Biosynthesis, Distinct Post-translational Modifications, and Functional Characterization of Lymphoma Proprotein Convertase*

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Proprotein convertases are responsible for the endoproteolytic processing of prohormones, neuropeptide precursors, and other proproteins within the constitutive and regulated secretory pathways. Cleavage occurs carboxyl-terminally of basic amino acid motifs, such as RX(K/R)R, RXXR, and (R/K)R. As already available for the other known mammalian members of this enzyme family, we here define structural and functional features of human lymphoma proprotein convertase (LPC). Analysis of expression of recombinant LPC in stably transfected Chinese hamster ovary cells reveals biosynthesis of a 92-kDa nonglycosylated precursor (proLPC) and a 102-kDa endoglycosidase H-sensitive glycosylated form of proLPC. Only the latter is further processed and after propeptide removal converted into a complexly N-glycosylated mature form of LPC of about 92 kDa. Co-expression experiments of truncated LPC with an active site mutant of LPC (LPC8265A) indicate that prodomain removal of LPC occurs via an autoproteolytic, intramolecular mechanism, as was demonstrated before for some of the other members of this enzyme family. Prodomain removal is shown to be required for LPC to exit the endoplasmic reticulum. As far as subcellular localization is concerned, immunocytochemical, ultrastructural, and biochemical analyses show that LPC is concentrated in the trans-Golgi network, associated with membranes, and not secreted. Carboxyl-terminal domains are critically involved in this cellular retention, because removal of both the hydrophobic region and the cytoplasmic tail of LPC results in secretion. Of interest are the observations that LPC is not phosphorylated like furin but is palmitylated in its cytoplasmic tail. Finally, substrate specificity of LPC is similar to that of furin but not identical. Whereas for furin a basic substrate residue at position P-2 is dispensable, it is essential for LPC. For optimal LPC substrate processing activity, an arginine at position P-6 is preferred over an arginine at P-4.

A wide variety of secreted proteins are initially synthesized as inactive, higher molecular mass precursor proteins. In eukaryotes, specific endoproteolytic proteolysis of many of those precursor proteins occurs at particular basic amino acid motifs (1) by proprotein convertases (PCs), a subgroup of the subtilisin-like serine proteases or subtilases (2). All known subtilases are multi-domain serine proteases consisting of a signal peptide preceding the pro, catalytic, middle, and cytoplasmic domains. Homology is highest in the catalytic domains (containing the fully conserved catalytic triad, aspartate-histidine-serine) but diverges toward the carboxyl-terminal domains. To date, seven mammalian proprotein convertases have been identified, including furin, PC1/PC3, PC2, PC4, PACE4, PC5/PC6, and LPC (3–5). The first known eukaryotic proprotein convertase with such a cleavage specificity was the kexin enzyme of the yeast Saccharomyces cerevisiae encoded by the KEX2 gene (6). On the basis of structural and functional analysis, human furin was identified as the first mammalian member of this novel enzyme family of proprotein convertases (7–9).

Recently, promoter regions of the FUR, PC1, and PC2 genes have been analyzed in more detail (10–12). Proprotein convertases in yeast and mammals are synthesized aszymogens that are proteolytically processed, removing the propeptide from the rest of the enzyme. For kexin (13) and furin (14–17) removal of the propeptide occurs autoproteolytically in the endoplasmic reticulum and is a prerequisite for exit of the mature enzyme out of the endoplasmic reticulum and transport to a late Golgi compartment (18–21).

Recently, we have identified the gene for human proprotein convertase LPC in the chromosome breakpoint region of a high grade lymphoma carrying a t(11;14)(q23;q32) translocation (5). Independently, human, rat, and mouse LPC were cloned from cell lines and designated PC8 (22), PC7 (23), and SPC7 (24), respectively. In the present study, we will only use LPC as an acronym to indicate the proprotein convertase. LPC is widely expressed but not as ubiquitously as furin. LPC RNA has been demonstrated both in early embryonal stages (24) as well as in several adult tissues and cell lines (5, 23). The catalytic domain of LPC exhibits substantial homology to other mammalian PCs (5), but based on phylogeny analysis it has been suggested that LPC is more related to kexin of yeast than are other mammalian members of this family (23).

1 The abbreviations used are: PC, proprotein convertase; LPC, lymphoma proprotein convertase; TGN, trans-Golgi network; PBS, phosphate-buffered saline; kb, kilobase; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; VV, Vaccinia virus; N-glycosylation, asparagine-linked glycosylation; endo-H, endoglycosidase H; endo-F, endoglycosidase F/N-glycosidase F; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino].

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tional characteristics have been described for most of the mammalian proprotein convertases. Being the most recent member, such detailed information, which is crucial for further studies, is not yet available for LPC. Therefore, the main objective of the present report is to define such structural and functional characteristics of LPC. Studies of LPC presented here include 1) its biosynthesis, 2) its post-translational processing and modifications, i.e. glycosylation, palmitoylation, and phosphorylation, 3) its subcellular localization, and 4) its proprotein processing activity as well as specificity.

MATERIALS AND METHODS

Plasmids and Secondary Structure Prediction—A GLC-1-M13 (lung tumor cell line) cDNA library in Agt11 was screened with a human LPC probe (5) and a 3.4-kb LPC cDNA was isolated and cloned as an EcoRI fragment in pGEM-3Zf(+) (3). For expression in the VV:T7/PK(15) system, a 2.6-kb SacI subfragment encompassing the complete LPC protein coding region was cloned into pGEM-3Zf(+). For expression in COS-1 and CHO-DHFR- cells, this 2.6-kb LPC cDNA subfragment was re-cloned into the EcoRI and XbaI sites of pcDNA3 (Invitrogen). LPC mutants lacking the carboxyl terminus (LPCΔcyt; introduction of a stop codon after Ser595), the hydrophobic domain as well as the carboxyl terminus (LPCΔcyt; introduction of a stop codon after Ser595), and the active site mutant of LPC (LPCS265A; Ser925→Ala), were generated using the altered sites in vitro mutagenesis system (Promega) according to the guidelines of the supplier. For this purpose the 2.6-kb LPC SacI cDNA subfragment was re-cloned into pSELECT (Promega). The sequences of the mutagenic primers (Pharmacia Biotech Inc.) used were 5′-GTATATTGGTTCACTGGATGAATGCTT-3′ (LPCΔcyt), 5′-ACCCTAAGACCTCACTGTCGGTGTTAG-3′ (LPCΔcyt+Δcyt), and 5′-GAGGGCCACACAGGTCGCCAGTCGACGCC-3′ (LPCS265A), with the mutated nucleotides underlined. The mutated nucleotides were confirmed by nucleotide sequence analysis according to the dideoxy-chain termination method.

Secondary structure predictions of LPC were based on results obtained with PROTEAN (DNASTAR Inc.), TMAP, 2 and Predictprotein.3

Biosynthesis of LPC

MP1 and MP2 directed against the multiple antigenic peptide conjugate and KP1 and KP2 directed against the keyhole limpet hemocyanin conjugate. All sera were tested in Western blotting, immunoprecipitation analysis, and indirect immunofluorescence microscopy. Sera KP1 and KP2 were also tested in immunoelectron microscopy. Rat anti-KDEL (Lys-Asp-Glu-Leu) antibody10 was kindly provided by Dr. G. Butcher (Cambridge, UK). Rabbit anti-γ-adaptin antibody was a generous gift from Dr. M. Robinson (Cambridge, UK) and was used for immunoelectron microscopy as described previously (26, 27). Mouse monoclonal MON-152 and rabbit polyclonal antibodies against human furin have been described (28). Fluorescein- or Texas red-conjugated goat-anti-rabbit Ig (Sigma) and donkey anti-rabbit IgG (Amersham Corp.) were used.

Cos-1 cells grown on glass chamber slides (Nunc) were washed two times with phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 30 min at room temperature, followed by incubation in 50 μM NH2Chi in PBS for 15 min at room temperature to quench unreacted aldehydes. Cells were then blocked in PBS containing 0.5% blocking reagent (Boehringer Mannheim) and 0.2% Triton X-100 (PBS-BT) for 30 min at room temperature. Incubation with primary antibodies (diluted 1:200–1:1000 in PBS-BT) continued for 1 h at room temperature. After rinsing cells three times, bound antibodies were detected with fluorescently labeled secondary antibodies by incubation for 1 h at room temperature. After two washes in PBS-BT, slides were dried under the microscope cover and analyzed with a Zeiss Axioshot microscope equipped with UV optics. Images were recorded with a CE200A CCD-camera (Photometrics) at a binning of 4 using Smartcapture (Digital Scientific) and IPLab Spectrum (Signal Analytics) software.

Cell Fractionation and Membrane Extraction—Cells were collected in PBS containing 1 mM EDTA at room temperature. During the rest of the procedure, incubations were carried out on ice unless mentioned otherwise. Cells were washed twice with PBS and once with 10 mM HEPES (pH 6.3) containing 5 mM EDTA. Cells were resuspended in 10 mM HEPES (pH 6.3) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml pepstatin and sheared by passing five times through a 25-gauge needle. The cell lysates were centrifuged at 2000 rpm for 10 min at 4 °C to remove intact cells and nuclei. The post-nuclear supernatant was diluted 2-fold and centrifuged at 4 °C for 30 min at 200,000 × gsw in an SW60 rotor (Beckmann). The membrane-containing pellets were drained for 5 min, resuspended in 2 volumes of 100 μM Na2CO3 (pH 11.5; 0 °C), transferred to new tubes, and then extracted in approximately 40 volumes of the same buffer for 30 min (29). Membranes were centrifuged again at 200,000 × gsw for 30 min. Both the supernatants and carbon-extracted membrane pellets were immediately neutralized with 100 mM HEPES (pH 6.3; final concentration) and analyzed by SDS-PAGE and Western blotting (ECL detection system, Amersham Corp.). Protein concentrations were determined using the BCA assay (Pierce) with bovine serum albumin as a standard.

Immunoelectron Microscopy—Cells were prepared for ultrastructural immunocytochemistry as described (30). Immunolabeling of anti-gamma-adaptin serum was performed using the protein A-gold technique (31). Sections were immunoreacted with 5 μl of primary antibody diluted in PBS containing 5 μl fetal calf serum and 0.1% bovine serum albumin. Cells were put on 300-mesh copper grids, washed and fixing the complexes with 1% glutaraldehyde in PBS for 10 min. The sections were observed in a Philips CM100 transmission electron microscope.

Radiolabeling, Immunoprecipitation, and Analysis of N-Glycosylation—Labeling of cells (5–10 × 106 cells/sample) with Tran32Slabeled (specific activity, >1000 Ci/mmol, ICN), substrate processing in PK15 cells, immunoprecipitation, analysis of N-linked glycosylation, and SDS-PAGE have been described (15). In experiments with brefidalin A (Boehringer Mannheim), CHO cells received 5 μCi/ml brefidalin A throughout the starvation, labeling, and chase periods. For [3H]palmitic acid labeling, cells were starved 4 h in minimal essential medium α containing 10 mM HEPES (pH 7.4) and then incubated for 2 h in the presence of 1 μCi/ml [3H](9,10)-palmitic acid (specific activity, 30–60 Ci/mmol; NEN Life Science Products) in minimal essential medium α containing 1% Me2SO and 0.1% bovine serum albumin. Cells were put on ice and washed once with 1 ml of ice-cold PBS, followed by lysis in 1 ml of DIPA buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 2 μg pepstatin,
Biosynthesis of LPC

Results

Biosynthesis, N-Linked Glycosylation, and Proteolytic Processing of LPC-Encoded Proteins—To study the biosynthesis of LPC, CHO cell lines were generated that stably expressed wild-type LPC. For comparative evaluation, a CHO cell line expressing recombinant furin was also included in these studies (25). After labeling of CHO-LPC cells for 1 h, antibodies KP1 as well as MP1 detected two proteins migrating at approximately 92 and 102 kDa (Fig. 1A). Both proteins are most likely LPC isofoms, as substantiated by similar immunoprecipitation analysis of deletion mutants of LPC (Δcyt and Δtm+cyt) lacking a part of the carboxyl-terminal region of LPC. Such analysis also revealed the biosynthesis of two proteins, and they had a higher electrophoretic mobility in accordance with the deleted sequences (see Fig. 6). When pulse-labeled CHO-LPC cells were subsequently chased for 2 h, one major immunoreactive protein of about 90–92 kDa was detected that now migrated more diffusely, whereas the intensity of the 102-kDa LPC protein had clearly decreased (Fig. 1A, lanes c). Specificity of the anti-LPC antibodies was demonstrated by concomitant analysis of the parental, nontransfected CHO cell line (Fig. 1A, lanes -). No LPC proteins were detected in these control experiments. Similar results were obtained with antibodies MP2 and KP2 (data not shown).

Pulse-chase analysis of cells that had been labeled for only 10 min also showed that anti-LPC antibodies immunoprecipitated two proteins of 92 and 102 kDa. They slowly disappeared in time (Fig. 1B). After a chase period of 40 min, a third protein with a molecular mass of about 90 kDa became visible. It subsequently increased in molecular mass, most likely as a result of further post-translational modifications, until it finally comigrated with the protein of about 92 kDa mentioned above (Fig. 1B). The 90-kDa protein was never detected in analyses of cells expressing an inactive LPC mutant in which the active site serine of the catalytic triad was mutated (LPCS265A, described below), indicating that the 90-kDa protein corresponds to a processed form of LPC that lacks the propeptide. Comigration of the 92-kDa protein already detectable after pulse labeling and the 92-kDa protein originating from the 90-kDa protein made it impossible to precisely follow the fate of the former.

N-Linked glycosylation of LPC was also studied (Fig. 2). Treatment of the LPC-encoded proteins with endoglycosidase H and F suggested that the initially synthesized 92-kDa protein is a nonglycosylated LPC proprotein and the 102-kDa protein is an endo-H-sensitive, N-glycosylated isoform of it. These results supported the conclusion that the 92- and 102-kDa proteins, detectable directly after pulse labeling, contain the same polypeptide backbone. This conclusion was further substantiated by analysis of deletion mutants of LPC, one lacking only the carboxyl terminus (LPCΔcyt), the other both the carboxyl terminus and the hydrophobic domain (LPCΔtm+cyt); described below). Also in cells expressing these mutants, two proteins were detected, although with smaller molecular masses in agreement with their deletions. Results obtained with the 90-kDa protein revealed complex N-glycosylation. Studies after various chase times demonstrated that the observed increase in molecular mass of the 90-kDa protein to about 92 kDa was most likely due to additional N-linked glycosylation (Fig. 2). Moreover, the composition of the N-linked sugars changed during the chase from a high mannose (shown by its sensitivity to endo-H) to a complex type (insensitivity to endo-H), suggesting that this protein represents processed LPC
(i.e. its propeptide removed) that is transported out of the endoplasmic reticulum into the Golgi complex. In contrast, the 102-kDa LPC proprotein remained endo-H-sensitive throughout the complete chase period, suggesting that it never left the endoplasmic reticulum. Similar observations were made in studies with the processing-defective LPC mutant LPCS265A (data not shown; see also below, immunofluorescence data in Fig. 3). It should also be noted that removal of the LPC prodomain is not inhibited by the fungal metabolite brefeldin A (data not shown). Moreover, brefeldin A reduces the complex N-glycosylation of mature LPC as was concluded from the change in appearance of the immunoprecipitated protein band from a diffuse to a compact band when brefeldin A was added. All these observations are in support of the conclusion that processing of LPC takes place in the endoplasmic reticulum.

Intracellular Localization of LPC—Immunofluorescence analysis of wild-type LPC and furin in transiently transfected CHO (data not shown) and COS-1 cells (Fig. 3, A and B) suggested that the intracellular location of the bulk of both proteins is predominantly restricted to the Golgi complex. Similar analysis of the processing-defective LPC mutant LPCS265A revealed retention of the mutant LPC protein in the endoplasmic reticulum as indicated by the colocalization with the staining obtained with antibodies recognizing endogenous KDEL sequences (Fig. 3, C and D). In previous studies (17), similar results were obtained for furin using processing-defective FUR mutants. These observations suggest that removal of the propeptide is a prerequisite for the LPC protein to leave the endoplasmic reticulum, as in the case of furin.

The intracellular localization of LPC was also studied at the electron microscopic level (Fig. 4). Initial observations of transfected AtT-20 cells showed that anti-LPC immunoreactivity colocalized to a large extent with TGN markers TGN38 (33) and recombinant furin (19, 20) in a juxtanuclear region that seemed to consist of several vesicle-like structures. Anti-furin immunoreactivity seemed to be concentrated somewhat more than that of LPC. To resolve the question of whether LPC was concentrated in the Golgi complex under steady state conditions, immunoelectron microscopy was performed on CHO cell lines stably expressing recombinant LPC. The results of these studies showed anti-LPC immunoreactivity in the TGN adjacent to the Golgi stack (Fig. 4a). LPC immunogold labeling could often be observed in association with the same coated buds and vesicles that were labeled with γ-adaptin in double-labeling experiments. Because γ-adaptin is an established marker for the TGN (26, 27), these results provide further evidence for the association of LPC with the TGN (Fig. 4b).

Plasma membrane labeling was occasionally detected at the cell surface (data not shown).

Maturation of LPC Involves Autocatalytical Removal of the Propeptide—For some of the mammalian members of this family of proprotein processing enzymes as well as for the yeast homolog kexin, it has been reported that enzyme maturation involves autocatalytical removal of the propeptide. In light of this, it was tested whether this also occurred according to such a mechanism in case of LPC (data not shown). In analogy to experiments described before (14–17), a processing-defective LPC mutant LPCS265A, having the active site Ser265 residue replaced by Ala, and the catalytically active mutant LPCDcyt, lacking the carboxyl terminus, were coexpressed in CHO-DFHR2 cells. Analysis of radiolabeled proteins in CHO-
FIG. 5. Cleavage of wild-type and mutant prorenin substrates by furin and LPC. PK(15) cells were infected with VV:TM recombinant Vaccinia virus and lipofected with Ren-2 cDNA in combination with LPC cDNA, FUR cDNA (15), or pGEM-3Z(f+) (endogenous control), respectively. Cells were labeled with 100 μCi/ml Tranylcypromine (8) for 1 h followed by a chase of 3 h. Intracellular LPC and furin as well as secreted (pro)renin were analyzed by SDS-PAGE, as described under "Materials and Methods." Ren-2 substrates are indicated with their P-1 to P-6 residues (wild-type Ren-2 substrate is DRVFTKR). A, top panel, endogenous processing; middle panel, LPC-mediated processing; bottom panel, furin-mediated processing. Relative positions of prorenin and renin are indicated. B, expression of immunoprecipitated LPC (upper panel, KP1) and furin (lower panel, polyclonal). The relative position of the molecular mass marker is indicated.

Processing studies with wild-type and mutant pro-von Willebrand factor confirmed these requirements (data not shown). Prorenin processing studies using cell lines constitutively expressing LPC of furin gave similar results. Extensive endogenous processing activity in the parental CHO cell line (as much as 50% processing with prorenin substrates RVFTKR and RVFTQKR) made it difficult to detect subtle differences (data not shown). In summary, these data establish that LPC is a genuine proprotein convertase with selectivity for multi-basic substrate residues. Furthermore, comparison of substrate processing for LPC and furin shows overlapping but not identical substrate profiles (Fig. 5, compare lanes RVFTQKR, DRVFTQKR, and DRRRRKR).

LPC Is Not Secreted from Cells and Is Associated with Membranes—In a pilot pulse-chase experiment (data not shown), it was tested whether LPC or a protein isoform of it is secreted from cells. In the case of furin, a 83-kDa truncated form lacking the transmembrane domain and cytoplasmic tail was readily detectable in the medium after a 60-min chase, as reported before (37). Similar secretion of LPC or a protein isoform thereof could not be demonstrated, suggesting potentially interesting differences between LPC and furin with respect to their carboxyl-terminal regions. To obtain insight in this matter, structural features in the carboxyl terminus of LPC were evaluated. Computer analysis (Predictprotein predictor algorithm) of the amino acid sequence data predicted a short hydrophobic domain in LPC (region Leu527–Leu543), possibly functioning as a membrane-anchoring domain by which LPC could be associated with membranes. This possibility was tested by analyzing mutant proteins of LPC lacking carboxyl-terminal sequences. Two LPC mutants were generated, one lacking only the cytoplasmic domain (LPCΔcyt) and the other lacking the cytoplasmic as well as the hydrophobic domain (LPCΔtm+cyt). Biosynthesis of both mutants was analyzed in transiently transfected CHO cells using anti-LPC antibody MP1 (Fig. 6). With mutant LPCΔcyt, three polypeptides of approximately 78 kDa (N-glycosylated proLPCΔcyt), 68 kDa (non-glycosylated proLPCΔcyt), and 66 kDa (LPC not yet completely glycosylated) were detected in cell lysates. No LPC proteins could be detected in the medium. With mutant LPCΔtm+cyt, a similar pattern of three polypeptides was detected, all with a slightly higher electrophoretic mobility due to the additional deleted sequences in this mutant. In the medium, a 74-kDa protein was detected, most likely representing a complex N-glycosylated form of processed LPCΔtm+cyt. These results indicate that the hydrophobic domain is critical for intracellular retention of the protein, suggesting that it acts...
supernatants of the control experiments. Sodium carbonate (pH 11.5; 0 °C) and analyzed by Western blotting using either LPC or furin were fractionated as described under "Materials and Methods." The resulting membrane fractions were extracted with sodium carbonate (pH 11.5; 0 °C) and analyzed by Western blotting together with corresponding amounts from supernatants S₁ and S₂. Lanes S₁, 200,000 × g, supernatant after first ultracentrifugation step (14 μg of protein). Lanes P, membranes after sodium carbonate (pH 11.5; 0 °C) extraction (6 μg of protein). Lanes S₂, 200,000 × g, supernatant after sodium carbonate (pH 11.5; 0 °C) extraction (16–19 μg of protein). Note that the S₂ lane in the LPC experiment is twice as broad as the other lanes. The relative positions of the molecular mass markers are indicated.

as a transmembrane-anchoring domain.

In cell fractionation studies using CHO-LPC cells, all of the LPC protein was found in the 200,000 × g, microsome pellet, as in CHO cells expressing furin. To test whether LPC is tightly associated with the membranes, microsomes were incubated with 100 mM Na₂CO₃ (pH 11.5; at 0 °C), which is an efficient method for selectively stripping extrinsic proteins off membranes without affecting the disposition of integral components, including transmembrane and lipid-anchored proteins (29). In control experiments, microsomes of both the parental CHO cell line and the CHO cell line expressing furin were studied. The results of the experiments showed that a portion of LPC was found in the supernatant after treatment with Na₂CO₃ (Fig. 7). In contrast, furin was not detected at all in the supernatants of the control experiments.

**LPC Is Not Phosphorylated but It Is Palmitoylated**—It has been demonstrated that the cytoplasmic tail of furin is phosphorylated and that phosphorylation plays a role in the recycling of furin between Golgi and post-Golgi compartments. In light of this, it was of interest to establish whether LPC was subjected to phosphorylation too. In a [³²P]orthophosphate labeling experiment of furin and LPC, however, only phosphorylation of furin was found (data not shown), suggesting that LPC is not phosphorylated.

The results of the phosphorylation studies prompted experiments to test whether the cytoplasmic tail of LPC was post-translationally modified in another way. Evaluation of the amino acid sequence data revealed that five cysteine residues are present in the cytoplasmic tail of LPC and that two of these are localized within 15 amino acid residues of the hydrophobic domain (see also Fig. 1). Cysteine residues on similar positions in several membrane-anchored proteins have been found to be targets for palmitoylation (38). To test the possibility of palmitoylation, CHO cell lines stably expressing either LPC or furin were cultured in the presence of [³H]palmitate. Using anti-LPC antibodies, a radiolabeled protein of about 92 kDa was detected (Fig. 8A). With anti-furin antibodies, no labeled proteins were detected. The acylated LPC protein migrated at the same molecular mass as mature, complexly N-glycosylated LPC, suggesting that only mature LPC was post-translationally modified with palmitate (Fig. 8B). In the evaluation of palmitoylation of processing-defective LPC mutant LPCS265A, no labeling was detected (Fig. 8C). Similarly, analysis of mutant LPCΔcyt did also not reveal any labeling, suggesting that palmitoylation occurs in the cytoplasmic tail of LPC (Fig. 8C). To substantiate that labeling of LPC with [³H]palmitate had indeed occurred through thioester bonds on cysteine residues, acylated LPC was treated with hydroxylamine. Fig. 8D shows that treatment of immunoprecipitated LPC with hydroxylamine directly after SDS-PAGE completely removed the [³H]palmitate but not the ³⁵S label from LPC. Incubation with a Tria/HCl buffer had no effect. These results strongly suggest that cysteine residues in the cytoplasmic tail of LPC are involved in post-translational palmitoylation of LPC.

**DISCUSSION**

**Biosynthesis, Proteolytic Processing, and Post-translational Glycosylation of LPC**—Our studies presented here establish various features of the biosynthesis of proprotein convertase LPC and reveal similarities as well as differences relative to furin. The results suggest that LPC expression involves the biosynthesis of a nonglycosylated proLPC of about 92 kDa and a 102-kDa glycosylated proLPC in the endoplasmic reticulum. Via autoproteolytic, intramolecular cleavage (like furin and some of the other members of this enzyme family), the 102-kDa proLPC protein is cleaved at its putative propeptide processing site (RRAKR⁻¹) into a 90-kDa LPC protein. This protein traverses through the Golgi complex, where it is complexly N-glycosylated into a 92-kDa mature enzyme that is concentrated in the TGN. The 92-kDa proLPC protein does not seem to become N-glycosylated nor proteolytically processed into mature LPC. Instead, it seems to be slowly degraded as indicated by our endo-H and endo-F experiments. The physiological role of this protein remains to be established. Our biosynthesis data presented here are in good agreement with predicted features. Based on nucleotide sequence data of the LPC cDNA, it can be deduced that human LPC is initially synthesized as a zymogen of 785 amino acids (5). Cleavage of the signal peptide is predicted to occur at Gly⁻¹⁰⁵ (Ref. 39 and TMAP prediction algorithm) and that of the propeptide at RRAKR⁻¹. The difference of 10 kDa between proLPC and processed LPC versus 6 kDa for profurin and processed furin can be explained by the larger propeptide of LPC (104 amino acid residues versus 81 amino acid residues for furin). Evaluation of comparative pulse-chase labeling and N-glycosylation analyses of LPC and furin in CHO cell lines shows that autoprocessing and complex N-glycosylation occur similarly for LPC and furin; however, they occur for LPC at a slower rate than for furin.

Finally, a major difference between LPC and furin pertains to secretion of the proteins. No LPC or fragments thereof could be detected in the media of cells expressing LPC. A mutant form of the LPC protein lacking the putative transmembrane and cytoplasmic domains (LPCΔtm + cyt), however, was found in the medium of cells expressing the mutant. These results are in contrast with those obtained for furin, which is shedded into the medium following an as yet unknown cleavage event, amino-terminal to its transmembrane domain (16, 17, 37). The results with truncated LPC proteins are in agreement with the prediction that LPC contains a membrane-anchoring domain of 17 amino acid residues (Protein prediction algorithm, Leu⁵²⁷–Leu⁵⁴⁵) followed by a tail of 101 amino acid residues (Glu⁵⁴⁴–Cys⁶⁴⁴) in LPC.

**Cleavage Selectivity in Substrate Processing by LPC**—Initial LPC substrate processing results in a VV:T7/PK(15) overexpression system demonstrate that LPC possesses a processing capability with cleavage specificity for sites with basic residues. Substrate processing results for furin were in accordance with established furin substrate sequence requirements as re-
Biosynthesis of LPC

Fig. 8. Palmitoylation of LPC. A, CHO-DHFR<sup>−</sup> cells stably expressing furin (Furin) or LPC (LPC) were labeled with 1 mCi/ml [35S]<sup>−</sup> Tran35Slabel or [3H]palmitic acid ([3H]palmitate) for 3 h. Subsequently, proteins were immunoprecipitated using antibody KP1 and analyzed by SDS-PAGE. Autoradiography was performed with the [35S]- and [3H]-containing gels for 5 h and 4 days, respectively.

B, C, and D, N-glycosylation analysis. CHO-LPC cells were radiolabeled with Tran35Slabel ([35S]) or [3H](9,10)-palmitic acid ([3H]palmitate) for 2 h. LPC was analyzed as described in the legend to Fig. 2 except that dithiothreitol was omitted. C, LPC is post-translationally palmitoylated in its cytoplasmic domain. CHO-DHFR<sup>−</sup> cells were transiently transfected with 2 μg of LPC, LPCS265A, or LPCΔcyt cDNA and labeled and immunoprecipitated the following day as described for panel A of this figure. Autoradiography was performed for 1 and 8 days for the [35S]- and [3H]-labeled LPC proteins, respectively. The relative positions of the molecular mass markers are indicated.

The 35S- and 3H-labeled LPC proteins, respectively. The relative positions of the molecular mass markers are indicated.

Ported for mouse Ren-2 prorenin (35, 36) and human pro-von Willebrand factor (15, 40). Briefly, for a substrate to be fully processed by furin, it requires an Arg at position P-1 and two of three basic residues at positions P-2, P-4, and P-6. This is illustrated by complete processing of prorenin substrates XRRXKR, RXRXXK, and RXXXKR.

Both LPC and furin show an absolute requirement for substrates having an Arg residue at the P-1 position. However, the additional contribution of basic residues at the P-2, P-4, and P-6 positions in LPC is different. Whereas a basic residue at the P-2 position is dispensable for furin, it is found here to be essential for LPC. Furthermore an Arg residue appears to be more important at position P-6 than at position P-4 in LPC (complete processing of substrate RXRRKR versus partial processing of XRRXKR), whereas the P-4 Arg is the second-most important residue (after P-1 Arg) in substrates cleaved by furin (35, 36). Although it should be kept in mind that substrate specificity in overexpression studies might differ from endogenous specificity, it strongly suggests that LPC and furin substrate specificities are similar but not identical.

Unfortunately, no three-dimensional crystal structures are available for the propropeptidases yet. Comparison of the homology models of the catalytic domains of both LPC and furin (2) revealed subtle differences between these domains of the two convertases (34) that might explain the requirement of additional basic substrate residues for LPC to efficiently bind and process substrates. One example is the substitution of Glu<sup>123</sup> in furin to Pro<sup>123</sup> in LPC. In furin, the Glu<sup>123</sup> was predicted to provide additional interaction, with a basic P-3 and/or P-5 residue (2), but in LPC the Pro<sup>123</sup> should provide no selectivity for basic P-3 or P-5 residues. In addition, it might reduce flexibility of the enzyme backbone and should reduce substrate binding in general. The Glu<sup>123</sup> → Pro substitution in LPC most likely renders the enzyme insensitive to repulsion with acidic S3 or S5 substrate residues. Hence LPC might efficiently process substrates having a REKR processing motif, such as is present in gp160. LPC is expressed in LoVo cells (23).

Because furin-deficient LoVo cells process gp160 (41), it is tempting to speculate that LPC is a candidate proprotein convertase for endogenous processing of gp160 in LoVo cells. Results of recent studies (42, 43) seem to confirm this.

Intracellular Localization and Palmitoylation—Our colocalization data suggest that LPC is transported to and concentrated in the TGN. Furthermore, the presence of both γ-adaptin and LPC in association with the same coated buds and vesicles in double-labeling experiments suggest that LPC is also transported out of the TGN in clathrin-coated vesicles. All resident proteins of the Golgi complex described so far are type I and type II transmembrane proteins. Retention in the Golgi complex is conferred by means of signals in the transmembrane, adjacent stalk, and cytoplasmic domains (44, 45). Recent data for kexin (46), TGN38 (47), and furin (19–21, 48) have demonstrated that accumulation in the TGN occurs through structural determinants in their cytoplasmic tails. Two independent signals were identified that target furin to the TGN. Firstly a YXXL motif was found to be important for retrieval of furin from the plasma membrane and has been found in TGN38 and many plasma membrane proteins that are internalized.

After SDS-PAGE, the gels were soaked in 1 M hydroxylamine (+NH₂OH) or 1 M Tris-HCl (−NH₂OH), respectively. 1/8 volume of a sample containing immunoprecipitated, 35S-labeled LPC was electrophoresed compared with 1 volume of 3H-labeled LPC. The gel was exposed for 8 days. The relative positions of the molecular mass markers are indicated.

R. Siezen, personal communication.
Biosynthesis of LPC

through coated pits. Secondly, a stretch of acidic amino acids was shown to be essential for concentration of furin in the Golgi complex. The phosphorylation state of serine residues in this motif modulates intracellular trafficking of furin (49, 50) and of the cation-dependent mannose 6-phosphate receptor (51). Because we could not demonstrate that LPC is phosphorylated, we conclude that it cannot be a determining factor in intracellular trafficking of LPC. Furthermore, the retrieval motif YXXL, which is present in furin and PC6B, is not present in LPC. These data suggest that other motifs in LPC may be involved in mediating sorting to and possibly exit from the plasma membrane. Two dileucine internalization motifs (52) present in the cytoplasmic tail of LPC remain to be established, the fact that this post-translational modification has been found for LPC opens new perspectives.

Finally, we present the first evidence here that LPC is post-translationally palmitoylated at its cytoplasmic tail. Acylation of proteins with either palmitate and/or myristate is a modification that in a growing number of proteins has been shown to be important for their proper localization and/or function (38). Although the cell biological implications of palmitoylation of LPC remain to be established, the fact that this post-translational modification has been found for LPC opens new perspectives.

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