Endothelial lipase is inactivated upon cleavage by the members of the proprotein convertase family

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Abstract Mature endothelial lipase (EL) is a 68 kDa glycoprotein. In HepG2 cells infected with adenovirus encoding human EL, the mature EL was detectable in the cell lysates and heparin-releasable fractions. In contrast, cell media of these cells contained two EL fragments: an N-terminal 40 kDa fragment and a C-terminal 28 kDa fragment. N-terminal protein sequencing of the His-tagged 28 kDa fragment revealed that EL is cleaved on the C terminus of the sequence RNKR^{330}↓, the consensus cleavage sequence for mammalian proprotein convertases (pPCs). Replacement of Arg-330 with Ser by site-directed mutagenesis totally abolished EL processing. EL processing could efficiently be attenuated by specific inhibitors of pPCs, α1-antitrypsin Portland (α1-PDX) and α1-antitrypsin variant AVRR. Coexpression of the pPCs furin, PC6A, and PACE4 with EL resulted in a complete conversion of the full-length EL to a truncated 40 kDa fragment. Exogenously added EL was also processed by cells, and the processing could be attenuated by α1-PDX. The expressed N-terminal 40 kDa fragment of EL (EL-40) harboring the catalytic site failed to hydrolyze [^{14}C]NEFA from [^{14}C]dipalmitoyl-PC-labeled HDL. EL-40 was incapable of bridging ^{125}I-labeled HDL to the cells and had no impact on plasma lipid concentration when overexpressed in mice. Thus, our results demonstrate that pPCs are involved in the inactivation process of EL.—Gauster, M., A. Hrzenjak, K. Schick, and S. Frank. **Endothelial lipase is inactivated upon cleavage by the members of the proprotein convertase family. *J. Lipid Res.* 2005.** 46: **977–987.**

Supplementary key words furin • PC6 • site-directed mutagenesis • high density lipoprotein • bridging • phospholipase activity

Endothelial lipase (EL) is a member of the triglyceride (TG) lipase gene family. EL is synthesized by various tissues and cell types, including endothelial cells. Like LPL (1) and hepatic lipase (2), EL exhibits high affinity for the cell surface heparan sulfate proteoglycan (HSPG) (3, 4). Interaction occurs via positively charged residues that are highly conserved among all three members of the family (5, 6). EL is synthesized as a 55 kDa protein that is secreted into medium as a 68 kDa protein upon maturation. In contrast to lysates, heparin-supplemented cell media of EL-expressing cells contain a 68 kDa band and an additional 40 kDa EL band, most likely generated by the cleavage of the 68 kDa form (6–8). EL exhibits pronounced phospholipase and low TG lipase activity (5, 6, 9). By virtue of its phospholipase activity, EL efficiently hydrolyzes phosphatidylcholine (PC) on HDL (9). This process liberates NEFAs, which are taken up by EL-expressing cells and are substrates for the biosynthesis of endogenously synthesized lipids (10). The EL-mediated depletion of HDL-PC alters the lipid composition and physical properties of HDL, resulting in a diminished ability of HDL to mediate scavenger receptor class B type I-dependent cholesterol efflux (11, 12). When overexpressed in cultured cells, EL facilitates HDL particle binding and uptake (3, 4) as well as the selective uptake of HDL-cholesteryl ester (4). Experiments in engineered mice with a disrupted native EL locus (13, 14) as well as in mice overexpressing human EL (13, 15) revealed an inverse relationship between HDL-cholesterol level and EL expression. Most recently, EL was found to facilitate the progression of atherosclerosis in apolipoprotein E-deficient mice (16).

Numerous biologically active proteins and peptides are synthesized as larger inactive precursors, which are proteolytically cleaved by cellular calcium-dependent serine endoproteases called proprotein convertases (pPCs). Seven members of the mammalian pPCs activate a wide variety of serum proteins, prohormones, receptors, and zymogens (reviewed in 17 and 18). Consistent with their shared structural similarity, the mammalian pPCs have conserved enzymatic properties, thus cleaving precursors at sites containing the consensus sequence K/R-Xn-K/R↓, where n

Abbreviations: EL, endothelial lipase; FCS, fetal calf serum; HBD, heparin binding domain; HSPG, heparan sulfate proteoglycan; MOI, multiplicity of infection; PC, phosphatidylcholine; α1-PDX, α1-antitrypsin Portland; PL, phospholipid; pPC, proprotein convertase; TG, triglyceride.

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Manuscript received 17 December 2004 and in revised form 11 January 2005.
Published, JLR Papers in Press, February 1, 2005.
DOI 10.1194/jlr.M400500-JLR200

indicates the number of spacer amino acid residues. Considering their tissue distribution, mammalian pPCs can be divided into two groups. The first includes convertases PC1 and PC2, expressed mainly in neuroendocrine tissue and sorted into secretory granules of the regulated secretory pathway, as well as PC4, expressed exclusively in reproductive germ cells (17, 18). The second group of pPCs, which includes furin, PACE4, PC6, and PC7, shows a wide distribution in tissues and cell lines (18–20). PACE4 and PC6A (a splice variant of PC6) are secretory enzymes (21–23), and furin, PC7, and PC6B (a splice variant of PC6) cycle between the *trans*-Golgi and the cell surface through the endocytic compartment (24). These pPCs are believed to be involved in the processing of precursors in the constitutive secretory pathway (25–27).

In the present study, we provide evidence that the full-length 68 kDa form of EL is cleaved by members of the mammalian pPCs, yielding a N-terminal 40 kDa fragment and a C-terminal 28 kDa fragment. The expressed N-terminal 40 kDa fragment, named EL-40, was incapable of hydrolyzing HDL-PC and bridging HDL to the cells. Importantly, the concentration of plasma lipids remained unaltered in mice overexpressing EL-40.

EXPERIMENTAL PROCEDURES

Plasmids

The full-length human EL cDNA was amplified using the sense primer EL 1 (5′-CGAGGGCAGATCTCGTTCTGG-3′), corresponding to nucleotides 34–54 of EL cDNA, the antisense primer EL-Not-His (5′-ACTCGAGCGGCCGCCGGGAAGCTCCACAG-3′), and the plasmid pCR2.1-EL (4) as a template. The antisense primer EL-Not-His constitutes part of the multiple cloning site of the plasmid pcDNA4/myc-His (Invitrogen, Carlsbad, CA) with the *Not*I and *Xho*I cleavage sites as well as nucleotides 1753–1739 of EL cDNA. After sequencing, the *Bgl*II/*Not*I-cleaved amplification product was subcloned into the *Bam*HI/*Not*I-cleaved pcDNA4/myc-His vector, resulting in the pcDNA4-EL-myc/His expression plasmid encoding EL with myc and His tags at the C terminus (EL-myc/His). To obtain an expression plasmid encoding a mutant EL-myc/His in which the codon 330 AG**G** (Arg) is replaced by AG**C** (Ser), part of the EL cDNA encompassing nucleotides 817–1272 was amplified using the sense primer EL 4 (5′-ATCACAGGTTTGGATCCTGCCG-3′), corresponding to nucleotides 817–838 of EL cDNA, the antisense mutant primer EL-mut (5′-TGCCCGGGTTTTTAGGTACATTTTGCTGTT**G**CTCTTGTTCC-3′), comprising nucleotides 1272–1241 of EL cDNA with a base substitution shown in boldface, and pcDNA4-EL-myc/His as a template. After subcloning into pCR2.1 and sequence verification, the 455 bp amplification fragment was excised with *Bam*HI and *Sma*I and ligated along with the 490 bp *Not*I-*Sma*I and 5.8 kb *Not*I-*Bam*HI fragments, respectively, obtained by the cleavage of pcDNA4-EL-myc/His. The resulting expression plasmid was pcDNA4-EL-MUT-myc/His. To obtain the expression plasmid pcDNA4-EL-40 encoding the N-terminal portion of EL with 330 amino acid residues (EL-40), part of the EL cDNA encompassing nucleotides 817–1242 was amplified using the sense primer EL 4 and the antisense primer EL-40 (5′-CGAGCGGCCGCCTCACCTCTTGTTCCTC-3′) and pcDNA4-EL-myc/His as a template. Primer EL-40 contained a stop codon after the sequence corresponding to nucleotides 1231–1242 of EL cDNA followed by a *Not*I site to allow subcloning. The resulting 425 bp fragment was cleaved with *Bam*HI and *Not*I and was finally ligated with the 5.8 kb *Not*I-*Bam*HI fragment from pcDNA4-EL-myc/His. PCR was performed using DyNAzyme and Phusion high-fidelity DNA polymerases (Finnzymes, Espoo, Finland) according to the manufacturer's protocols. Plasmids pcDNA3-α1-PDX [encoding rat α1-antitrypsin Portland (α1-PDX) (28)], pcDNA3-AVRR (encoding the α1-antitrypsin variant containing in the reactive site loop the AVRR pPC consensus sequence), pRcCMV-PC6A (encoding a splice variant of PC6 devoid of a transmembrane domain), pALTERMAX-PACE4A-I (encoding human PACE4A), and pRcCMV-furin (encoding truncated soluble furin lacking a transmembrane domain) have been described (21, 29). These plasmids were kindly provided by Dr. Akihiko Tsuji (Faculty of Engineering, University of Tokushima, Japan).

Construction and purification of the recombinant adenovirus

The cDNA sequence encoding EL-40 was excised from the plasmid pcDNA4-EL-40 and subcloned into the *Cla*I/*Kpn*I-cleaved plasmid pAvSvCv (30). The resulting shuttle plasmid was cotransfected with pJM17 (30) into Hek-293 cells. Recombinant adenovirus EL-40-Ad was prepared exactly as described (4).

Preparation of an antiserum to the C-terminal domain of EL

A polypeptide containing amino acids 384–446 of human EL was expressed, purified, and applied for immunization of a rabbit, exactly as described previously (4, 31). An antiserum dilution of 1:500 was found to be optimal for immunoblotting.

Cell culture, recombinant adenovirus infection, and transfection

HepG2 and Hek-293 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS) under standard cell culture conditions. For infection, HepG2 cells were plated onto 24-well trays (2×10^5 cells/well). After 48 h, cells were washed with PBS and infected with a multiplicity of infection (MOI) of 60 of EL-Ad (adenovirus encoding human EL) (4) in DMEM for 90 min. Thereafter, infection medium was removed and cells were incubated in the serum-free medium Panserin 401 or DMEM (Pan Biotech, Aidenbach, Germany) for 24 h, either in the absence or presence of a specific inhibitor of furin, α1-PDX (Affinity Bioreagents, Golden, CO). Hek-293 cells were plated onto 60 mm collagen-coated dishes (1.3×10^6 cells/dish) and transfected using the Profection mammalian transfection system (Promega, Madison, WI) with 5 μg of pcDNA4-EL-myc/His, pcDNA4-EL-MUT-myc/His, pcDNA4-EL40, and pcDNA4 (mock). Stable cell lines were selected with 33 μg/ml zeozin (Invitrogen) and screened for EL expression by Western blot analysis. To assess the effect of the specific pPC inhibitors as well as of pPCs on EL processing, Hek-293 cells stably expressing EL-myc/His were plated onto collagen-coated 12-well dishes and transfected with the following plasmids: pcDNA3-α1-PDX, pcDNA3-AVRR, pALTERMAX-PACE4A-I, pRcCMV-PC6A, pRcCMV-furin, and pcDNA4 (mock). Twenty hours after transfection, cells were washed with PBS and incubated for 10 h in DMEM without FCS. Heparin-supplemented cell media were further analyzed by Western blot as described bellow.

Exogenous EL cleavage

The heparin-supplemented media (10 U/ml heparin) obtained from Hek-293 cells stably expressing EL-myc/His were pressure-dialyzed against PBS at 4°C. Concentrated material (50 μl) containing both the full-length EL and the 40 kDa EL fragment was mixed with 200 μl of DMEM and incubated with HepG2 cells in the absence and presence of α1-PDX (2 μM). Af-

ter the indicated periods of time, heparin (100 U/ml) was added into media. Media were collected after an additional 15 min incubation at 37°C and analyzed by Western blot.

Western blot analysis of cell media, heparin-releasable fraction, cell lysate, and postheparin mouse plasma

Cell media and heparin-supplemented cell media collected 30 min after the addition of 10 or 100 U/ml heparin from infected or transfected cells were collected into prechilled tubes, spun, concentrated in a SpeedVac (160 μl → 40 μl), supplemented with 10 μl of 5× loading buffer [20% (w/v) glycerol, 5% (w/v) SDS, 0.15% (w/v) bromophenol blue, 63 mmol/l Tris-HCl, pH 6.8, and 5% (v/v) β-mercaptoethanol], and boiled for 10 min before loading. Samples were analyzed by SDS-PAGE (12% gel) and with subsequent immunoblotting using EL-specific antibodies exactly as described (10). To analyze the cell surface-bound EL, cells were washed with PBS and subsequently incubated with 220 μl of DMEM + heparin (100 U/ml) at 37°C for 30 min. The heparin-releasable fractions were collected and further processed for Western blotting as described above for the cell medium. Thereafter, cells were washed with PBS, lysed with 250 μl of 1× loading buffer, and subjected to Western blot analysis. Mouse plasma (30 μl) was incubated overnight at 4°C with Hi-Trap heparin-Sepharose (Amersham Biosciences, Uppsala, Sweden) in the presence of a protease inhibitor cocktail (Sigma) and subsequently washed three times with PBS. After centrifugation, the heparin-Sepharose pellet was suspended in 1× loading buffer.

Expression, purification, and sequencing of the EL cleavage products

A confluent culture of Hek-293 cells stably expressing EL-myc/His was incubated with 24 ml of Panserin 401 medium for 24 h. During the last 30 min of incubation, heparin (10 U/ml) was added into medium. Medium was collected into prechilled tubes and spun. One milliliter of medium was supplemented with 100 μl of Talon metal affinity resins (BD Biosciences, Palo Alto, CA). After rotation-incubation at 20°C for 30 min, Talon was washed three times with PBS and Talon-bound fraction was eluted with 50 μl of EDTA (100 mM). The Talon eluates obtained from 10 ml of conditioned media were pooled and concentrated using an Ultrafree-0.5 centrifugal filter device (Millipore, Bedford, MA). Concentrated eluates were mixed with loading buffer, boiled, and loaded onto one lane of a 12% SDS-PAGE gel. After fractionation for 1.5 h at 150 V, proteins were transferred in 50 mM boric acid and 10% (v/v) methanol, pH 9, onto polyvinylidene difluoride sequencing membranes (Millipore). Proteins were visualized by Coomassie blue staining, and the bands corresponding to 40 kDa EL and 28 kDa EL-myc/His were subjected to sequencing by Edman degradation (N-terminal protein sequencing).

Phospholipase activity assay

The assay was done as described previously (4, 10). Briefly, PC substrate was made by mixing [^{14}C]dipalmitoyl-PC (New England Nuclear, Boston, MA), lecithin (1 mg/ml), and the substrate buffer Tris/TCNB [100 mM Tris-HCl, pH 7.4, 1% (v/v) Triton X-100, 5 mM $CaCl_2$, 200 mM NaCl, and 0.1% (w/v) NEFA-free BSA] and subsequently dried under nitrogen. The dried phospholipids (PLs) were reconstituted in the substrate buffer. Aliquots (190 μl) of the heparin-supplemented media were mixed with 10 μl of the substrate and incubated at 37°C for 1 h. The reaction was terminated by the addition of 1 ml of 0.2 M HCl and extraction with hexane-isopropanol (3:2, v/v; 0.1% HCl). Aliquots (500 μl) of the upper phase were dried in a SpeedVac and reconstituted in 100 μl of hexane-isopropanol (3:2, v/v). After separation by TLC (hexane-diethyl ether-acetic acid, 70:29:1, v/v), the liberated [^{14}C]NEFAs were quantitated in a scintillation counter (Beckman).

Isolation of human HDL

HDL (subclass 3, d = 1.125–1.21 g/ml) was prepared by sequential density ultracentrifugation of plasma obtained from normolipidemic blood donors and dialyzed against PBS (pH 7.4) exactly as described previously (11).

HDL labeling procedure

Labeling of HDL with [^{14}C]PC was performed as follows: 2 μCi of [^{14}C]PC was dried under nitrogen, redissolved in 30 μl of ethanol, and added to a solution containing HDL (3 mg of protein) and lipoprotein depleted serum (LPDS) (700 μl) in a final volume of 1.7 ml in PBS. Subsequently, this mixture was incubated under argon in a shaking water bath at 37°C. After 16 h of

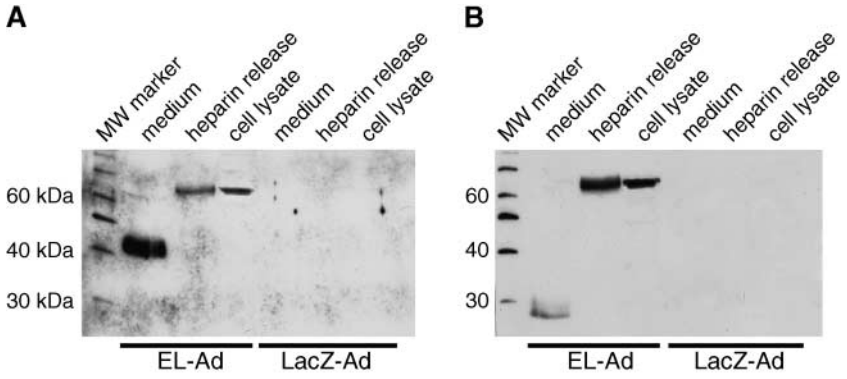

Fig. 1. Western blot analysis of endothelial lipase (EL) expression in HepG2 cells infected with EL-Ad and LacZ-Ad using N-EL (polyclonal antibody directed against a peptide in the N-terminal region of EL; A) and C-EL (antibody raised against a peptide in the C-terminal domain of EL; B) antibodies. HepG2 cells were plated onto 24-well trays and infected with EL-Ad and LacZ-Ad at a multiplicity of infection (MOI) of 60. After 24 h of incubation with the serum-free medium, medium was collected and cells were washed with PBS and incubated with 100 U/ml heparin for 30 min at 37°C to obtain the heparin-releasable fraction. Cells were subsequently washed with PBS and lysed in loading buffer. Aliquots of the media, heparin-releasable fractions, and lysates were fractionated by SDS-PAGE (12% gels), and EL-specific bands were detected using N-EL and C-EL antibodies and ECL assay. MW marker, the molecular mass marker MagicMark (Invitrogen).

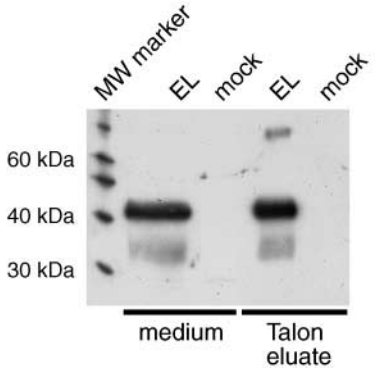

Fig. 2. Western blot analysis of EL fragments in the medium and Talon eluate of Hek-293 cells stably expressing EL-myc/His. Hek-293 cells stably expressing EL-myc/His and mock-transfected cells were cultured in Panserin for 24 h followed by the addition of heparin to a final concentration of 10 U/ml and incubation for another 30 min at 37°C. One milliliter of the heparin-supplemented medium was incubated with 100 μl of Talon metal affinity resin at 20°C for 30 min followed by extensive PBS washing and elution with 50 μl of EDTA (100 mM). EL fragments were detected in the aliquots of medium and Talon eluates by Western blot using a mixture of N-EL and C-EL antibodies. MW marker, the molecular mass marker MagicMark (Invitrogen).

incubation, labeled HDL was reisolated by density gradient ultracentrifugation in a TLX120 benchtop ultracentrifuge with a TLA100.4 rotor (Beckman). The HDL band was aspirated and desalted by size exclusion chromatography using 10DG columns (Bio-Rad). The specific activity obtained by this procedure was 2,100 cpm/μg HDL protein.

Iodination of HDL was performed as described previously (11) using NBr succinimide as the coupling reagent. Briefly, 0.5 mCi of $Na^{125}I$ (New England Nuclear, Stevenage, UK) was used to label 3 mg of HDL protein. Labeled HDL was stored at 4°C under an argon atmosphere and used within 1 week.

TLC analysis of ^{14}C-labeled lipids in cell media and cells expressing EL-myc/His, EL-40, and mock-transfected cells

Hek-293 cells stably expressing EL-myc/His, EL-40, and mock-transfected cells were plated onto 24-well trays and incubated in DMEM + 10% FCS. After 24 h, cells were washed with PBS and incubated in 500 μl of Panserin 401. After an additional 24 h, the indicated amounts of [^{14}C]HDL-PC were added into conditioned media and incubated at 37°C for 5. At the end of this incubation, media were collected and cells were incubated with DMEM containing 100 U/ml heparin to remove labeled HDL from the cell surface. Afterward, cells were washed with PBS. The lipids were extracted twice from the media and cells with hexane-isopropanol (3:2, v/v), dried in the SpeedVac, and redissolved in chloroform before application onto TLC plates. Hexane-diethyl ether-glacial acetic acid (70:29:1, v/v) was used as a mobile phase. The signals corresponding to PL, TG, and FFA were visualized by I_2, and lipid spots were cut out of the TLC plates and measured by scintillation counting.

^{125}I-HDL binding at 4°C

HepG2 cells were plated onto 24-well dishes (2×10^5 cells/well) and infected 40 h later with EL-Ad, EL-40-Ad, and LacZ-Ad at an MOI of 60. After infection, cells were incubated with 0.5 ml of the serum-free medium (Panserin 401) at 37°C for 24 h. Afterward, cells were put on ice and the indicated amounts of ^{125}I-HDL were added directly into cell medium, followed by incubation at 4°C for 1 h. After 1 h of incubation at 4°C, medium was aspirated and cells were washed extensively with ice-cold PBS and lysed with 0.5 ml of 0.3 M NaOH at 20°C. The cell-associated radioactivity and protein content (32) were measured in an aliquot of the cell lysate.

Experiments in mice

Female 12 week old C57BL×CBA mice were housed at 22°C under a constant light/dark cycle and had free access to water and chow diet. During injection into the tail vein, as well as during bleeding by retro-orbital puncture, the mice were anesthetized with isoflurane (Pharmacia and Upjohn, Guyancourt, France). Four mice per group were injected via the tail vein with 4×10^9 plaque-forming units of EL-Ad, EL-40-Ad, and LacZ-Ad. For blood sampling, mice were fasted overnight and preheparin samples were taken 1 day before and at day 4 after virus injection. After collecting preheparin plasma at day 4, mice were injected with 150 U/kg heparin. Postheparin plasma samples were collected 5 min after heparin injection.

Plasma lipid analysis

Plasma lipid concentrations were measured using commercially available assay kits: TGs (Triglycerides Enzymatique PAP 150; bio-

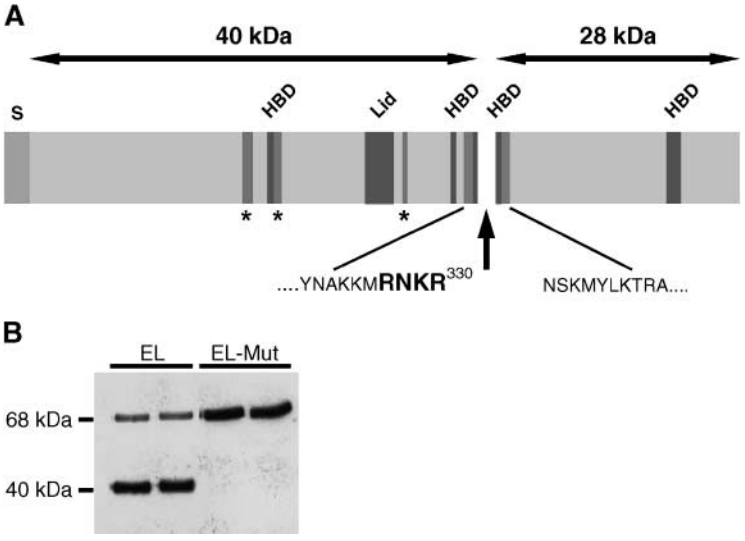

Fig. 3. Schematic presentation of the EL cleavage site together with the N- and C-terminal cleavage products (A) and effect of the Arg-330→Ser substitution on EL cleavage (B). A: The cleavage recognition sequence for proprotein convertases is indicated in boldface letters, and a vertical arrow indicates the cleavage site. Heparin binding domains are indicated by HBD, and the catalytic residues are indicated by asterisks. The numbering includes 20 amino acid residues of the signal peptide (S). Lid domain covers the catalytic site and confers substrate specificity to the enzyme. B: Heparin-supplemented medium of Hek-293 cells stably expressing EL- and EL-MUT-myc/His was concentrated in a SpeedVac and analyzed by Western blot as described for Fig. 1 using N-EL antibody and the ECL system.

Merieux sa, Lyon, France), phospholipids (Phospholipid B; Wako, Neuss, Germany), total cholesterol (Cholesterol Liquicolor; Rolf Greiner BioChemica, Flacht, Germany), and HDL-cholesterol (Precipitant and Standard; Human Gesselschaft fuer Biochemica and Diagnostica mbH,Wiesbaden, Germany).

Statistics

Results are expressed as means ± SD. Significance of differences was examined using Student's *t*-test.

RESULTS

Distribution of EL in the media, heparin-releasable fractions, and lysates of HepG2 cells infected with EL-Ad

The results presented in **Fig. 1** show Western blot analyses of cell media, heparin-releasable fractions, and cell lysates of HepG2 cells infected with EL-Ad encoding full-length EL. Using N-EL polyclonal antibody directed against a peptide in the N-terminal region of EL, a 68 kDa EL band representing mature EL was detected in the cell lysates and heparin-releasable fractions. In contrast, our antibody identified in the cell media a single protein band of ~40 kDa, most likely representing a cleavage product of the full-length EL. In the control samples obtained by the infection of cells with LacZ-Ad, neither full-length nor truncated EL could be detected, indicating the specificity of our antibody. Analysis of the samples described above by C-EL antibody, raised against a peptide in the C-terminal domain of EL, a 28 kDa band, representing the C-terminal cleavage product of EL (28 kDa EL), was detected exclusively in the cell media.

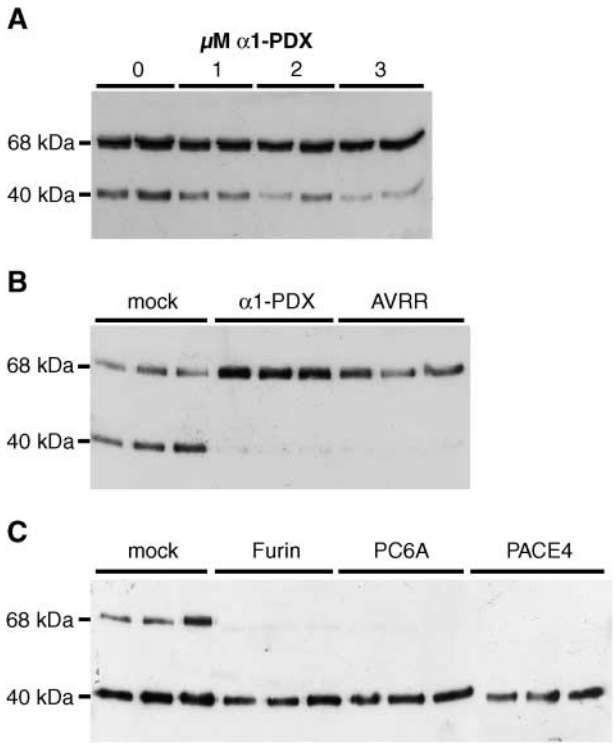

Fig. 4. Western blot analysis of heparin-supplemented media of EL-expressing cells in the presence of exogenously added α1-antitrypsin Portland (α1-PDX; A) and upon overexpression of α1-PDX and AVRR (B) and furin, PC6A, and PACE4 (C). A: HepG2 cells infected with EL-Ad with a MOI of 60 were incubated in the presence of the indicated amounts of α1-PDX in DMEM for 16 h. Heparin (100 U/ml) was added into medium during the final 30 min of incubation, yielding heparin-supplemented medium. B and C: Hek-293 cells stably expressing EL-myc/His were plated onto 12-well trays and transfected with plasmids encoding α1-PDX, AVRR, or plasmid without cDNA (mock; B) or with plasmids encoding furin, PC6A, PACE4, or plasmid without cDNA (C). After 24 h, cells were washed with PBS and incubated for another 10 h in DMEM. Aliquots of heparin-supplemented media were subsequently concentrated in a SpeedVac and analyzed by Western blot as described for Fig. 1.

Expression of myc/His-tagged EL in Hek-293 cells

To identify the cleavage site in 68 kDa EL, EL was stably expressed as a myc/His-tagged protein (EL-myc/His) in Hek-293 cells to allow His tag-mediated purification of the C-terminal cleavage product. As detected by a mixture of both N- and C-terminal EL antibodies, the cell media of stably expressing cells contained both EL cleavage products, the 40 kDa EL as well as the myc/His-tagged 28 kDa EL fragment (28 kDa EL-myc/His) of ~32 kDa (**Fig. 2**). To further characterize the different EL fragments, conditioned media were incubated with Talon affinity resins followed by Western blot analysis of the Talon eluates. As shown in Fig. 2, not only the C-terminal 28 kDa EL-myc/His but also N-terminal 40 kDa EL lacking the myc/His tag could be isolated by Talon affinity chromatography from the conditioned media. Additionally, the mature 68 kDa EL could also be detected in the EL-enriched Talon eluates, indicating a slight heparin-independent release of the full-length EL from the cell surface into the cell medium.

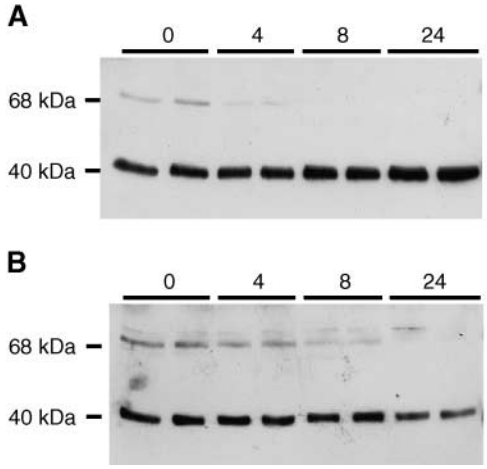

Fig. 5. Western blot analyses of heparin-supplemented media from HepG2 cells incubated with exogenously added EL in the absence (A) and presence (B) of α1-PDX. HepG2 cells (2×10^5) were plated onto 24-well trays. After 48 h, cells were washed extensively with PBS and incubated with EL prepared as described in Experimental Procedures in 250 μl of DMEM in the presence or absence of α1-PDX (2 μM final concentration). After the indicated periods, heparin (100 U/ml) was added to the media and heparin-supplemented media was collected after 15 min at 37°C. Aliquots of the heparin-supplemented media were analyzed by Western blot as described in Experimental Procedures.

Identification of the protease cleavage site

The N-terminal protein sequencing of the Talon-purified 40 kDa EL band revealed amino acid sequence SPVPF corresponding to amino acids 21–25 of the published EL sequence (5, 6), representing the N terminus of secreted EL. The N-terminal amino acid sequence of the Talon-purified 28 kDa EL-myc/His fragment corresponded to amino acids 331–336 of EL, providing evidence for the cleavage of a 68 kDa EL fragment after Arg-330 on the C-terminal end of the sequence KMRNKR↓ (**Fig. 3A**). It is important to note that this sequence represents the optimal consensus sequence for cleavage by several mammalian pPCs (20). However, an additional pPC consensus cleavage sequence (RKNR) is located 15 amino acid residues upstream of the defined cleavage site. Therefore, we tested whether EL cleavage occurs exclusively after Arg-330. For this purpose, Arg-330 was replaced with Ser using site-directed mutagenesis. As shown in Fig. 3B, the replacement of Arg-330 with Ser completely abrogated EL cleavage, confirming the exclusive cleavage of EL at position 330. Accordingly, in the heparin-supplemented media of Hek-293 cells stably expressing mutant EL (EL-MUT), only the full-length 68 kDa EL fragment was detectable. In contrast, cells stably expressing wild-type EL (EL) contained both the 68 kDa protein and the 40 kDa fragment in the heparin-supplemented media.

Detection of potential pPC candidates for the processing of EL

Considering the fact that mutation of Arg-330 at position P1 of the consensus sequence, which is essential for cleavage by pPCs, abolished the EL cleavage, it is tempting to suggest the involvement of pPCs in the processing of EL. Therefore, we attempted to detect potential pPC candidates for the processing of EL. We first tested whether engineered α1-antitrypsin, α1-PDX, which is a specific inhibitor of furin, PC6, and PACE4, affects the cleavage of EL. For this purpose, EL-Ad-infected HepG2 cells were incubated in the presence of increasing α1-PDX concentrations followed by Western blot analysis of the heparin-supplemented cell media. **Figure 4A** shows a substantial concentration-dependent inhibition of EL cleavage by α1-PDX, as demonstrated by a decrease in the amount of 40 kDa EL. This indicated a role of furin, PC6, or PACE4 in EL cleavage. Efficient inhibition of EL cleavage was also achieved by the expression of α1-PDX and an α1-antitrypsin variant (AVRR), a specific inhibitor of furin, PC6, but not PACE4, in Hek-293 cells stably expressing EL (Fig. 4B). As demonstrated in Fig. 4C, furin, PC6A, and PACE4 cleaved EL with similar efficiency upon expression in Hek-293 cells, resulting in the complete conversion of full-length EL in the 40 kDa fragment.

Extracellular cleavage of EL

Because the cleavage products of EL, the 40 and 28 kDa fragments, were detectable only in the cell supernatants and not in the cell lysates of HepG2 cells infected with EL-Ad, we assumed that cleavage occurs after secretion. To substantiate this assumption, aliquots of an EL preparation containing both the full-length and truncated forms of EL were added to HepG2 cells and incubated in the absence and presence of α1-PDX. As shown in **Fig. 5A**, the intensity of the full-length 68 kDa EL decreased during incubation with cells, whereby the rate of decrease could be

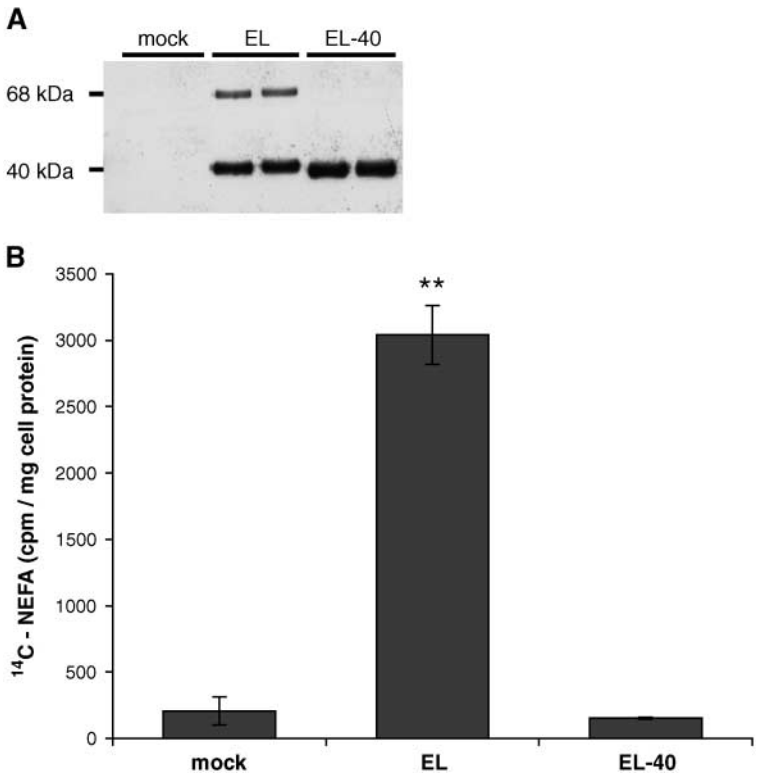

Fig. 6. Western blot analysis (A) and phospholipase activity (B) of heparin-supplemented cell media of Hek-293 cells stably expressing EL-myc/His, EL-40, and mock-transfected cells. Confluent cultures of Hek-293 cells stably expressing EL-myc/His, EL-40, and mock-transfected cells were washed with PBS and incubated in DMEM without fetal calf serum. After 24 h, heparin (10 U/ml) was added into medium, which after an additional incubation for 30 min at 37°C was collected in pre-chilled tubes and spun to remove cellular debris. Aliquots were analyzed by Western blot using N-EL antibody (A) and by phospholipase assay as described in Experimental Procedures (B). Results (cpm/mg cell protein) are means ± SD of two experiments performed in triplicate dishes. ** $P \leq 0.01$ (compared with mock and EL-40).

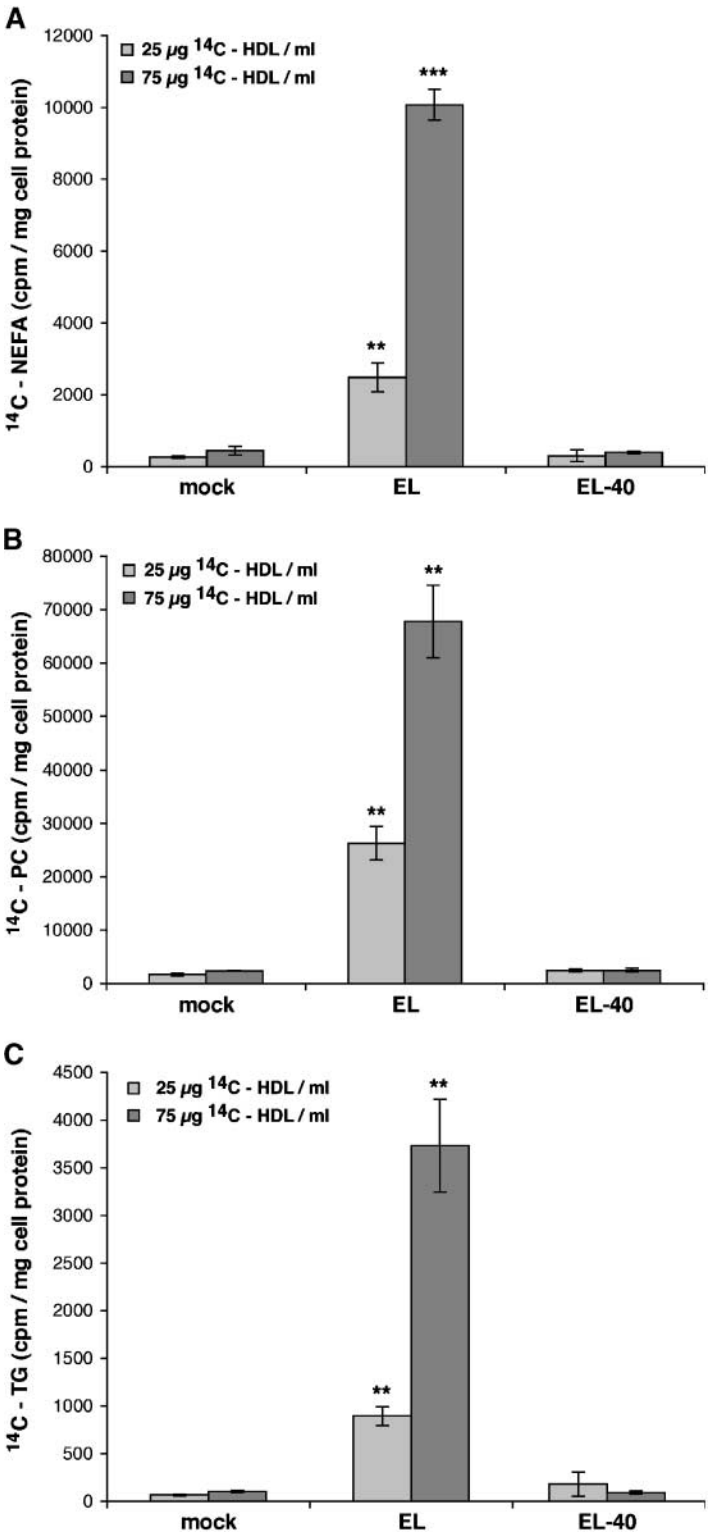

Fig. 7. Quantification of [^{14}C]NEFA in cell supernatant (A), [^{14}C]phospholipid (PL; B), and [^{14}C]triglyceride (TG; C) in cell extracts of Hek-293 cells stably expressing EL-myc/His, EL-40, and mock-transfected cells incubated with [^{14}C]HDL-PC. Confluent cultures of Hek-293 cells stably expressing EL-myc/His, EL-40, and mock-transfected cells were washed with PBS and incubated with the serum-free medium Panserin 401. After 24 h, the indicated amounts of [^{14}C]HDL-PC (µg HDL protein/ml medium) were added into cell media and incubated for 5 h at 37°C. After incubation, the cell medium was collected and lipids were extracted with hexane-isopropanol (3:2, v/v). After heparin release (100 U/ml) and extensive PBS washing, cellular lipids were extracted and analyzed along with lipids from cell media by TLC using hexane-diethyl ether-glacial acetic acid (70:29:1, v/v) as a mobile phase. Lipid spots were visualized by I_2 staining, subsequently cut out from the TLC plate, and quantified by scintillation counting. The cellular proteins were measured in lipid-depleted cells by the method of Lowry (32). Results are means ± SD of two experiments performed in triplicate dishes. *** $P \leq 0.001$ (compared with mock and EL-40). ** $P \leq 0.01$ (compared with mock and EL-40).

attenuated with α1-PDX (Fig. 5B). These results clearly demonstrated that pPC-mediated EL cleavage could occur in the extracellular compartment.

Phospholipase activity of the N-terminal 40 kDa EL (EL-40)

To examine the phospholipase activity of the N-terminal 40 kDa cleavage product of EL without background contamination with the full-length EL, a construct encoding the N-terminal 40 kDa fragment of EL, named EL-40, was stably expressed in Hek-293 cells. Heparin-supplemented media obtained from EL-40-, EL-, and mock-transfected cells were analyzed by Western blotting (**Fig. 6A**) and assayed for phospholipase activity (Fig. 6B). As expected, the heparin-supplemented media from EL-40-expressing cells contained a single 40 kDa fragment, whereas both the 40

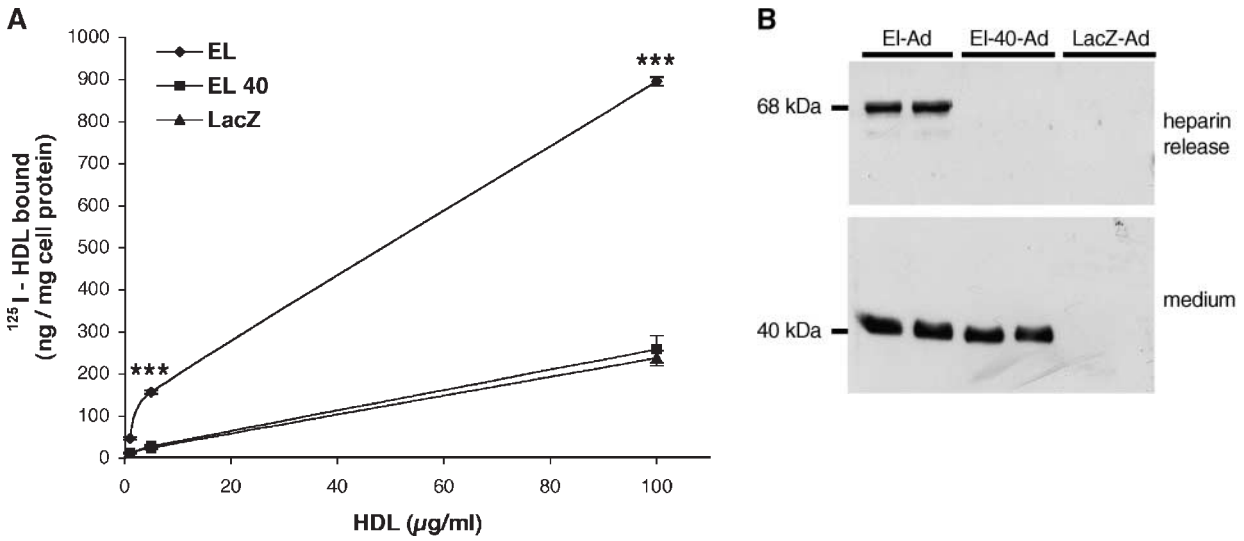

Fig. 8. ^{125}I-HDL binding at 4°C by HepG2 cells infected with EL-Ad, EL-40-Ad, and LacZ-Ad (A) and Western blot analyses of the EL fraction (B) bound to the cell surface and in the cell medium of cells infected as described in A. HepG2 cells (2×10^5 cells/well) were plated onto 24-well dishes and infected with EL-Ad, EL-40-Ad, and LacZ-Ad at a MOI of 60. After infection, cells were incubated with 0.5 ml of the serum-free medium (Panserin 401) at 37°C for 24 h. Afterward, cells were put on ice and the indicated amounts of ^{125}I-HDL were added directly into cell medium, followed by incubation at 4°C for 1 h. After 1 h of incubation at 4°C, medium was aspirated and cells were washed extensively with ice-cold PBS and lysed with 0.5 ml of 0.3 M NaOH at 20°C. The cell-associated radioactivity and protein content were measured in an aliquot of the cell lysate. Results are means ± SD for one representative experiment out of two performed in triplicate dishes. *** $P \leq 0.001$ (compared with LacZ and EL-40). B: Media and heparin-releasable fraction of HepG2 cells infected and cultured as described for A were collected and analyzed by Western blot as described for Fig. 1A.

and 68 kDa fragments were detected in the heparin-supplemented media of EL-expressing cells. As revealed by the phospholipase activity assay, the amount of [^{14}C]NEFA hydrolyzed from [^{14}C]dipalmitoyl-PC by heparin-supplemented media from cells expressing exclusively EL-40 was similar to that in control (mock) media and profoundly lower than that in media obtained from EL-expressing cells.

To rule out the possibility that the lack of [^{14}C]PC hydrolysis by EL-40 was attributable to the presentation of [^{14}C]PC in the form of Triton X-100 mixed micelles, we tested the ability of EL-40 to hydrolyze [^{14}C]NEFA from [^{14}C]PC-labeled HDL ([^{14}C]PC-HDL), a natural substrate of EL. For this purpose, Hek-293 cells stably expressing EL and EL-40 as well as mock-transfected control cells were incubated with [^{14}C]PC-HDL. TLC analysis of the cell media revealed that in contrast to uncleaved EL, EL-40 was incapable of releasing [^{14}C]NEFA from [^{14}C]PC-HDL (**Fig. 7A**). Correspondingly, the amounts of [^{14}C]PL (Fig. 7B) and [^{14}C]TG (Fig. 7C) in the cell lysates of EL-40-expressing cells were similar to those in control cells and profoundly lower compared with EL-expressing cells. These results clearly demonstrate the inability of EL-40 to release [^{14}C]NEFA from [^{14}C]PC-HDL.

Bridging function of EL-40

EL was demonstrated previously to mediate HDL binding to cells independent of its enzymatic activity (3, 4). To examine the ability of EL-40 in this process, HepG2 cells were infected with EL-40-Ad, EL-Ad, and LacZ-Ad. After 24 h, ^{125}I-labeled HDL was added directly to prechilled cell media at 4°C. As shown in **Fig. 8A**, the amount of ^{125}I-labeled HDL bound to the cell surface of EL-40-Ad-infected cells after incubation for 1 h was similar to that in LacZ-Ad-infected cells and significantly lower compared with EL-Ad-infected cells. These results demonstrated the inability of EL-40 to bridge HDL to the cell surface. This finding was underscored by results of Western blot analysis (Fig. 8B). EL-40 was exclusively found in the cell media (lower panel) but not in the heparin-releasable fractions (upper panel) of EL-40-Ad-infected cells. In contrast, the full-length EL could be detected only in the heparin-releasable fractions. Minimal amounts of the full-length EL, which is released spontaneously into medium, without the addition of heparin, could be detected in the cell media by incubation with Talon affinity resins (Fig. 2, Talon eluate).

Effect of EL-40 on plasma lipids in mice

Finally, we examined whether overexpression of EL-40 affects the concentration of plasma lipids in mice. For this purpose, plasma lipids obtained 4 days after virus injection from fasted female mice overexpressing EL-40, EL, and LacZ were quantitated. Whereas in mice overexpressing EL, plasma PL, TG, total cholesterol, and HDL-cholesterol were profoundly decreased (85, 63, 77, and 99%, respectively), lipid parameters were unaffected in EL-40-overexpressing mice and were similar to those of LacZ control mice (**Fig. 9A**). Again, these results provided further evidence for the inactivity of EL-40. Western blot analysis of the heparin-Sepharose-treated postheparin plasma samples suggested an efficient cleavage process of EL upon

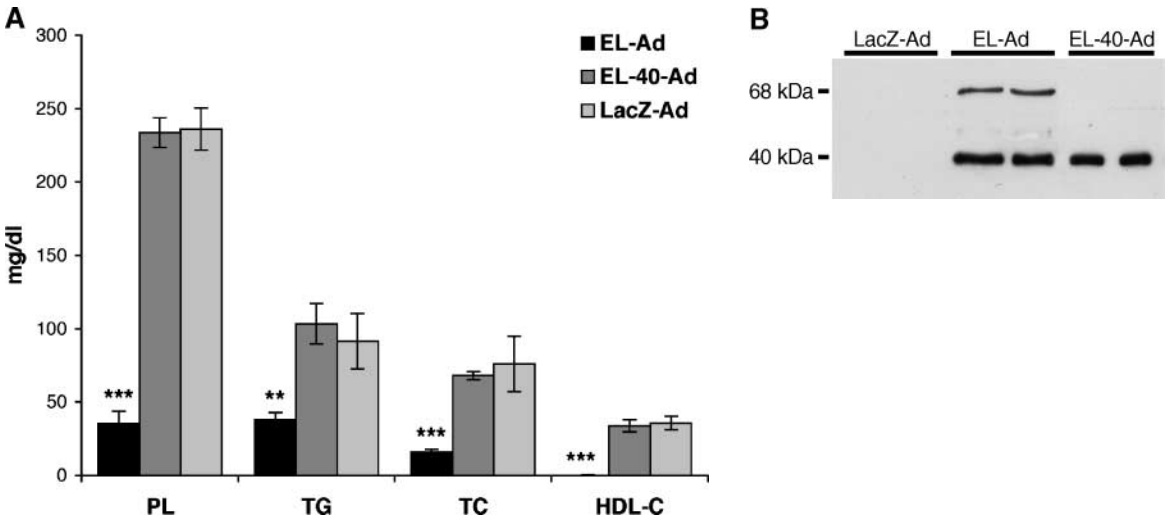

Fig. 9. Quantification of preheparin plasma lipids (A) and Western blot analysis of postheparin plasma (B) of mice injected with EL-Ad, EL-40-Ad, and LacZ-Ad. A: Four female mice per group were injected via the tail vein with 4×10^9 plaque-forming units of EL-Ad, EL-40-Ad, and LacZ-Ad. For blood sampling, mice were fasted overnight and preheparin samples were taken at day 4 after virus injection. Plasma PL, TG, total cholesterol (TC), and HDL-cholesterol (HDL-C) were determined using commercially available assay kits. Results are means ± SD. *** $P \leq 0.001$ (compared with LacZ and EL-40). ** $P \leq 0.01$ (compared with LacZ and EL-40). B: After collection of preheparin plasma, mice were injected with 150 U/kg heparin. Postheparin plasma samples were collected 5 min after heparin injection followed by heparin-Sepharose treatment and Western blot.

expression in mice (Fig. 9B), similar to that observed in cultured HepG2 or Hek-293 cells.

DISCUSSION

EL is synthesized as a 55 kDa protein that is secreted after maturation, including N-linked glycosylation (7), as a full-length protein of 68 kDa. Several studies have shown that heparin-supplemented media of cells expressing either recombinant EL (7) or endogenous EL (6, 8) contained in addition to the full-length EL of 68 kDa also a N-terminal 40 kDa fragment interpreted as a putative EL cleavage product.

The aim of this study was to identify the cleavage site within the mature protein and the proteases involved in EL processing as well as to assess the functional properties of the N-terminal 40 kDa EL fragment. Using specific antibodies directed specifically against the N- and C-terminal domains of EL, we detected in the cell media, but neither in the lysates nor in the heparin-releasable fractions, two proteolytic fragments of EL: the N-terminal 40 kDa fragment and the C-terminal 28 kDa fragment.

Overexpression of myc/His-tagged EL in Hek-293 cells and subsequent purification of the myc/His-tagged C-terminal cleavage fragment, followed by N-terminal protein sequencing, identified the cleavage site in EL at Arg-330 on the C-terminal end of the sequence RNKR↓. This sequence represents the optimal consensus sequence for cleavage by pPCs. Interestingly, a similar sequence (RKNR), which deviates only slightly from the other one, is located 15 amino acid residues upstream of the cleavage site. To rule out the possibility that EL cleavage occurs at both positions, Arg-330 was replaced with Ser, and the mutated EL obtained was resistant to cleavage. This clearly demonstrated that the C-terminal end of Arg-330 was the unique cleavage site. The lack of cleavage in the upstream consensus sequence might be attributable to an Asn residue instead of a basic residue at the P2 position, which does not fulfill the requirements for optimal hydrolysis by pPCs (20, 33). Additionally, it is possible that some structural features other than the cleavage site sequence are critical for substrate processing. Accordingly, the efficiency of cleavage might also be affected by the surrounding peptide sequences or by the positioning of the cleavage site within the three-dimensional scaffold of the protein substrate.

An unexpected finding of the present study was the efficient retention of the N-terminal 40 kDa EL lacking the myc/His tag by Talon affinity resins. Talon affinity resins used in the present study contained a Co^{2+} ion in an electronegative pocket of a specific metal chelator. Accordingly, these affinity resins should specifically bind polyhistidine-tagged proteins. Because polyhistidine sequence is not present in EL, it is conceivable that some of the 15 histidine residues that are unevenly distributed throughout the N-terminal 40 kDa fragment of EL exhibit high affinity for the Talon affinity resins.

It has previously been reported that pPCs have overlapping substrate specificity and are widely distributed among cell lines (17, 18, 20). As revealed by PCR analysis, Hek-293 cells express furin, PC6, PACE4, and PC7 (29). To identify the respective members of the pPC family responsible for EL processing in our cell system, we tested the ability of specific pPC inhibitors to diminish or abolish the processing of EL. Because EL cleavage was efficiently abolished

by α1-PDX, a specific inhibitor of furin, PC6, and PACE4 but not of PC7 (34), we excluded the possibility that PC7 was involved in the processing of EL in Hek-293 cells. EL cleavage could also be abolished with another α1-antitrypsin variant (AVRR), a specific inhibitor of furin and PC6 but not of PACE4. Thus, our results excluded a contribution of PACE4 to the EL processing in Hek-293 cells. Although furin and PC6 turned out to be candidates for EL cleavage based on their sensitivity to applied inhibitors, overexpression of pPCs in Hek-293 cells stably expressing EL clearly demonstrated that PACE4, in addition to PC6A and furin, also efficiently cleaved EL. Accordingly, the lack of EL cleavage in the presence of AVRR might be attributable to a low, insufficient expression level of PACE4 compared with furin and PC6 in Hek-293 cells. It is conceivable, therefore, that PC7, when overexpressed, would also efficiently cleave EL. Our results showed that furin, PC6A, and PACE4 have the ability to mediate the cleavage of EL. However, the relative contributions of the respective pPCs to the cleavage of EL seem to be related to their relative abundance and activity in EL-expressing cells.

Because both EL-processing products, the N-terminal 40 kDa fragment and the C-terminal 28 kDa fragment, could not be detected in the cell lysate, we assumed the cleavage to occur after secretion. Consistent with this assumption was the finding that the full-length EL was cleaved when added exogenously into cell medium of HepG2 cells and that the cleavage could be attenuated by α1-PDX. Furin has been shown to reside mainly in the *trans*-Golgi and endocytic compartments (33). However, significant amounts of furin have been found at the cell surface, where it is anchored to the cell membrane via its transmembrane domain (35). There, furin may mediate the processing of EL, as described for the processing of cellular, bacterial, and viral protein precursors (19, 35). Similarly, extracellular EL processing could be mediated by PC6A and PACE4, which are bound to HSPG after secretion (21).

We found that both processing products of EL were identified in the cell media and could not be detected in the heparin-releasable fractions, suggesting decreased affinity toward the cell surface compared with the full-length EL. The processing of EL occurs in one of four heparin binding domains (HBDs), which are clusters of basic residues. Upon cleavage, two basic residues, Lys-329 and Arg-330, remain exposed on the novel C-terminal end, from which they could be removed by extracellular HSPG-bound carboxypeptidases (36), resulting in a massive truncation of this HBD. A potential explanation for the decreased affinity of the EL-processing products toward the cells might be that remaining intact HBDs, two in the N-terminal cleavage product and one in the C-terminal cleavage product, are insufficient to mediate their binding to the cells. However, additional studies are required to determine the relative activities of HBDs in EL. An alternative explanation might be that upon cleavage the three-dimensional scaffold of EL is altered, interfering with the interaction with HSPG.

Experiments addressing the functionality of the N-terminal processing product of EL (EL-40), which harbors the catalytic site, revealed that EL-40 is incapable of liberating NEFA from HDL-PC. EL-40 was incapable of bridging HDL to the cell surface and had no impact on plasma lipids upon overexpression in mice. These findings are consistent with the results of a recent study (37) showing that the C-terminal domain of EL is essential for the ability of EL to bind and hydrolyze HDL.

The inactivation of EL by pPCs raises the question of the biological significance of this processing event. Only enzymatically active EL has a significant impact on plasma lipids when expressed in wild-type mice (38). The pPC-mediated separation of the functionally distinct domains of EL and concomitant loss of function might be a regulatory mechanism that reduces the bioavailability of active EL. The expression of EL in endothelial cells has been shown to be upregulated by the inflammatory cytokines IL-1β and TNF-α (5, 39) as well as by shear and cyclic stresses (39). Interestingly, furin was also found to be upregulated by shear stress in endothelial cells (40). Although the regulation of pPC expression by inflammatory cytokines has not been addressed to date, it is tempting to speculate that a coordinated upregulation of EL and pPCs attenuates the overexpression of active EL and in turn prevents the extreme reduction in HDL plasma level. Additionally, the modulation of EL activity by pPCs may regulate the supply of EL-expressing cells with NEFA and lyso-PC, thus preventing the overloading of cells with lipolytic products during conditions that upregulate the expression of EL.

In summary, we found that EL is cleaved by members of the pPC family after Arg-330, yielding the N-terminal 40 kDa and C-terminal 28 kDa fragments. The N-terminal 40 kDa fragment, named EL-40, was incapable of hydrolyzing HDL-PC or bridging HDL to the cells. Moreover, overexpression of EL-40 in mice had no impact on plasma lipids.

This work was supported by Grants SFB-F717 (to S.F.) and SFB-701 from the Austrian Science Fund, by Grant 10172 (to S.F.) from the Austrian National Bank, and by Franz-Lanyar-Foundation Grants 279 and 294 (to S.F.). The authors are grateful to Dr. Akihiko Tsuji (University of Tokushima, Japan) for providing expression plasmids encoding furin, PC6A, PACE4, α1-PDX, and the AVRR variant. The authors thank Dr. Ernst Steyrer for critical reading of the manuscript and helpful suggestions. The authors thank Michaela Tritscher for excellent technical assistance and A. Hermann for help with care of the mice.

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