-fragment.

To quantify these effects, LPL-bridging experiments were performed after ANGPTL3 treatment using ^{125}I-labeled LDL. At 4 °C, the amount of radiolabeled LDL associated with the ANGPTL3-treated cells was significantly lower than that of control cells. This difference was mainly due to the reduced heparin-sensitive pool, which represents the LPL-mediated LDL binding (Fig. 2*B*). Consistent with the binding results, the amount of cell surface full-length LPL released by heparin treatment was significantly reduced in the presence of ANGPTL3 (Fig. 2*B, lower panel*). Thus, ANGPTL3 blocks the noncatalytic function of full-length LPL by enhancing its processing and hence dissociation from the cell surface.

To confirm that the amount of the cell surface LPL was altered in the presence of ANGPTL3, cell surface biotinylation was performed in Huh7 cells. The biotinylated cell surface proteins were captured using streptavidin beads and subjected to protein analysis. Using endogenous cell surface protein, scavenger receptor class B, type I, was used as a loading control, and the data in Fig. 2*C* revealed that ANGPTL3 appreciably decreased the full-length LPL-Myc on the cell surface by about 50% with no cleavage products detected. In the same setting, mANGPTL3 significantly enhanced LPL cleavage in the culture medium.

To test whether ANGPTL3 indeed promotes LPL cleavage, conditioned medium containing only the full-length LPL-Myc was obtained by incubating the LPL-Myc cells with heparin (10

A

pCMV-shRNA	shEGFP	shEGFP	shFurin	shFurin	shPACE4	shPACE4	shPCSK5	shPCSK5
Control medium	+	-	+	-	+	-	+	-
mANGPTL3 medium	-	+	-	+	-	+	-	+
Lane	1	2	3	4	5	6	7	8

PCSK mRNA expression

Lane	1	2	3	4	5	6	7	8
Furin	100	103	15	13	101	115	100	104
PACE4	100	102	98	104	23	25	112	100
PCSK5	100	100	89	100	100	100	13	14

B

IRES-GFP	+	-	-	-	+	-	-	-
pCMV-Furin-IRES-EGFP	-	+	-	-	-	+	-	-
pCMV-PACE4-IRES-EGFP	-	-	+	-	-	-	+	-
pCMV-PCSK5-IRES-EGFP	-	-	-	+	-	-	-	+
mANGPTL3 medium	+	+	+	+	-	-	-	-
Lane	1	2	3	4	5	6	7	8

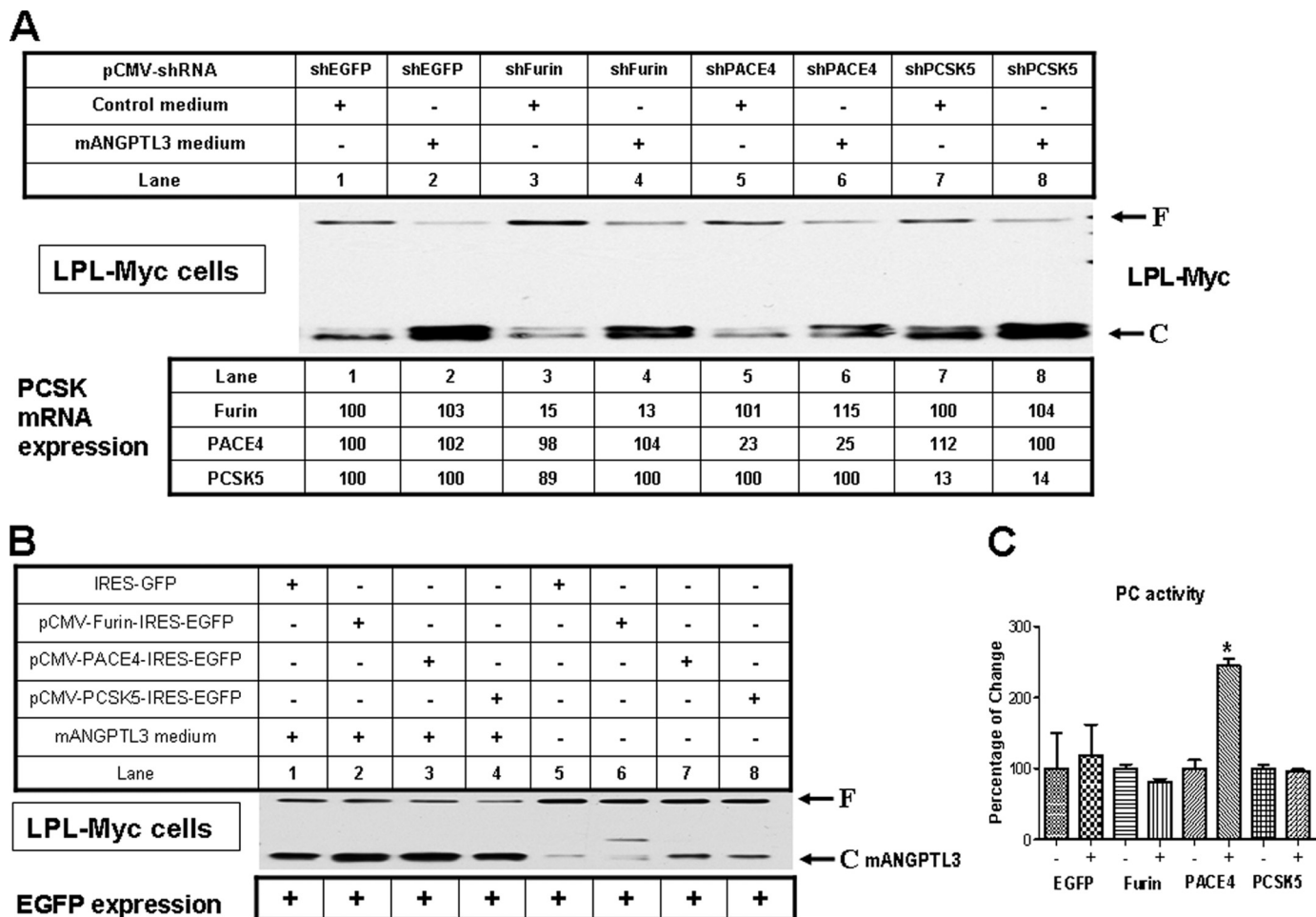| EGFP expression	+	+	+	+	+	+	+	+

C

FIGURE 4. **PC-dependent effect of ANGPTL3 on LPL cleavage.** *A,* LPL-Myc protein in the medium was analyzed after knockdown of individual PCs using the indicated shRNA constructs. Real time PCR analysis of each PC expression is shown in the *lower panel*. Data are presented as the percentage of PC expression from the EGFP-transfected cells. *B,* LPL-Myc protein in the medium was analyzed after transfection with indicated constructs. The EGFP expression was measured in the cell lysates. *C,* PC activity was measured in a test tube after mixing the indicated medium as described under "Experimental Procedures." The data are presented as the percentage change of activity in the absence of ANGPTL3. A soluble furin was used for the assay. *Error bars* represent S.D. *, $p < 0.05$. This experiment was repeated three times with similar results.

age-resistant mutant of LPL (LPL-R324A-Myc), a PC recognition site mutant, was expressed in HEK293 cells, ANGPTL3 no longer affected the conversion of the full-length LPL-R324A-Myc mutant into the C-fragment (Fig. 3*B, lanes 4* and *5*). Also, the amount of LPL released into the medium is similar, indicating the induced dissociation of LPL from the cell surface by ANGPTL3 depends on its cleavage. Therefore, ANGPTL3 does not dissociate the cell surface LPL by itself, but rather it enhances the ability of PCs to cleave LPL at the consensus PC site (25), resulting in the release into the medium of the cleaved C-fragment of cell surface LPL.

Both EL and HL are two plasma lipases closely related to LPL. We tested whether ANGPTL3 was a general PC activator for these lipases. Cotransfection of constructs expressing mANGPTL3 or EL-Myc (at a ratio of 1:1) had no effect on the secretion of either the full-length or the C-terminal EL (Fig. 3*C*); however, there was about 35% inhibition of EL activity in the conditioned medium after ANGPTL3 treatment (supplemental Fig. 6). Coexpression of ANGPTL3 with HL had no effect on either secretion (Fig. 3*D*) or activity (supplemental Fig. 7). Expression of mANGPTL3 was confirmed by Western

blotting. These results are consistent with a previous observation that ANGPTL3 had minimal effects on HL activity *in vivo* (12). Thus, ANGPTL3 specifically enhances LPL cleavage by PCs but not the cleavage of other lipases in the same family.

PACE4- and Furin-dependent Effects—At least furin, PACE4, and PCSK5 within the PC family are able to cleave LPL, and all of them are expressed in HEK293 cells (Fig. 4*A, lower panel*). We thus tested whether ANGPTL3 was a general activator of these endogenous PCs. Constructs expressing small hairpin RNA (shRNA) against furin, PACE4, and PCSK5 were transfected into the LPL-Myc cells. A vector expressing shRNA against EGFP was used as control. The LPL protein was then examined in conditioned media. Knockdown of endogenous PACE4 and to a lesser extent furin, but not PCSK5, markedly blunted the effects of ANGPTL3 on LPL cleavage (Fig. 4*A*). The knockdown efficiency of endogenous PCs was confirmed by real time PCR with 85% furin, 77% PACE4, and 86% PCSK5, and the control vector had no effects of individual PC expression at 48 h after transfection (Fig. 4*A, lower panel*). Therefore, at least in HEK293 cells, ANGPTL3 likely enhances LPL cleavage mainly through the action of PACE4 or furin.
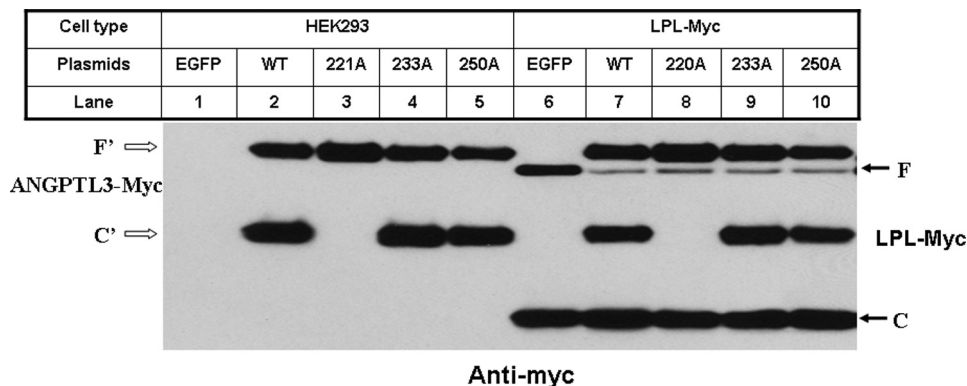
FIGURE 5. **ANGPTL3 cleavage-independent effect on LPL cleavage.** The HEK293 cells or LPL-Myc cells were transfected with a cleavage-resistant ANGPTL3 (R221A) and other mutants. Immunoblot analysis of LPL-Myc and their mutants in culture media was shown using the anti-Myc antibody. This experiment was repeated three times with similar results. *F* and *C* denote the full length and the C terminus of LPL-Myc. *F'* and *C'* denote the full length and the C terminus of ANGPTL3-Myc.
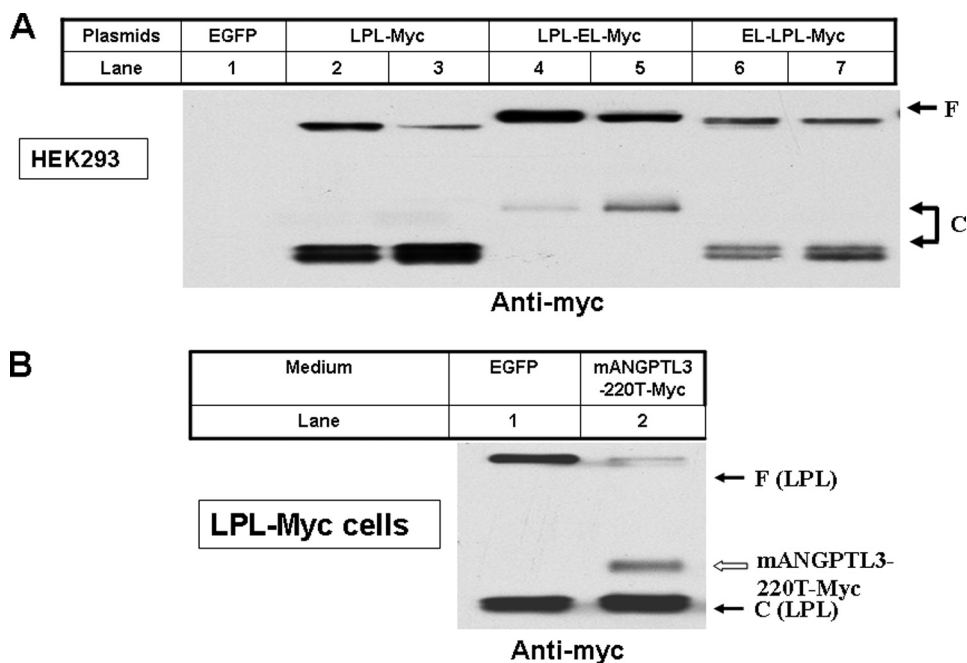

FIGURE 6. **Structural basis of ANGPTL3-enhanced LPL cleavage.** The HEK293 cells were transfected with control plasmid, and constructs expressing LPL-Myc or EL and LPL chimera (*A*) and ANGPTL3–220T-Myc (*B*) are shown. Conditioned media were collected, and the degree of LPL cleavage in the culture media was examined by immunoblotting. This experiment was repeated three times with similar results.

To further demonstrate that ANGPTL3 could enhance PC activity against LPL, we transfected the LPL-Myc cells with plasmids expressing EGFP, furin, PCSK5, or PACE4 and incubated them with the ANGPTL3 medium for 6 h. As shown in Fig. 4*B*, all of them could promote LPL cleavage in the absence of ANGPTL3. In the presence of ANGPTL3, cleavage of LPL was further enhanced by coexpression of furin and PACE4 (Fig. 4*B*). An additional band of LPL was observed when furin was overexpressed but was absent in the presence of ANGPTL3. The expression of each PC was confirmed by measuring EGFP signal in the cell lysates (Fig. 4*B*, *lower panel*). In a cell-free system, ANGPTL3 also enhanced the *in vitro* PACE4 activity by ~2-fold against a peptide substrate but not the activity of PCSK5 or a soluble form of furin (Fig. 4*C*). Therefore, ANGPTL3 can enhance the activity of PACE4.

Cleavage of ANGPTL3 Is Not Required and Has No Effect—We previously demonstrated that cleavage of ANGPTL3 can be blocked by profurin and that ANGPTL3 has a consensus PC recognition site like LPL (18). We thus sought to test whether cleavage of ANGPTL3 is necessary for its enhancement effect on LPL cleavage. Cleavage-resistant mutant of murine ANGPTL3 (R221A) and other adjacent arginine mutants were expressed in the LPL-Myc cells by transfection. As shown in Fig. 5, the cleavage-resistant mutant of ANGPTL3 had similar effects on LPL cleavage as the wild type ANGPTL3, and other mutants of ANGPTL3 that were cleaved also promoted LPL cleavage to a similar extent. Of note, the amount of ANGPTL3 proteins in the conditioned medium was similar to that of LPL-Myc. The levels of ANGPTL3 proteins were not affected by the presence or absence of LPL. Thus, ANGPTL3 can effectively enhance LPL cleavage, even when it is present at equal concentrations to LPL. The extent of ANGPTL3 cleavage does not seem to influence the cleavage efficacy of LPL by PCs.

Structural Basis for the Interaction between LPL and ANGPTL3— To understand the structural basis of ANGPTL3-enhanced LPL cleavage, we used the chimeric proteins of LPL and EL to test whether N-and/or C-terminal domains of LPL were required for ANGPTL3-enhanced cleavage. As shown in Fig. 6*A*, ANGPTL3 promoted the conversion of both the full-length LPL-EL-Myc and EL-LPL-Myc into cleavage fragments, suggesting both domains are required for the maximal effect of ANGPTL3 on LPL cleavage. We tried to detect the interaction between LPL, PACE4, and ANGPTL3 by coimmunoprecipitation, but we did not reveal any stable interaction between them (data not shown). However, we could not rule out transient and unstable interactions among them, as has been documented for ANGPTL3 and LPL in several cell-free systems (26, 27).

To test which domain of ANGPTL3 could promote LPL cleavage, a mutant of ANGPTL3 containing the N terminus of ANGPTL3 with a Myc-His tag (amino acids 1–220, ANGPTL3–220T-Myc) was expressed in the LPL-Myc cells. It effectively enhanced the conversion of the full-length LPL into the C-fragment (Fig. 6*B*). Therefore, it is likely that the N ter-
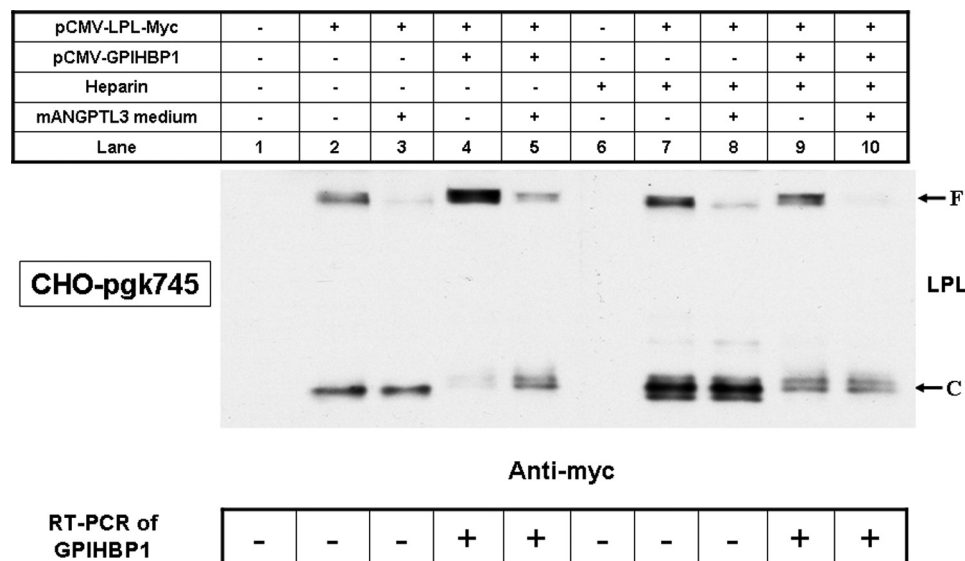
pCMV-LPL-Myc	-	+	+	+	+	-	+	+	+	+
pCMV-GPIHBP1	-	-	-	+	+	-	-	-	+	+
Heparin	-	-	-	-	-	+	+	+	+	+
mANGPTL3 medium	-	-	+	-	+	-	-	+	-	+
Lane	1	2	3	4	5	6	7	8	9	10

CHO-pgk745

← F

LPL

← C

Anti-myc

RT-PCR of GPIHBP1	-	-	-	+	+	-	-	-	+	+

FIGURE 7. **ANGPTL3-enhanced LPL cleavage in the presence of GPIHBP1.** HSPG-defective CHO-pgk45 cells were transfected with either control or GPIHBP1 in the presence or absence of heparin or the mANGPTL3 medium. The degree of LPL cleavage in culture media was examined by immunoblotting. This experiment was repeated three times with similar results.

A working model

Medium or plasma

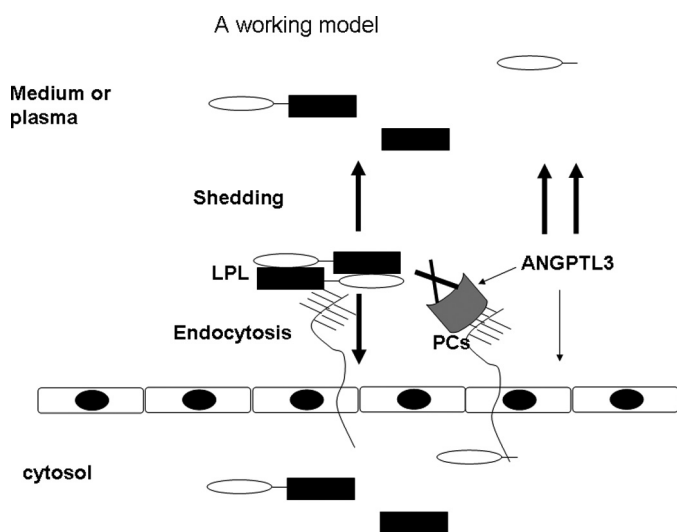Shedding

LPL

Endocytosis

ANGPTL3

PCs

cytosol

FIGURE 8. **Our working model.** The cell surface LPL can be uptaken by the cell through endocytosis or dissociated from cell surface. ANGPTL3 promotes PC activity against LPL and enhances the dissociation of LPL from the cell surface.

minus of ANGPTL3 mediates its effect on LPL cleavage, which is consistent with its inhibitory effects on LPL activity and plasma triglyceride raising effects by this form of ANGPTL3 as reported by others (28).

ANGPTL3 Also Enhances the Release of LPL from GPIHBP1—GPIHBP1 has been revealed to provide an important platform, independent of HSPG, for the LPL-mediated processing of chylomicrons in capillaries (29). In a cell-free system, a soluble fragment of GPIHBP1 blocks the inhibitory effect of ANGPTL3 on LPL activity (11). We examined whether ANGPTL3 could also promote LPL conversion in the presence of GPIHBP1 in a mutant CHO-K1 cell line lacking any cell surface HSPG. As shown in Fig. 7, ANGPTL3 enhanced the cleavage of LPL even in the presence of GPIHBP1. The expression of GPIHBPI was confirmed by heparin treatment as well as RT-

PCR (Fig. 7, *lower panel*). Similar results were obtained in the wild type CHO-K1 cells (supplemental Fig. 8). Therefore, in addition to HSPG, ANGPTL3 also enhances LPL cleavage even in the presence of GPIHBP1.

DISCUSSION

The major findings of our study are summarized below. 1) The cleavage of LPL by PCs is an inactivation process, similar to that seen for EL cleavage (20). 2) ANGPTL3 is a potent inhibitor of LPL in the presence of cells, likely through LPL cleavage at physiological concentrations. 3) ANGPTL3 can promote LPL cleavage by PACE4 and furin but not PCSK5. 3) The effect of ANGPTL3 is specific to LPL but not EL or HL. 4) The N-terminal domain of ANGPTL3 is required for its effect on LPL cleavage. 5) ANGPTL3 blocks the catalytic and noncatalytic functions of LPL. 6) ANGPTL3 determines the fate of the cell surface LPL through a PC-mediated cleavage. 7) ANGPTL3 enhances LPL cleavage in the presence either HSPG and/or GPIHBP1. Our current working model is that ANGPTL3 enhances PC activity against LPL, which controls the shedding of cell surface LPL by dissociation instead of by endocytosis (Fig. 8). This provides first mechanistic insight into how the shedding of cell surface LPL is regulated at the molecular level.

Most of LPL protein in human plasma before heparin treatment is cleaved, and using an anti-5D2 antibody, the uncleaved and cleaved fragments exhibit molecular masses of 52 and 18 kDa, respectively.[3] This cleavage pattern matches well the LPL-Myc cleavage shown throughout this study. As such, it is very likely LPL is cleaved by PCs in humans. The physiological importance of LPL cleavage remains to be elucidated. Most of the plasma LPL protein before heparin treatment is inactive, which is consistent with our finding that the cleavage of LPL by PCs is an inactivation process. According to our working model, plasma triglyceride levels may not only reflect the expression of LPL but also the interaction between ANGPTL3, PCs, and LPL. This may explain the absence of correlation between ANGPTL3 and plasma triglyceride concentrations in healthy individuals (5, 6).

Our work provides an alternative mechanism by which ANGPTL3 may effectively inhibit LPL activity *in vivo*. Compared with the direct inhibition mechanism that occurs at high concentrations, much lower concentrations of ANGPTL3 are required for the herein proposed indirect mechanism. Also, GPIHBP1 has little effect on ANGPTL3-enhanced LPL cleavage. Considering that plasma ANGPTL3 level is far below the IC_{50} value of ANGPTL3 and that active LPL binds to GPIHBP1,

---

[3] J. Liu, H. Afroza, D. J. Rader, and W. Jin, unpublished data.

the indirect effect of ANGPTL3 is more likely to be the major physiological mechanism by which it inhibits LPL activity in animals and humans. Further experiments are needed to test this hypothesis in physiological settings. Although ANGPTL3 can inhibit both EL and LPL activities, it does so by very different mechanisms. ANGPTL3 directly inhibits EL activity, which is enhanced by PC-directed ANGPTL3 cleavage. In contrast, ANGPTL3 indirectly inhibits LPL activity by enhancing LPL cleavage, which is insensitive to the status of ANGPTL3 processing. The structural basis within lipases for these seemingly fundamentally different mechanisms remains to be determined. Finally, it should be mentioned that PACE4, like PCSK5, is mostly activated at the cell surface, where it is anchored through its C-terminal Cys-rich domain via HSPGs (30). Thus, the need for a cell surface for the cleavage of LPL into the C-fragment by PACE4 may also reflect the zymogen activation site of this convertase, providing a nucleation point for the interaction between the enzyme and its substrates like LPL.

By analogy with the paradigm of 7B2 and PC2 (31), the interaction of ANGPTL3 and furin or PACE4 provides another example that PCs can be spatially and temporally regulated by the dynamics of interactions between converting enzymes and their binding proteins. 7B2 enhances the activation of PC2 and preserves its proteolytic activity during secretion by serving as a chaperone (31). In the case of ANGPTL3 and furin or PACE4, only LPL but not EL cleavage is enhanced by ANGPTL3, providing a new model of how protease targets a specific cell surface substrate. It will be interesting to test whether ANGPTL3 can also enhance PC activity against other potential substrates. This layer of specificity is critical to resolving certain issues in the study of proteases. Specifically, furin, PACE4, and PCSK5 recognize similar dibasic sequences and are widely expressed, leading to the suggestion or assumption that they have redundant functions. Yet knock-out of any one of these individual PCs is lethal, which argues against the assumption of redundancy (32). Here, our data suggest that PACE4 and furin act differently against LPL from PCSK5 in the presence ANGPTL3. This may serve as a new template for understanding how PCs work in a target-specific manner. As such, PC activity may also be fine-tuned toward a specific target, which cannot be compensated by other PCs. This may explain the dramatic phenotypes seen in the individual PC knock-out animals. Finally, it should be mentioned that PACE4 is highly expressed in skeletal muscle (33), but not PCSK5, a situation resembling that of LPL, likely rationalizing its preferred cleavage by PACE4. One of the immediate implications of our findings may allow the development of a specific assay for PACE4 that would differentiate its activity from those of other PCs, a possibility we are currently exploring.

REFERENCES

1. Nakayama, K., Bayasgalan, T., Yamanaka, K., Kumada, M., Gotoh, T., Utsumi, N., Yanagisawa, Y., Okayama, M., Kajii, E., Ishibashi, S., and Iwamoto, S. (2009) *J. Med. Genet.* **46,** 370–374
2. Hegele, R. A., Ban, M. R., Hsueh, N., Kennedy, B. A., Cao, H., Zou, G. Y., Anand, S., Yusuf, S., Huff, M. W., and Wang, J. (2009) *Hum. Mol. Genet.* **18,** 4189–4194
3. Wang, J., Ban, M. R., Zou, G. Y., Cao, H., Lin, T., Kennedy, B. A., Anand, S., Yusuf, S., Huff, M. W., Pollex, R. L., and Hegele, R. A. (2008) *Hum. Mol. Genet.* **17,** 2894–2899
4. Romeo, S., Yin, W., Kozlitina, J., Pennacchio, L. A., Boerwinkle, E., Hobbs, H. H., and Cohen, J. C. (2009) *J. Clin. Invest.* **119,** 70–79
5. Shimamura, M., Matsuda, M., Yasumo, H., Okazaki, M., Fujimoto, K., Kono, K., Shimizugawa, T., Ando, Y., Koishi, R., Kohama, T., Sakai, N., Kotani, K., Komuro, R., Ishida, T., Hirata, K., Yamashita, S., Furukawa, H., and Shimomura, I. (2007) *Arterioscler. Thromb. Vasc. Biol.* **27,** 366–372
6. Yilmaz, Y., Ulukaya, E., Atug, O., and Dolar, E. (2009) *Eur. J. Gastroenterol. Hepatol.* **21,** 1247–1251
7. Miida, T., and Hirayama, S. (2010) *Curr. Opin. Lipidol.* **21,** 70–75
8. Shan, L., Yu, X. C., Liu, Z., Hu, Y., Sturgis, L. T., Miranda, M. L., and Liu, Q. (2009) *J. Biol. Chem.* **284,** 1419–1424
9. Stejskal, D., Karpísek, M., Humenanská, V., Solichová, P., and Stejskal, P. (2007) *Gen. Physiol. Biophys.* **26,** 230–233
10. Robciuc, M. R., Tahvanainen, E., Jauhiainen, M., and Ehnholm, C. (2010) *J. Lipid Res.* **51,** 824–831
11. Sonnenburg, W. K., Yu, D., Lee, E. C., Xiong, W., Gololobov, G., Key, B., Gay, J., Wilganowski, N., Hu, Y., Zhao, S., Schneider, M., Ding, Z. M., Zambrowicz, B. P., Landes, G., Powell, D. R., and Desai, U. (2009) *J. Lipid Res.* **50,** 2421–2429
12. Köster, A., Chao, Y. B., Mosior, M., Ford, A., Gonzalez-DeWhitt, P. A., Hale, J. E., Li, D., Qiu, Y., Fraser, C. C., Yang, D. D., Heuer, J. G., Jaskunas, S. R., and Eacho, P. (2005) *Endocrinology* **146,** 4943–4950
13. Ge, H., Cha, J. Y., Gopal, H., Harp, C., Yu, X., Repa, J. J., and Li, C. (2005) *J. Lipid Res.* **46,** 1484–1490
14. Wicher, I., Sattler, W., Ibovnik, A., Kostner, G. M., Zechner, R., and Malle, E. (1996) *J. Immunol. Methods* **192,** 1–11
15. Semenkovich, C. F., Wims, M., Noe, L., Etienne, J., and Chan, L. (1989) *J. Biol. Chem.* **264,** 9030–9038
16. Jin, W., Fuki, I. V., Seidah, N. G., Benjannet, S., Glick, J. M., and Rader, D. J. (2005) *J. Biol. Chem.* **280,** 36551–36559
17. Griffon, N., Jin, W., Petty, T. J., Millar, J., Badellino, K. O., Saven, J. G., Marchadier, D. H., Kempner, E. S., Billheimer, J., Glick, J. M., and Rader, D. J. (2009) *J. Biol. Chem.* **284,** 23322–23330
18. Jin, W., Wang, X., Millar, J. S., Quertermous, T., Rothblat, G. H., Glick, J. M., and Rader, D. J. (2007) *Cell Metab.* **6,** 129–136
19. Nour, N., Mayer, G., Mort, J. S., Salvas, A., Mbikay, M., Morrison, C. J., Overall, C. M., and Seidah, N. G. (2005) *Mol. Biol. Cell* **16,** 5215–5226
20. Mayer, G., Hamelin, J., Asselin, M. C., Pasquato, A., Marcinkiewicz, E., Tang, M., Tabibzadeh, S., and Seidah, N. G. (2008) *J. Biol. Chem.* **283,** 2373–2384
21. Jin, W., Sun, G. S., Marchadier, D., Octtaviani, E., Glick, J. M., and Rader, D. J. (2003) *Circ. Res.* **92,** 644–650
22. Fuki, I. V., Blanchard, N., Jin, W., Marchadier, D. H., Millar, J. S., Glick, J. M., and Rader, D. J. (2003) *J. Biol. Chem.* **278,** 34331–34338
23. Liu, H., Talmud, P. J., Lins, L., Brasseur, R., Olivecrona, G., Peelman, F., Vandekerckhove, J., Rosseneu, M., and Labeur, C. (2000) *Biochemistry* **39,** 9201–9212
24. Li, Y., Sun, L., Xu, H., Fang, Z., Yao, W., Guo, W., Rao, J., and Zha, X. (2008) *Acta Biochim. Biophys. Sin.* **40,** 459–465
25. Seidah, N. G., and Chrétien, M. (1999) *Brain Res.* **848,** 45–62
26. Yau, M. H., Wang, Y., Lam, K. S., Zhang, J., Wu, D., and Xu, A. (2009) *J. Biol. Chem.* **284,** 11942–11952
27. Lee, E. C., Desai, U., Gololobov, G., Hong, S., Feng, X., Yu, X. C., Gay, J., Wilganowski, N., Gao, C., Du, L. L., Chen, J., Hu, Y., Zhao, S., Kirkpatrick, L., Schneider, M., Zambrowicz, B. P., Landes, G., Powell, D. R., and Sonnenburg, W. K. (2009) *J. Biol. Chem.* **284,** 13735–13745
28. Ono, M., Shimizugawa, T., Shimamura, M., Yoshida, K., Noji-Sakikawa, C., Ando, Y., Koishi, R., and Furukawa, H. (2003) *J. Biol. Chem.* **278,** 41804–41809
29. Beigneux, A. P., Davies, B. S., Gin, P., Weinstein, M. M., Farber, E., Qiao,

X., Peale, F., Bunting, S., Walzem, R. L., Wong, J. S., Blaner, W. S., Ding, Z. M., Melford, K., Wongsiriroj, N., Shu, X., de Sauvage, F., Ryan, R. O., Fong, L. G., Bensadoun, A., and Young, S. G. (2007) *Cell Metab.* **5,** 279–291

30. Seidah, N. G., Mayer, G., Zaid, A., Rousselet, E., Nassoury, N., Poirier, S., Essalmani, R., and Prat, A. (2008) *Int. J. Biochem. Cell Biol.* **40,** 1111–1125

31. Seidah, N. G., Benjannet, S., Hamelin, J., Mamarbachi, A. M., Basak, A., Marcinkiewicz, J., Mbikay, M., Chrétien, M., and Marcinkiewicz, M. (1999) *Ann. N.Y. Acad. Sci.* **885,** 57–74

32. Scamuffa, N., Calvo, F., Chrétien, M., Seidah, N. G., and Khatib, A. M. (2006) *FASEB J.* **20,** 1954–1963

33. Seidah, N. G., Chrétien, M., and Day, R. (1994) *Biochimie* **76,** 197–209