Dynamic palmitoylation of lymphoma proprotein convertase prolongs its half-life, but is not essential for *trans*-Golgi network localization

Jan-Willem H. P. VAN DE LOO[1], Meike TEUCHERT, Ilse PAULI, Evelyn PLETS, Wim J. M. VAN DE VEN and John W. M. CREEMERS[2]

Laboratory for Molecular Oncology, Center for Human Genetics, University of Leuven and Flanders Interuniversity Institute for Biotechnology, Herestraat 49, 3000 Leuven, Belgium

Proprotein convertases are responsible for the endoproteolytic activation of proproteins in the secretory pathway. The most recently discovered member of this family, lymphoma proprotein convertase (LPC), is a type-I transmembrane protein. Previously, we have demonstrated that its cytoplasmic tail is palmitoylated. In this study, we have identified the two most proximal cysteine residues in the cytoplasmic tail as palmitoylation sites. Substitution of either cysteine residue by alanine interfered with palmitoylation of the other. Palmitoylation of LPC was found to be sensitive to the protein palmitoyltransferase inhibitor tunicamycin but not cerulenin. It was also insensitive to the drugs brefeldin A, monensin and cycloheximide, indicating that the modification occurs in a late exocytic or endocytic compartment. Turnover of palmitoylated LPC is significantly faster ($t_{1/2} \approx$ 50 min) than that of the LPC polypeptide backbone ($t_{1/2} \approx 3$ h), suggesting that palmitoylation is reversible. Abrogation of palmitoylation reduced the half-life of the LPC protein, but did not affect steady-state localization of LPC in the *trans*-Golgi network. Finally, LPC could not be detected in detergent-resistant membrane rafts. Taken together, these results suggest that dynamic palmitoylation of LPC is important for stability, but does not function as a dominant trafficking signal.

Key words: fatty acid, post-translational modification, stability.

INTRODUCTION

Specific endoproteolytic activation of many growth and differentiation factors, blood coagulation factors, plasma proteins, neuropeptides, peptide hormones and their receptors, viral proteins and bacterial toxins occurs C-terminally of basic amino acid motifs, by a family of subtilisin-like serine proteases known as proprotein convertases (PCs). So far, seven mammalian PCs have been described: furin, PC1/PC3, PC2, PC4, PACE4 (paired basic amino acid-cleaving enzyme 4), PC5/PC6 and lymphoma PC(LPC)/PC7/PC8 [1–4]. Structurally, PCs share a similar multi-domain organization, including an N-terminal signal peptide preceding the essential pro-, catalytic and middle domains, followed by unique C-terminal domains. PCs in yeast and mammals are synthesized as zymogens. Autoproteolytic cleavage of the propeptide occurs in the endoplasmic reticulum (furin, PC1, LPC and PACE4 [5–12]) or in a late Golgi compartment (PC2 [13]) and initiates enzyme activation and transport. Proteolytic activation of substrates occurs in the *trans*-Golgi network (TGN), in endosomes, at the cell surface, in secretory granules or in combinations thereof [2,3]. Since many substrates of PCs have similar processing sites, insight into the intracellular trafficking of PCs is crucial to understand the selectivity or redundancy of these enzymes *in vivo*. For instance, the sorting into secretory granules of the neuroendocrine-specific enzymes PC1 and PC2 is reflected by their cleavage of specific substrates, as demonstrated in a PC1-deficient patient [10] and in PC2-knockout mice [14].

The latest member of the family, LPC, was first discovered in the chromosome breakpoint region of a high-grade lymphoma carrying a t(11;14)(q23;q32) translocation [15]. LPC is the least-conserved family member and phylogenetic analysis indicates that LPC is more related to kexin in yeast than any other mammalian PC [16,17]. LPC is widely expressed, during both embryogenesis and adult life [17–19a]. Substrate specificity is similar to, but not identical with, other PCs [11,20]. LPC is a type-I transmembrane protein that localizes to the constitutive secretory pathway and is concentrated in the TGN, were it overlaps partially with furin and TGN38 (a TGN protein of 38 kDa) [11,21]. The cytoplasmic tail of LPC does not contain a tyrosine-based internalization motif nor an acidic cluster motif (with or without a pair of phosphorylated serines), which has been shown to be critical for correct localization of furin and PC6B [2,22]. Intracellular trafficking and TGN localization must therefore depend on other, as yet unknown, sequences.

Previously, we have reported that LPC is palmitoylated post-translationally in its cytosolic domain [11]. Palmitoylation involves the post-translational attachment of the C_{16} saturated fatty acid palmitic acid to the thiol group of a specific cysteine residue in soluble and transmembrane-containing proteins via an acyl-thioester bond in the endoplasmic reticulum, Golgi complex or at the plasma membrane [23–26]. In contrast with the chemical stability of the acyl-amide bond in N-myristoylation [27], the chemically labile thioester bond allows regulated cycles of palmitoylation and depalmitoylation that may control a protein's subcellular localization. In the case of the cation-dependent mannose-6-phosphate receptor (CD-MPR), palmitoylation modulates sorting from endosomes to the TGN [28]. Palmitoylation of the transferrin receptor might reduce its endocytosis rate [29,30]. For several viral spike proteins, palmitoylation is necessary for envelopment and egress of virus particles [24]. For a number of G-protein-linked receptors, dynamic palmitoylation modulates their phosphorylation status, internalization rate and thus agonist-mediated desensitization [25,31]. Furthermore, palmitoylation of the α subunits of heterotrimeric G-proteins has been suggested to alter their lateral mobility in the plasma membrane, thereby enabling interaction

Abbreviations used: PC, proprotein convertase; LPC, lymphoma proprotein convertase; TGN, *trans*-Golgi network; CD-MPR, cation-dependent mannose-6-phosphate receptor; CHO, Chinese hamster ovary; NRK, normal rat kidney; DHFR, dihydrofolate reductase; DRM, detergent-resistant membrane raft; PLAP, placental alkaline phosphatase.

[1] Present address: European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy.

[2] To whom correspondence should be addressed (e-mail john.creemers@med.kuleuven.ac.be).

with adenylate cyclase and other proteins in specific signal-transduction subdomains of the plasma membrane (caveolae) [25,31]. More recently, cholesterol transport by caveolin-1 from the endoplasmic reticulum to caveolae was shown to depend on the palmitoylation status of caveolin-1 [32].

Here, we describe the identification of palmitoylation sites in the cytoplasmic tail of LPC, the characterization of the dynamics of this modification and its functional relevance.

EXPERIMENTAL

Plasmids and mutagenesis

Cloning of wild-type LPC and mutant LPC-Δcyt (lacking the cytoplasmic tail; stop codon after Ser^{548}) has been described in [11]. Mutations in the LPC cDNA were generated using the *in vitro* site-directed mutagenesis system (Promega) according to the guidelines of the suppliers. To facilitate the multiple substitution of cysteine residues to alanine, an *Acc*III site was introduced between Cys^{558} and Cys^{563}, making use of codon degeneracy of Ser^{560}. The following constructs were produced: LPC-C1-5 (substitution of all five cytoplasmic Cys residues, $Cys^{558,563,585,628,644}$), LPC-C1-4 ($Cys^{558,563,585,628}$), LPC-C1-3 ($Cys^{558,563,585}$), LPC-C3-5 ($Cys^{585,628,644}$), LPC-C1-2 ($Cys^{558,563}$), LPC-C1 ($Cys^{558}$) and LPC-C2 ($Cys^{563}$). In constructs LPC-$H^{567}$, LPC-$P^{583}$, LPC-$G^{599}$, LPC-$D^{610}$, LPC-$D^{627}$ and LPC-D^{634}, stop codons were introduced immediately following the indicated amino acid. In addition, two constructs, F-LPC and F-LPC-C1-2 were generated with a FLAG epitope (amino acids DYKDDDDK) between their pro- and catalytic domains using overlap-extension PCR [33]. FLAG-tagged LPC was processed and N-glycosylated to the same extent as its non-tagged counterpart in our assays (results not shown). After cleavage of the prodomain, the FLAG epitope resides at the N-terminus of the LPC enzyme. All mutations were confirmed by nucleotide sequence analysis according to the dideoxy chain-termination method. Human anti-(placental alkaline phosphatase) (*PLAP*) cDNA was provided kindly by Deborah Brown (State University of New York, New York, NY, U.S.A.).

Cell lines and DNA transfer

Chinese-hamster ovary-K1 (CHO; ATCC CRL-9618) and CHO-dihydrofolate reductase $(DHFR)^-$ cell lines (ATCC CRL 9096) were maintained as described previously [11]. Normal rat kidney (NRK) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal-calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin (all from Life Technologies). For transfections, typically $(5–8) \times 10^5$ cells were transfected with 2 μg of DNA and 6 μl of LIPOFECTAMINE (Life Technologies) according to the manufacturer's guidelines. CHO-$DHFR^-$ cells stably expressing human wild-type LPC or mutants thereof have been described in [11]. NRK cells stably overexpressing F-LPC and F-LPC-C1-2 were generated as described previously [34].

Antibodies

Rabbit anti-LPC antibodies MP1 and KP1 were used as described before [11]. Guinea-pig anti-TGN38 serum was a gift from Wolfgang Garten (Philipps-Universitat, Marburg, Germany). Rabbit anti-PLAP antibody was provided kindly by Deborah Brown. Mouse FLAG-1 antibody was obtained from Sigma, fluorescein-conjugated rabbit anti-guinea-pig Ig was from Dako and goat anti-mouse Ig Alexa 594 was from Molecular Probes. Pig anti-rabbit Ig and mouse anti-rabbit Ig conjugated to horseradish peroxidase (Dako) were used with the ECL detection system (Amersham Pharmacia Biotech).

Indirect immunofluorescence microscopy

NRK cells stably expressing F-LPC or F-LPC-C1-2 were seeded on coverslips 18–24 h prior to fixation. NRK cells were fixed for 5 min at room temperature in methanol/acetone (1:1, v/v). Cells were incubated with primary antibodies (diluted 1:500 in PBS) for 1 h at room temperature. Bound antibodies were detected with fluorescently labelled secondary antibodies by incubation for 1 h at room temperature. Slides were mounted in Mowiol (Calbiochem) and analysed with a Zeiss Axiophot microscope equipped with UV optics. Images were recorded with a CE200A CCD camera (Photometrics) using Smartcapture (Digital Scientific) and IPlab Spectrum (Signal Analytics) software.

Radiolabelling and immunoprecipitation

Labelling of cells [$(8–10) \times 10^5$ cells/sample] with 100 μCi/ml L-[^{35}S]methionine (specific radioactivity > 1000 Ci/mmol; ICN), immunoprecipitation and SDS/PAGE have been described previously [11]. In the case of overnight labelling, 5% dialysed serum (Life Technologies) was added to the cells. Brefeldin A (Roche Diagnostics; stock 5 mg/ml in DMSO), cycloheximide (Sigma), monensin (Sigma; stock 5 mg/ml in DMSO), tunicamycin (Roche Diagnostics; stock 10 mg/ml in DMSO) and cerulenin (Sigma; stock 2 mg/ml in DMSO) were used throughout the starvation, labelling and chase periods.

For [^{3}H]palmitic acid labelling, cells were starved for 1 h in minimal essential medium-α containing 10 mM Hepes (pH 7.4) and then labelled for 1.5 h in the presence of 1 mCi/ml [9,10-^{3}H] palmitic acid (specific activity 30–60 Ci/mmol; NEN Life Science Products) in minimal essential medium-α containing 1% DMSO and 0.1% BSA. 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 μM pepstatin/1 mM iodoacetamide/100 units/ml Trasylol/1 mM PMSF). Cell lysates were precleared by centrifugation at 4 °C for 30 min at 19200 ***g*** in an Eppendorf centrifuge and then by incubation of the supernatants with 5 μl of rabbit serum bound to 10 mg of Protein A–Sepharose CL-4B (Amersham Pharmacia Biotech) for 60 min. LPC was immunoprecipitated with 5 μl of rabbit polyclonal anti-LPC serum. Samples labelled with [^{3}H]palmitic acid were incubated for 1 min at 95 °C in sample buffer containing 40 mM dithiothreitol. Quantification of gels containing ^{3}H-labelled proteins was done with a Kodak Image Station 440 (Kodak) and a Personal Densitometer (Molecular Dynamics), and ^{35}S-labelled proteins were quantified with a PhosphorImager (Molecular Dynamics).

Preparation of detergent-resistant membrane rafts (DRMs)

DRMs were prepared essentially as described by Brown and Rose [35]. Briefly, 10^6 cells were labelled with [^{35}S]methionine (1 h pulse/1 h chase) and lysed on ice in 1 ml of extraction buffer [25 mM Hepes, pH 7.5/150 mM NaCl/1% Triton X-100/protease-inhibitor cocktail (complete EDTA-free, Roche Diagnostics)]. Insoluble material was collected by scraping and, together with the extracted material, it was centrifuged for 2 min in a microfuge at 16600 ***g***. Supernatant (900 μl) was collected in a clean tube containing 100 μl of lysis buffer (50 mM Tris/HCl, pH 8.8/5 mM EDTA/1% SDS). The pellet was centrifuged again and the remaining supernatant was removed. The pellet was resuspended subsequently in 100 μl of lysis buffer by extensive

vortexing and 900 μl of extraction buffer was added. Both supernatant and pellet fraction were used for immunoprecipitation.

RESULTS

LPC is palmitoylated on Cys^{558} and Cys^{563} in the cytosolic domain

We have described previously that the cytosolic domain of LPC contains hydroxylamine-sensitive palmitoylation, indicating that this modification occurred on cysteine residues [11]. The cytoplasmic tail contains five cysteines, at positions 558, 563, 585, 628 and 644 (referred to as Cys1, Cys2, Cys3, Cys4 and Cys5, respectively). In order to identify the cysteine residues that are palmitoylated, a series of mutants with deletions in the cytosolic domain was generated and studied in a palmitoylation assay using transiently transfected CHO-K1 cells (Figure 1). Only the processed form of LPC was palmitoylated, indicating that palmitoylation occurred after propeptide cleavage. Truncation of the cytosolic tail up to His^{567}, leaving only four residues C-terminally of Cys2, had no effect on palmitoylation. Only when the entire cytoplasmic tail was deleted was the incorporation of palmitic acid blocked. This suggests that Cys1 and/or Cys2 are crucial for palmitoylation, although palmitoylation of additional cysteines could not be excluded. Therefore, a second set of mutants was generated in which cysteine residues in the cytosolic domain of LPC were replaced systematically by alanine residues (Figure 2). In transiently transfected CHO-K1 cells, we observed a dramatic reduction of [^{3}H]palmitate-labelled LPC when either Cys1 or Cys2 was mutated (Figure 2B). Mutation of the other three cysteines, either separately or in combination, did not affect the labelling of LPC with [^{3}H]palmitate. It is of interest to note that despite replacement of all five cysteine residues in the cytosolic domain, residual palmitoylation was visible, suggesting that other residues became acylated, albeit to a low extent. Densitometric analyses of these experiments showed that palmitoylation of mutant LPC-C1-2 was reduced by more than 90 % relative to wild-type LPC. Moreover, comparison of cysteine mutants LPC-C1, LPC-C2 and LPC-C1-2 showed that for efficient labelling both cysteines had to be present and

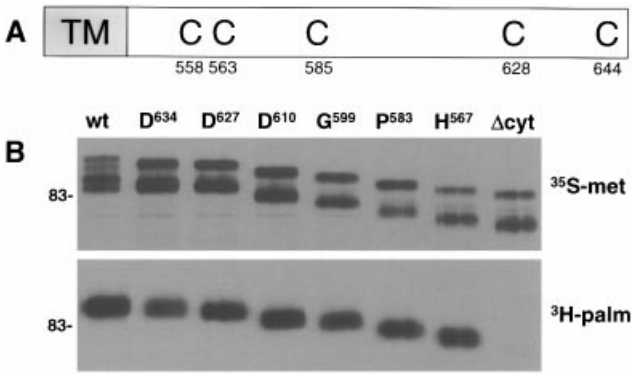

Figure 1 Palmitoylation of LPC is independent of sequences downstream of His^{567}

(**A**) Schematic presentation of the relative positions of the cysteine residues in the cytoplasmic tail of LPC. TM, transmembrane domain. (**B**) Immunoprecipitation (antibody MP1) of lysates of CHO-DHFR$^-$ cells transiently transfected with 2 μg of LPC or various mutant LPC cDNAs with incremental deletions of the cytosolic domain. Cells were radiolabelled for 90 min with [9,10-^{3}H]palmitic acid (^{3}H-palm) or L-[^{35}S]methionine (^{35}S-met). The relative position of the molecular-mass marker is indicated.

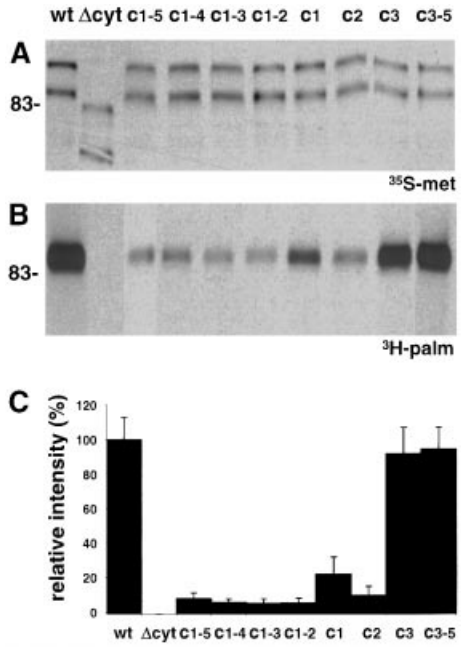

Figure 2 LPC is palmitoylated at cysteine residues Cys^{558} and Cys^{563} in its cytosolic tail

Immunoprecipitation (antibody MP1) of lysates of CHO-DHFR$^-$ cells transiently transfected with 2 μg of LPC (wt), LPC-Δcyt or LPC cDNAs with mutations in one or more cysteine residues. Cells were radiolabelled for 90 min with [9,10-^{3}H]palmitic acid (**A**) or L-[^{35}S]methionine (**B**). The relative positions of the molecular-mass markers are indicated. (**C**) Quantification of palmitoylation. The intensities of palmitoylation were normalized for total protein concentration and compared with wild-type LPC $\times$ 100 %. The results are means $\pm$ S.D. ($n = 4$).

that mutation of Cys2 had a more deleterious effect than mutation of Cys1. Similar results were obtained with CHO cell lines stably expressing these cysteine mutants (results not shown). Together, our data indicate the presence of a motif in the juxtamembraneous region of the cytosolic tail of LPC, inducing the palmitoylation of two cysteine residues, Cys^{558} and Cys^{563}, independently of C-terminal sequences in the cytosolic domain.

Palmitoylation of LPC is sensitive to tunicamycin but not cerulenin and occurs in a late compartment of the exocytic pathway

In order to characterize the LPC palmitoyltransferase, we tested the inhibitory effects of cerulenin and tunicamycin, the only two cell-permeant drugs that are known to inhibit palmitoylation [36]. As can be seen from Figures 3(A) and 3(B), palmitoylation of LPC was inhibited by tunicamycin, but not cerulenin, in a dose-dependent manner with inhibitory concentrations starting at $\approx$ 10 μg/ml. This is four times higher than the concentration reported to block co-translational N-glycosylation of LPC [20] and 10–100 times higher than the concentration that is sufficient to block N-glycosylation in general [36]. Therefore, the lack of inhibition at lower concentrations of tunicamycin demonstrates that this is not an indirect effect caused by inhibition of intracellular transport of non-glycosylated LPC. Analysis of whole-cell lysates (Figure 3C) demonstrated that tunicamycin blocked labelling of all major proteins with [^{3}H]palmitate, whereas cerulenin specifically inhibited palmitoylation of some, but not all, proteins.

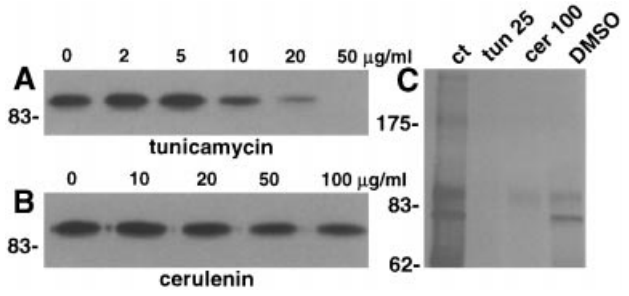

Figure 3 Palmitoylation of LPC is inhibited by tunicamycin but not cerulenin

CHO-DHFR$^-$ cells stably expressing LPC were radiolabelled for 2 h with 1 mCi/ml [9,10-^{3}H] palmitic acid in the presence of increasing doses of tunicamycin (**A**) or cerulenin (**B**), lysed and immunoprecipitated with antibody KP1. Whole-cell lysates (**C**) from control wells, which received no drug (ct), 25 μg/ml tunicamycin (tun 25), 100 μg/ml cerulenin (cer 100) or 5% DMSO (solvent) throughout the labelling procedure, were methanol-precipitated.

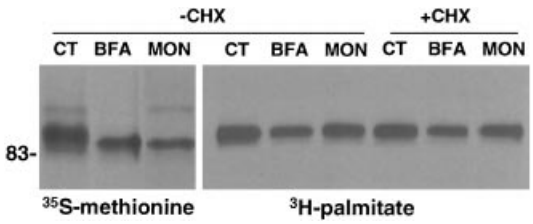

Figure 4 Palmitoylation occurs in a late compartment

CHO-DHFR$^-$ cells stably expressing LPC were radiolabelled for 90 min in the absence (CT) or presence of 5 μg/ml brefeldin A (BFA) or 5 μg/ml monensin (MON), with (+CHX) or without (−CHX) 100 μg/ml cycloheximide. LPC was immunoprecipitated with antibody KP1. The relative position of the molecular-mass marker is indicated.

To obtain insight in the subcellular compartment of the LPC palmitoyltransferase, we used the transport inhibitors brefeldin A and monensin. Both fungal antibiotics effectively block the anterograde transport of secretory proteins through the Golgi apparatus, thereby interfering with complex N-glycosylation of such proteins. Brefeldin A causes a dramatic and rapid retrograde transport of *cis*-Golgi cisternae back to the endoplasmic reticulum via tubular extensions as well as extensive tubulation of the *trans*-Golgi and TGN compartments. However, retrograde microtubule-mediated transport from the plasma membrane is not affected [37]. The ionophore monensin blocks transport from *cis*- and *medial*-cisternae to the *trans*-cisternae of the Golgi [38]. As is shown in Figure 4, both transport inhibitors affected complex N-glycosylation of newly synthesized [^{35}S]methionine-labelled LPC, resulting in a higher electrophoretic mobility of processed LPC in SDS/PAGE. In contrast, it had no effect on palmitoylation or the electrophoretic mobility of [^{3}H]palmitate-labelled LPC, suggesting that palmitoylation of LPC occurs in a compartment beyond the *cis*-Golgi. This was substantiated further by the observation that palmitoylation of LPC was not affected significantly by pretreatment of the cells with the protein-translation inhibitor cycloheximide, indicating that palmitoylation of LPC is independent of protein synthesis.

Palmitoylation of LPC is reversible

Palmitoylation can be reversible, creating a dynamic system to regulate protein–protein or protein–lipid interactions [25,26,31].

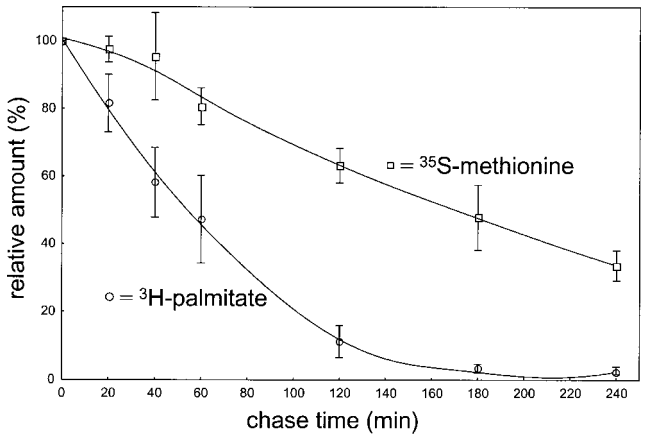

Figure 5 LPC is reversibly palmitoylated

CHO-DHFR$^-$ cells stably expressing LPC were radiolabelled for 1 h with 1 mCi/ml [9,10-^{3}H]-palmitic acid (○) or overnight with 100 μCi/ml L-[^{35}S]methionine (□) and chased for the times indicated. LPC was immunoprecipitated with antibody KP1. The results are means ± S.D. ($n = 5$).

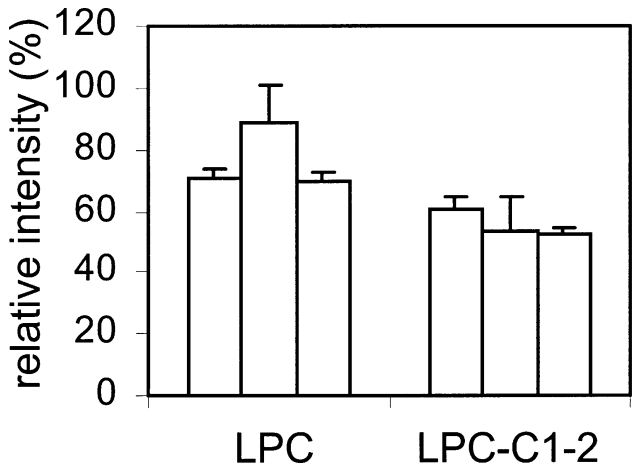

Figure 6 Palmitoylation prolongs the half-life of LPC

Three independent clones of CHO-DHFR$^-$ cells stably expressing wild-type LPC or LPC-C1-2 were labelled overnight with L-[^{35}S]methionine and chased for 75 min. LPC was immunoprecipitated with antibody KP1, separated by SDS/PAGE and the amount of radiolabelled LPC quantified on a PhosphorImager and presented as the ratio of (1 h chase/0 h chase) × 100%. The results are means ± S.D. ($n = 3$). The statistical significance of the difference between LPC and LPC-C1-2 was $P < 0.001$ (two-tailed Student's t test).

In order to investigate the possibility of reversible palmitoylation of LPC, we determined the half-life of palmitoylated LPC. To allow comparison with the LPC peptide backbone, we incubated the cells for 16 h in the presence of [^{35}S]methionine to obtain steady-state labelling. [^{3}H]Palmitate-labelled LPC rapidly decreased by 90% over the first 2 h, with a half-life of ≈ 50 min (Figure 5). Small amounts remained detectable during the last 2 h of the chase, which was probably due to the re-use of [^{3}H]palmitic acid [36,39]. The amount of [^{35}S]methionine-labelled LPC decreased linearly during the chase, with a half-life of ≈ 3 h. These results suggest strongly that palmitoylation of LPC is reversible.

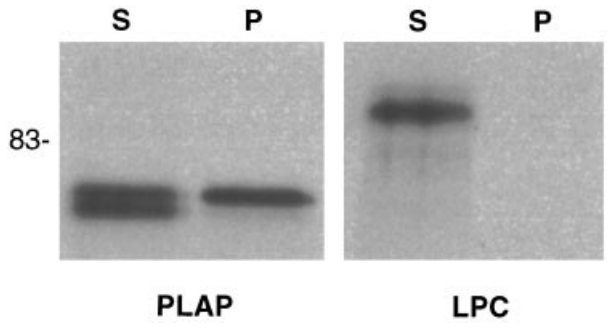

Figure 7 LPC is not localized in DRMs

CHO-K1 cells transiently transfected with *PLAP* cDNA and CHO-DHFR$^-$ cells stably expressing wild-type LPC were labelled with L-[^{35}S]methionine and extracted with Triton X-100 as described in the Experimental section. PLAP and LPC were immunoprecipitated with antibodies anti-PLAP and KP1, respectively. The DRMs were present in the pellet fraction. S, supernatant; P, pellet.

Palmitoylation affects the half-life of LPC, but does not affect TGN localization

Having established the reversible nature of palmitoylation of LPC, we investigated its possible function. As expected, no differences were found in the relative rate of autoprocessing and transport to the TGN between wild-type LPC and non-palmitoylated mutant LPC-C1-2 (results not shown), since palmitoylation occurs in a late-exocytic or endocytic compartment. Subsequently, we compared the stability of LPC and LPC-C1-2 stably expressed in CHO cell lines (Figure 6). After a 75 min chase, an average of only 25% of LPC had been degraded, whereas more than 40% of LPC-C1-2 was degraded. Although small differences between independent clones were observed, the reduced stability was persistently observed in all clones, with clear statistical significance (two-tailed Student's t test, $P < 0.001$).

Since palmitoylation might affect intracellular trafficking, we studied the localization of LPC and LPC-C1-2 in stably transfected CHO and NRK cells (results not shown). Both LPC and LPC-C1-2 were concentrated in a juxtanuclear region, largely co-localizing with TGN38, a marker protein for the TGN. We therefore conclude that palmitoylation of LPC is not a dominant TGN sorting signal.

LPC is not localized in DRMs

At least half of the proteins in DRMs are palmitoylated, including some transmembrane proteins [40]. Triton X-100-resistant membrane fractions were prepared, using the glycosylphosphatidylinositol-anchored protein PLAP as a marker. PLAP becomes resistant to Triton X-100 extraction in the Golgi apparatus during transport to the cell surface [35]. This coincides with a lower electrophoretic mobility as a result of complex N-glycosylation. As is shown in Figure 7, the lower-molecular-mass PLAP protein (localized in the endoplasmic reticulum) was extracted completely, whereas most of the higher-molecular-mass form remained insoluble. LPC, on the other hand, was only found in the soluble fraction, indicating that it is not present in DRMs.

DISCUSSION

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 [25–27]. Palmitoylation of LPC is unique within the PC family: furin has been shown not to be palmitoylated [11], PC6B does not contain cysteine residues in its cytosolic domain, and the other members do not have cytosolic domains.

Analysis of the five cysteine residues in the cytosolic domain of LPC has demonstrated that the cysteine residues adjacent to the transmembrane domain, Cys^{558} and Cys^{563}, are the two major, if not only, palmitoylation sites (Figures 1 and 2). However, some palmitoylation occurred even in mutants lacking all cytoplasmic cysteines. This residual palmitoylation was more extensive when Cys residues were mutated into Ser residues (results not shown). Similarly, hydroxylamine-insensitive labelling with [^{3}H]palmitate was found after mutagenesis of Cys into Ser residues in the transferrin receptor [29], suggesting that serine residues can be acylated. Since palmitoylation of wild-type LPC was entirely hydroxylamine-sensitive [11], this indicates that the residual palmitoylation on residues other than cysteines was also caused by the Cys-to-Ala mutations. Although alanines themselves cannot be palmitoylated, it is possible that flanking serines are utilized. Three serine residues are in close proximity to Cys^{558} and Cys^{563}, one of which is located in between the two cysteine residues. These data suggest that the juxtamembraneous region of the cytoplasmic tail contains a palmitoylation motif and that, in the absence of the cysteine residues, other residues are palmitoylated. It was also noticed that mutation of either Cys^{558} or Cys^{563} almost completely abolished palmitoylation, suggesting that acylation of both cysteines is synergistic. This contrasts with the CD-MPR, where the loss of palmitoylation of one cysteine was compensated by increased palmitoylation of the other [28].

Protein palmitoyltransferase activity has been demonstrated in endoplasmic reticulum, Golgi/TGN and plasma-membrane fractions [41,41a,42], but the corresponding genes have not been cloned yet [43]. As a first step towards characterizing the LPC palmitoyltransferase activity, we determined its inhibitor profile and investigated the subcellular site of palmitoylation. The LPC palmitoyltransferase activity was selectively inhibited by tunicamycin, but not cerulenin (Figure 3). Since cerulenin inhibits palmitoylation of most proteins, this observation will be instrumental in the identification of the physiological LPC palmitoyltransferase. We also conclude that LPC is most probably palmitoylated in a late compartment of the exocytic pathway, possibly the TGN, based on the following results: (i) palmitoylation was insensitive to the translation inhibitor cycloheximide (Figure 3); (ii) palmitoylation of an endoplasmic-reticulum-localized mutant LPC-S^{265}A was not detected [11]; (iii) palmitoylation and N-linked glycosylation of palmitoylated LPC were insensitive to the effects of the transport inhibitors brefeldin A and monensin (Figure 3); and (iv) analysis of N-linked glycosylation of palmitoylated LPC revealed a complexly N-glycosylated protein [11] that was detectable with a [^{3}H]palmitate pulse as short as 15 min (results not shown).

Early studies on protein acylation have concentrated on cellular and viral integral membrane proteins that are part of the plasma membrane [24]. However, the discovery that the activity of cytosolic proteins in signalling pathways is dynamically regulated by palmitoylation has led to an increased number of studies that focus on its reversible character [26,31]. We have measured the half-life of the palmitate moiety in LPC and compared it with the half-life of the protein backbone. [^{3}H]Palmitic acid turned over with a shorter half-life (50 min) than that of the ^{35}S-labelled protein (3 h; Figure 5). Comparison of the half-lives of the palmitate moiety in other proteins with the half-lives of their respective polypeptide backbones indicates that there is large variation: by a factor of two for the transferrin receptor ($t_{1/2} \approx 6$ h [44]), a factor of 20 for the CD-MPR ($t_{1/2} \approx$

2 h [28]) and by a factor of 75 or more for $p21^{N-ras}$ ($t_{1/2} \approx 20$ min [45]) and ankyrin ($t_{1/2} \approx 50$ min [39]). Two protein palmitoyl thioesterases that deacylate proteins have been identified, palmitoyl-protein thioesterase 1 (PPT1) and acyl-protein thioesterase 1 (APT1) [43]. PPT1 is concentrated in lysosomes, but it has been shown recently in overexpression experiments to enhance deacylation of a number of plasma-membrane proteins [46]. APT1 has been shown to play a role in deacylation of plasma-membrane-associated Ha-Ras and $G_s\alpha$ subunit and the plasmalemmal caveolae-localized endothelial nitric oxide synthase [47,48]. It is not known if either of these protein palmitoyl thioesterases is involved in the deacylation of LPC.

Mutation of Cys^{558} and Cys^{563} resulted in a decreased half-life, suggesting an important role in the intracellular trafficking and/or targeting. However, steady-state concentration in the TGN was not affected (results not shown), indicating that it is not a dominant sorting signal. Concentration of LPC in the TGN might be achieved by a combination of retention in the TGN and retrieval from the plasma membrane after endocytosis. Surface appearance of LPC has been demonstrated recently by antibody-uptake experiments [49]. Despite the presence of small amounts of LPC at the cell surface under steady-state conditions, abnormal cycling between the TGN and the plasma membrane might have a pronounced effect on the half-life of LPC. For instance, it has been shown that palmitoylation of the CD-MPR prevents the receptor from entering the lysosomes during its trafficking between the plasma membrane, endosomes and the TGN [28].

A large number of proteins in DRMs are palmitoylated, including some transmembrane proteins [40]. Membrane rafts are enriched in sphingolipids and cholesterol and have several important functions, including protein and lipid sorting in the exocytic and endocytic pathways [50,51]. How palmitoylation can target a transmembrane protein to DRMs is not entirely clear, but it might favour the packing of this domain in the liquid-ordered (l_o) phase in the lipid bilayer of rafts. Our finding that LPC is not present in DRMs is in accordance with results obtained for two other palmitoylated transmembrane-containing proteins, the transferrin receptor and the CD-MPR [40]. Alternatively, it is possible that the palmitate moiety functions in conjunction with a basic region flanking the two cysteines (six out of 10 amino acids C-terminal of Cys^{563} are basic). Insertion of the palmitoylated Cys residues into the lipid bilayer will bring these positively charged residues in close proximity to the membrane where they can interact with negatively charged phospholipid head groups. Dynamic palmitoylation might therefore act as a switch to regulate targeting to specific microdomains in a membrane.

This work was supported in part by the Geconcerteerde onderzoeksacties 1997–2001. M.T. is holder of an EMBO Long Term postdoctoral fellowship. J.W.M.C. is a postdoctoral fellow of the Fonds voor Wetenschappelijk Onderzoek, Vlaanderen. We thank Deborah Brown for providing us with PLAP reagents and Wolfgang Garten for the anti-TGN38 antibody.

REFERENCES

1 Nakayama, K. (1997) Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. Biochem. J. **327**, 625–635
2 Molloy, S. S., Anderson, E. D., Jean, F. and Thomas, G. (1999) Bi-cycling the furin pathway: from TGN localization to pathogen activation and embryogenesis. Trends Cell Biol. **9**, 28–35
3 Seidah, N. G. and Chretien, M. (1999) Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Res. **848**, 45–62
4 Steiner, D. F. (1998) The proprotein convertases. Curr. Opin. Chem. Biol. **2**, 31–39
5 Leduc, R., Molloy, S. S., Thorne, B. A. and Thomas, G. (1992) Activation of human furin precursor processing endoprotease occurs by an intramolecular autoproteolytic cleavage. J. Biol. Chem. **267**, 14304–14308
6 Vey, M., Schafer, W., Berghofer, S., Klenk, H. D. and Garten, W. (1994) Maturation of the trans-Golgi network protease furin: compartmentalization of propeptide removal, substrate cleavage, and COOH-terminal truncation. J. Cell Biol. **127**, 1829–1842
7 Creemers, J. W., Vey, M., Schafer, W., Ayoubi, T. A., Roebroek, A. J., Klenk, H. D., Garten, W. and Van de Ven, W. J. (1995) Endoproteolytic cleavage of its propeptide is a prerequisite for efficient transport of furin out of the endoplasmic reticulum. J. Biol. Chem. **270**, 2695–2702
8 Zhou, Y. and Lindberg, I. (1994) Enzymatic properties of carboxyl-terminally truncated prohormone convertase 1 (PC1/SPC3) and evidence for autocatalytic conversion. J. Biol. Chem. **269**, 18408–18413
9 Jutras, I., Seidah, N. G., Reudelhuber, T. L. and Brechler, V. (1997) Two activation states of the prohormone convertase PC1 in the secretory pathway. J. Biol. Chem. **272**, 15184–15188
10 Jackson, R. S., Creemers, J. W., Ohagi, S., Raffin-Sanson, M. L., Sanders, L., Montague, C. T., Hutton, J. C. and O'Rahilly, S. (1997) Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. Nat. Genet. **16**, 303–306
11 van de Loo, J. W., Creemers, J. W., Bright, N. A., Young, B. D., Roebroek, A. J. and Van de Ven, W. J. (1997) Biosynthesis, distinct post-translational modifications, and functional characterization of lymphoma proprotein convertase. J. Biol. Chem. **272**, 27116–27123
12 Nagahama, M., Taniguchi, T., Hashimoto, E., Imamaki, A., Mori, K., Tsuji, A. and Matsuda, Y. (1998) Biosynthetic processing and quaternary interactions of proprotein convertase SPC4 (PACE4). FEBS Lett. **434**, 155–159
13 Muller, L. and Lindberg, I. (1999) The cell biology of the prohormone convertases PC1 and PC2. Prog. Nucleic Acid Res. Mol. Biol. **63**, 69–108
14 Furuta, M., Yano, H., Zhou, A., Rouille, Y., Holst, J. J., Carroll, R., Ravazzola, M., Orci, L., Furuta, H. and Steiner, D. F. (1997) Defective prohormone processing and altered pancreatic islet morphology in mice lacking active SPC2. Proc. Natl. Acad. Sci. U.S.A. **94**, 6646–6651
15 Meerabux, J., Yaspo, M. L., Roebroek, A. J., Van de Ven, W. J., Lister, T. A. and Young, B. D. (1996) A new member of the proprotein convertase gene family (LPC) is located at a chromosome translocation breakpoint in lymphomas. Cancer Res. **56**, 448–451
16 Seidah, N. G., Hamelin, J., Mamarbachi, M., Dong, W., Tardos, H., Mbikay, M., Chretien, M. and Day, R. (1996) cDNA structure, tissue distribution, and chromosomal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. Proc. Natl. Acad. Sci. U.S.A. **93**, 3388–3393
17 Siezen, R. J. and Leunissen, J. A. (1997) Subtilases: the superfamily of subtilisin-like serine proteases. Protein Sci. **6**, 501–523
18 Constam, D. B., Calfon, M. and Robertson, E. J. (1996) SPC4, SPC6, and the novel protease SPC7 are coexpressed with bone morphogenetic proteins at distinct sites during embryogenesis. J. Cell Biol. **134**, 181–191
19 Bruzzaniti, A., Goodge, K., Jay, P., Taviaux, S. A., Lam, M. H., Berta, P., Martin, T. J., Moseley, J. M. and Gillespie, M. T. (1996) PC8, a new member of the convertase family. Biochem. J. **314**, 727–731
19a Erratum (1996) Biochem. J. **316**, 1007
20 Munzer, J. S., Basak, A., Zhong, M., Mamarbachi, A., Hamelin, J., Savaria, D., Lazure, C., Benjannet, S., Chretien, M. and Seidah, N. G. (1997) *In vitro* characterization of the novel proprotein convertase PC7. J. Biol. Chem. **272**, 19672–19681
21 Wouters, S., Leruth, M., Decroly, E., Vandenbranden, M., Creemers, J. W., van de Loo, J. W., Ruysschaert, J. M. and Courtoy, P. J. (1998) Furin and proprotein convertase 7 (PC7)/lymphoma PC endogenously expressed in rat liver can be resolved into distinct post-Golgi compartments. Biochem. J. **336**, 311–316
22 Xiang, Y., Molloy, S. S., Thomas, L. and Thomas, G. (2000) The PC6B cytoplasmic domain contains two acidic clusters that direct sorting to distinct trans-Golgi network/endosomal compartments. Mol. Biol. Cell **11**, 1257–1273
23 Olson, E. N. and Spizz, G. (1986) Fatty acylation of cellular proteins. Temporal and subcellular differences between palmitate and myristate acylation. J. Biol. Chem. **261**, 2458–2466
24 Schlesinger, M. J., Veit, M. and Schmidt, M. F. G. (1993) Palmitoylation of cellular and viral proteins, in Lipid Modifications of Proteins (Schlesinger, M. J., ed.), pp. 1–134, CRC Press, Boca Raton
25 Milligan, G., Parenti, M. and Magee, A. I. (1995) The dynamic role of palmitoylation in signal transduction. Trends Biochem. Sci. **20**, 181–187
26 Dunphy, J. T. and Linder, M. E. (1998) Signalling functions of protein palmitoylation. Biochim. Biophys. Acta **1436**, 245–261
27 Boutin, J. A. (1997) Myristoylation. Cell Signal. **9**, 15–35

28 Schweizer, A., Kornfeld, S. and Rohrer, J. (1996) Cysteine34 of the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is reversibly palmitoylated and required for normal trafficking and lysosomal enzyme sorting. J. Cell Biol. **132**, 577–584

29 Alvarez, E., Girones, N. and Davis, R. J. (1990) Inhibition of the receptor-mediated endocytosis of diferric transferrin is associated with the covalent modification of the transferrin receptor with palmitic acid. J. Biol. Chem. **265**, 16644–16655

30 Jing, S. Q. and Trowbridge, I. S. (1990) Nonacylated human transferrin receptors are rapidly internalized and mediate iron uptake. J. Biol. Chem. **265**, 11555–11559

31 Mumby, S. M. (1997) Reversible palmitoylation of signaling proteins. Curr. Opin. Cell Biol. **9**, 148–154

32 Uittenbogaard, A. and Smart, E. J. (2000) Palmitoylation of caveolin-1 is required for cholesterol binding, chaperone complex formation, and rapid transport of cholesterol to caveolae. J. Biol. Chem. **275**, 25595–25599

33 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**, 51–59

34 Teuchert, M., Berghofer, S., Klenk, H. D. and Garten, W. (1999) Recycling of furin from the plasma membrane. Functional importance of the cytoplasmic tail sorting signals and interaction with the AP-2 adaptor medium chain subunit. J. Biol. Chem. **274**, 36781–36789

35 Brown, D. A. and Rose, J. K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell **68**, 533–544

36 Patterson, S. I. and Skene, J. H. (1995) Inhibition of dynamic protein palmitoylation in intact cells with tunicamycin. Methods Enzymol. **250**, 284–300

37 Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H. P., Yuan, L. C. and Klausner, R. D. (1990) Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. Cell **60**, 821–836

38 Mollenhauer, H. H., Morre, D. J. and Rowe, L. D. (1990) Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. Biochim. Biophys. Acta **1031**, 225–246

39 Staufenbiel, M. (1987) Ankyrin-bound fatty acid turns over rapidly at the erythrocyte plasma membrane. Mol. Cell Biol. **7**, 2981–2984

40 Melkonian, K. A., Ostermeyer, A. G., Chen, J. Z., Roth, M. G. and Brown, D. A. (1999) Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated. J. Biol. Chem. **274**, 3910–3917

41 Liu, L., Dudler, T. and Gelb, M. H. (1996) Purification of a protein palmitoyltransferase that acts on H-Ras protein and on a C-terminal N-Ras peptide. J. Biol. Chem. **271**, 23269–23276

41a Erratum (1999) J. Biol. Chem **274**, 3252

42 Das, A. K., Dasgupta, B., Bhattacharya, R. and Basu, J. (1997) Purification and biochemical characterization of a protein-palmitoyl acyltransferase from human erythrocytes. J. Biol. Chem. **272**, 11021–11025

43 Resh, M. D. (1999) Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim. Biophys. Acta **1451**, 1–16

44 Omary, M. B. and Trowbridge, I. S. (1981) Covalent binding of fatty acid to the transferrin receptor in cultured human cells. J. Biol. Chem. **256**, 4715–4718

45 Magee, A. I., Gutierrez, L., McKay, I. A., Marshall, C. J. and Hall, A. (1987) Dynamic fatty acylation of p21N-ras. EMBO J. **6**, 3353–3357

46 Cho, S., Dawson, P. E. and Dawson, G. (2000) *In vitro* depalmitoylation of neurospecific peptides: implication for infantile neuronal ceroid lipofuscinosis. J. Neurosci. Res. **59**, 32–38

47 Duncan, J. A. and Gilman, A. G. (1998) A cytoplasmic acyl-protein thioesterase that removes palmitate from G protein alpha subunits and p21(RAS). J. Biol. Chem. **273**, 15830–15837

48 Yeh, D. C., Duncan, J. A., Yamashita, S. and Michel, T. (1999) Depalmitoylation of endothelial nitric-oxide synthase by acyl-protein thioesterase 1 is potentiated by $Ca^{(2+)}$-calmodulin. J. Biol. Chem. **274**, 33148–33154

49 Jean, F., Thomas, L., Molloy, S. S., Liu, G., Jarvis, M. A., Nelson, J. A. and Thomas, G. (2000) A protein-based therapeutic for human cytomegalovirus infection. Proc. Natl. Acad. Sci. U.S.A. **97**, 2864–2869

50 Brown, D. A. and London, E. (1998) Functions of lipid rafts in biological membranes. Annu. Rev. Cell Dev. Biol. **14**, 111–136

51 Harder, T. and Simons, K. (1997) Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. Curr. Opin. Cell Biol. **9**, 534–542

Received 27 July 2000/8 September 2000; accepted 5 October 2000