Small intestinal lactase-phlorizin hydrolase (LPH) is synthesized as a large precursor (prepro-LPH) of 1926 amino acids. In the endoplasmic reticulum, prepro-LPH is split by signal protease. The resulting pro-LPH is cut to mature LPH directly (human) or via a 180-kDa intermediate (rabbit), most likely in the trans-Golgi network or in a later compartment. Antibodies directed against different regions of rabbit pro-LPH locate the cleavage site resulting in the 180-kDa intermediate between amino acid residues 79 and 286. This stretch contains a typical signal sequence which is split at position 19–20 (1, 2); it ends with a membrane-spanning domain (1, 3) and a cytosolic C-terminal sequence (4). Between the signal sequence and the ends with a membrane-spanning domain (1, 3) and a cytosolic C-terminal sequence (4). Between the signal sequence and the membrane anchor, four homologous regions (I to IV) can be recognized (1). Only region III (lactase), region IV (phlorizin hydrolase) (3), the membrane-spanning segment, and the cytosolic C terminus make the “mature” LPH which can be isolated from the brush-border membrane. Processing of pro-LPH to LPH takes place intracellularly (5–7) (although pancreatic proteases also may contribute to it (8, 9)), in one step in human (5, 7, 10) or two steps in the rabbit (11, 12). In the latter species, an intermediate is formed (apparent molecular mass 180 kDa), which is split to yield mature LPH of 135 kDa, beginning at position 867 of prepro-LPH (1). In human, pro-LPH gives rise in one step to mature LPH, the final secondary N terminus being at position 868 (13, 2). In the rabbit and human, the amino acid sequence immediately upstream of the N terminus of mature LPH is SKTR and SKVR, respectively (1). We will not deal here with the processing at this position.

In the present paper we have investigated the first proteolytic processing step of rabbit pro-LPH, i.e. the conversion of pro-LPH to the intermediate 180-kDa form. The converting protease(s) responsible for this step have been identified as furin, PC1/3, and/or PC6A. The presence of their transcripts in the rabbit small intestine was confirmed by a polymerase chain reaction approach and by Northern analysis. By in situ hybridization, we show that all three proprotein convertases are expressed in enterocytes, the very cells in which LPH is expressed and processed.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of the highest possible purity and were purchased from Fluka (Buchs, Switzerland) unless otherwise indicated.

pSCTmLPH (human prepro-LPH (14)) and pR8-IR (rabbit prepro-LPH (15)) were prepared in our laboratory. The cDNAs coding for different proprotein convertases (PCs) were kindly provided by the following people: pSCTmfurin (mouse furin (16) (subcloned into the vector pSCT Gal-X-556 (17))), pCMVPC6A (mouse PC6A (18)), and pCMVPC6B (mouse PC6B (19)) were provided by Dr. K. Nakayama, University of Tsukuba, Japan. pCD-RPC3 (rat PC1/3 (20)) and pCD-RPC2 (rat PC2 (20)) were from Dr. N. P. Birch, University of Auckland, New Zealand. pRcCMV-PC1/3 (mouse PC1/3 (21)) and pRcCMV-PC2 (mouse PC2 (22)) were obtained from Dr. N. G. Seidah, Clinical Research Institute of Montreal, Canada.

Organ Culture and Immunoisolations—Explants from the small intestine of New Zealand White rabbits were cultured as described previously (11). The different LPH forms were immunoprecipitated as described by Lottaz et al. (23), using region-specific polyclonal guinea pig anti-rabbit LPH sera directed against amino acid residues 21–78 (region Ia), 287–373 (region Ib), 670–750 (region II) (24) and against mature LPH (3). The products were analyzed by SDS-PAGE in 5% polyacrylamide gels (25).

Transient Transfection of COS 7 Cells—COS 7 cells (cultured in DMEM/10% FCS; Life Technologies, Inc.) were transiently transfected as described by Chen and Okayama (26). One day before the transfection, the cells were seeded into 6-well plates, so that they were approx-

2 P. Keller, unpublished results.

3 E. Sterchi, personal communication.
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imately 80% confluent the next day. During the last 30 min prior to transfection, the cells were incubated in fresh 1.5-mL DMEM/10% FCS at 37 °C and 3.5% CO2. The calcium phosphate-DNA mixture was prepared by mixing 5 μg of plasmid DNA with 100 μl of 250 mM CaCl2 and 100 μl of 2 × BBS (50 mM BES, 280 mM NaCl, 1.5 mM Na2HPO4, with NaOH adjusted to exactly pH 6.95) and incubating for 15 min at room temperature. 150 μl of this mixture were then slowly added to the cells, and incubation was continued for 24 h at 37 °C and 3.5% CO2. On the following day, the cells were washed twice with PBS, refed with 2 ml of fresh medium, and incubated for 24 h at 37 °C and 5% CO2.

For immunofluorescense (see below), the cells were collected 48 h after transfection by detachment with a rubber policeman. Cells were solubilized in 5 × Laemmli sample buffer, boiled for 2 min, and then used for SDS-PAGE analysis.

For metabolic labeling studies, the cells were cultured for another 24 h in fresh medium. 72 h after transfection, the cells were washed with MEM, and then incubated in 2 ml of labeling medium (MEM supplemented with 1% dialyzed FCS) for 60 min at 37 °C and 5% CO2. After depletion of methionine, the cells were pulse-labeled in 1 ml of labeling medium, containing 25 μCi of [35S]methionine (Amersham) for 1 h. The cells were washed twice with PBS and were then either directly collected or chased in DMEM/10% FCS supplemented with 10 mM cold methionine. (In some experiments, BFA was added to all media at a concentration of 5 μg/ml.) The labeled cells were collected and sublimed in 400 μl of lysis buffer (25 mM Tris, pH 8.0, 50 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, 0.01 volume of 100 mM phenylmethylsulfonfyl fluoride, 0.01 volume of inhibitor mixture (0.25 mM pepstatin, 0.06 mg/ml aprotinin, 1.1 mg/ml leupeptin, 4.7 mg/ml benzamidine, 0.24 mg/ml bestatin, 0.3 mg/ml E-64, and 38.3 mg/ml o-phenanthroline)). Immunoprecipitations, using polyclonal guinea pig-anti rabbit LPH (see above) or monoclonal mouse-anti human LPH (27) antibodies, were performed as described by Lottaz et al. (23).

Immunofluorescense—Protein from polyacrylamide gels was transferred onto polyvinylidene difluoride membranes (Immobilon P, Millipore) using semidyry blotting in a Sartoblot II-S apparatus (Sartorius) with the buffer system of Kyhs-Andersen (28). After incubation in TTW5 (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.04% NaN3 and 0.05% Tween 20) for 20 min at room temperature, the membrane was incubated with the first antibody (serum dilution 2000:1 in TTW5) for 60 min, then was washed four times for 5 min with TTW5, incubated with the secondary antibody (goat anti-guinea pig IgG- alkaline phosphatase-conjugated, Inotech/KPL; 1000: diluted in TTW5) for 60 min, and then washed again four times for 5 min with TTW5. The blot was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Inotech/KPL; 1000: diluted in TTW5) for 60 min, and then washed for 24 h at room temperature. The slides were washed twice with 2× SSC, once with 0.5× SSC, twice with 0.1× SSC (all at 45 °C) and then incubated with 20 μg/ml RNase A (Sigma) for 12 min at 37 °C. The slides were incubated with streptavidin-peroxidase for 12 min at 42 °C. The peroxidase substrate (Ventana Medical Systems), aminoethylcarbazone, and H2O2 were applied for 20 min at 42 °C. The slides were removed from the machine and slightly counterstained with Mayer’s hemalum solution (Merck) prior to mounting with aquarmount solution (BDH).

RESULTS

Protein from pro-LPH in Rabbit Small Intestinal Cultures—Fig. 1A shows that in the rabbit, as reported by others (see e.g. Refs. 11 and 12), pro-LPH is processed to mature LPH via an intermediate form of 180 kDa. We have raised polyclonal antibodies against different, only once occurring sequences of rabbit pro-LPH (see Fig. 1C). Antibodies against a peptide corresponding to the 59 N-terminal amino acid residues of pro-LPH (segment Ia, amino acid residues 21–78), which did precipitate pro-LPH, failed to precipitate the 180-kDa intermediate form and mature LPH (Fig. 1B, lane Ia); the intermediate form was instead precipitated by antibodies raised against a larger segment of region I (i.e. segment Ib, amino acid residues 287–373; Fig. 1B, lane Ib) and a second segment of region II (amino acid residues 670–750; Fig. 1B, lane II). Antibodies directed against mature LPH precipitated all LPH forms (Fig. 1B, lane m). Hence, the site of the first split in rabbit pro-LPH must be located between positions 78 and 287 of pro-LPH.

The 220- to 180-kDa Conversion in COS 7 Cells Expressing PC-3—The conversion of pro-LPH to mature LPH in rabbit small intestinal mucosa in vivo has been studied using primary cultures of intestinal biopsies of adult New Zealand White rabbits. In such cultures, only 10% of the pro-LPH is released, mainly as the 180-kDa intermediate form (Clausen et al. (29)). To further characterize this conversion, COS 7 cells were stably transfected with the cDNA encoding rabbit pro-LPH (Fig. 1D). The conversion was analyzed at 15, 30, and 60 min after addition of the marker, and the results are shown in Fig. 2. The most prominent bands at 60 min after addition of the marker correspond to the 180-kDa intermediate form, the 120-kDa mature form, and a smaller band of about 90 kDa. The latter band is probably a degradation product of the 180-kDa intermediate form. The results indicate that pro-LPH is efficiently processed in COS 7 cells, and that the conversion is complete within 1 h after addition of the marker.

Most prohormones and neuroendocrine precursor proteins of the regulated pathway are processed at K–R– or R– sequences (— indicating the cleavage site), while the precursors of some growth factors and a few plasma membrane proteins (that are delivered via the constitutive pathway) are processed at more complex multibasic cleavage sites of the general type RX(K/R)R—. However, cleavage after RXRR— has been reported in a number of cases (see e.g. Refs. 37 and 38).

As to pro-LPH, in the stretch between positions 78 and 287 (i.e. between the sequences of the peptides used to raise anti-
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**Fig. 1. SDS-PAGE analysis of LPH synthesized by explants of rabbit small intestine.** A, pieces from the proximal small intestine of a 6-month-old male rabbit were metabolically labeled for 1 h and then chased for 1 to 20 h as indicated. The different LPH forms were immunosolubilized using an antibody directed against mature LPH (3). B, pieces of medial small intestine from a 3.5-year-old male rabbit were continuously labeled for 6 h. The different LPH forms were then isolated using antibodies directed against segments Ia, Ib, and II and against mature LPH (m). C, schematic drawing of rabbit pro-pre-LPH. Putative cleavage sites and peptides used to raise antibodies are indicated below and above the figure, respectively.

Bodies in the experiment above, see Fig. 1C, rabbit pro-LPH contains two sequences of the RXRR- type, which are potential cleavage sites for furin or other PCs: -Arg-Ala-Ser-Arg \(^{191}\) and -Arg-Cys-Tyr-Arg \(^{143}\). Neither site is present in the human enzyme, which is split to final LPH without going through this intermediate. Human pro-LPH, however, does have two dibasic sites between regions Ia and Ib that could in principle serve as a cleavage signal -Arg-Arg115. Human pro-LPH, however, does have two dibasic sites between regions Ia and Ib that could in principle serve as a cleavage signal -Arg-Arg115.

**COS cells transiently transfected with human prepro-LPH cDNA produce, but proteolytically process pro-LPH to mature LPH only to a very low extent (23, 39).** This cell line, therefore, is suitable to study the effect of PCs on the processing of pro-LPH. We thus coexpressed in COS 7 cells rabbit or human prepro-LPH along with individual PCs (furin, PC1/3, PC2, PC6A, PC6B; see Table I) of rat and mouse origin.

The results of the coexpression experiments (Fig. 2 and Table I) clearly show that coexpression of rabbit pro-LPH with furin and, to a lesser extent, PC1/3 or PC6A leads to the appearance of a band of approximately 180 kDa. Furin converts more than 55% of 220-kDa pro-LPH into a band with a size identical with the 180-kDa intermediate found in organ culture, whereas PC1/3 and PC6A convert about 38% and 18% of pro-LPH, respectively. All other proteases tested do not cleave the 220-kDa protein in significant amounts. Note that the processing of pro-LPH in general is slow, since in organ cultures even after a 20-h chase there is still some pro-LPH left (cf. Fig. 1A).

Interestingly, human pro-LPH (which is directly converted into mature LPH) is not cleaved by any of the PCs tested, providing a “negative control” that furin or PC1/3 or PC6A might indeed be the protease(s) generating the 180-kDa intermediate in rabbit LPH. When human pro-pre-LPH is cotransfected with PCs, a band of approximately 150 kDa is sometimes detectable (Fig. 2). This band, however, is also visible in cells transfected with human prepro-LPH alone, indicating that human pro-LPH might be processed to some extent (or degraded) by an intrinsic protease of the COS 7 cells.

Since processing of pro-LPH is known to take place in the trans-Golgi network (TGN) or in a later compartment (23, 40, 41), we wondered whether this was also true in COS 7 cells overexpressing furin. While the intracellular location of endogenous furin has not been reported (due to its very low level of expression), recombinant furin, expressed after transfection, has been localized to the Golgi complex by immunofluorescence (42, 43) and found to be concentrated in the TGN by immunoelectron microscopy (44). If this is true also for transfected COS 7 cells, then the processing of rabbit pro-LPH should be pre-
Furin, PC1/3, PC2, and PC6A/B are clearly all expressed in enterocytes of the small intestine. Since furin is the enzyme that cleaves rabbit pro-LPH most efficiently, we looked at its expression by Northern blotting (not shown). Rabbit furin is encoded by two mRNAs of approximately 5 and 6 kilobases, respectively, the former being the more abundant. These sizes are slightly larger than those found for furin in mouse and rat (46).

Expression of Furin, PC1/3, PC6A/B, and PC2 in Enterocytes—In order to decide which of the PCs were good candidates for the in vivo processing of rabbit pro-LPH in the enterocytes, we have performed in situ hybridization experiments (Fig. 5 and Table I). As the individual probes show considerable sequence identity (see above), in order to reinforce specificity the tissue slices were treated with RNase A after the hybridization step. Furin, PC1/3, PC2, and PC6A/B are clearly all expressed in enterocytes (Fig. 5, A–D). Generally, furin and PC1/3 transcripts are present at much higher levels than those of PC2 or PC6A/B. Their distribution and relative abundance, however, varies considerably along the intestine (see Table II). In addition, we have also looked at the distribution of these PCs in the stomach, the pancreas, and the liver (Fig. 5, E–L, Table II). As negative controls, tissue slices were treated with RNase A prior to the application of the probe (compare panels F and J in Fig. 5), or they were hybridized with a probe to a transcript that is not expressed in the respective tissue (LPH in the stomach, Fig. 5H).

These experiments clearly show that furin, PC1/3, and PC6A transcripts are present in the enterocytes of the small intestines.
tine. Thus, one or more of these enzymes are most likely to process rabbit pro-LPH to the 180-kDa intermediate.

**DISCUSSION**

Small intestinal rabbit pro-LPH, contrary to human pro-LPH, is subjected to at least two proteolytic processing steps, rather than one, on its way from the endoplasmic reticulum to the brush-border membrane. In the rabbit, an intermediate of 180 kDa is formed. The objective of the present work has been that of identifying the protease(s) involved in the formation of this intermediate from pro-LPH.

Our conclusions are that rabbit enterocytes process pro-LPH to the 180-kDa intermediate by way of furin and possibly PC1/3 and/or PC6A for the following reasons. First (Fig. 1B), rabbit intestinal pro-LPH is cut within a protein region where furin, PC1/3, and PC6A consensus sequences occur, namely -Arg-Cys-Tyr-Arg114- and -Arg-Ala-Ser-Arg191-. Of these sequences, the latter is the more probable since cysteine residues have been found to be excluded from position -3 (47, 48). Neither of these two potential cleavage sequences occurs in human pro-LPH in which the consensus sequences indicated above do not occur. Fourth, by reverse transcription-PCR, we show that furin, PC1/3, and PC6A/B transcripts are present in the small intestine and, more specifically, as shown by in situ hybridization (Fig. 5), in enterocytes (the very cells which express and proteolytically process pro-LPH). The distribution of these transcripts varies along the length of the intestine (as does the distribution of pro-LPH transcripts), but generally, stronger signals are detected for furin and PC1/3 than for PC6A/B. Fifth (Fig. 3), we also show that the processing of pro-LPH to the 180-kDa intermediate by way of furin occurs in the TGN, or, thereafter, that is in the same or in a closely related compartment where we have localized the proteolytic processing of human pro-LPH to the mature enzyme (23).

Which One of These Proteases Is the Most Likely to Split Rabbit Pro-LPH to the 180-kDa Intermediate?—Furin shows the highest cleavage efficiency of pro-LPH in COS 7 cells, and its transcripts are found at high levels in the enterocytes. PC1/3 is also highly expressed in the enterocytes, its cleavage efficiency, however, being only one-third of that of furin. PC6A cleaves pro-LPH with intermediate efficiency, but is only poorly expressed in the enterocytes. Therefore, we believe that furin is the most likely enzyme to split pro-LPH in vivo.
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**TABLE II**

Cellular localization of PC transcripts as detected by in situ hybridization

After hybridization with the respective probes, all tissue slices were treated with RNase A to eliminate nonspecific signals (see "Results").

| Tissue       | Furin Transcripts detected by in situ hybridization in | PC 1/3 | PC2 | PC6A/B |
|--------------|-------------------------------------------------------|--------|-----|--------|
| Duodenum     | Enterocytes                                           | Enterocytes* | Enterocytes† | Enterocytes* |
| Jejunum      | Enterocytes                                           | Enterocytes | Enterocytes | Enterocytes |
| Ileum        | Enterocytes                                           | Enterocytes | Enterocytes | Enterocytes |
| Colon        | Not detectable                                        | Enterocytes | Not detectable† | Not detectable* |
| Stomach      | Surface mucous cells                                 | Enterodendocrine cells | Surface mucous cells† | Enterodendocrine cells |
| Pancreas      | Exocrine cells                                        | Exocrine cells | Exocrine cells | Exocrine cells |
| Liver        | Hepatocytes                                           | Hepatocytes* | Not determined | Hepatocytes* |

* Very weak signal, but significantly above background.
† Only a very faint labeling near the limit of detection was observed.
§ Weak signal, but significantly above background.
# However, Northern blot analysis fails to show expression (46).

Walker et al. (49) have suggested that furin may show a broader specificity when overexpressed. However, this observation does not change our conclusions, since rabbit pro-LPH is still efficiently processed even in the presence of a 5000-fold lower amount of furin CDNA (not shown). In addition, experiments in Ltk− cells which endogenously express furin, but not the other PCs (46), show that overexpressed rabbit, but not human pro-LPH, is processed to the 180-kDa intermediate in these cells.

PC6A and PC6B contain the same catalytic domain. Interestingly, only PC6A, which is a soluble enzyme, cleaves rabbit pro-LPH, whereas PC6B, which contains a C-terminal membrane anchor (19), does not. Since pro-LPH is anchored to the same membrane as PC6B, this may indicate a steric problem in the processing of pro-LPH by PC6B or absence of direct physical interaction. Furin, which also contains a membrane anchor, cleaves pro-LPH, which may indicate that the interaction between pro-LPH and furin does not suffer from a similar steric problem. Alternatively, the cleavage of pro-LPH may be due to the truncated, soluble form of the enzyme (see eg. Ref. 50). Whatever form of furin splits pro-LPH, furin and pro-LPH must at some point colocalize.

In order to see whether furin cleaves rabbit pro-LPH at the authentic site in COS 7 cells, we have analyzed the different LPH forms obtained in organ cultures and in COS 7 cells on the same polycrylamide gel. The migration behavior of pro-LPH and the 180-kDa intermediate was indistinguishable between the two samples, both before and after deglycosylation with N-glycosidase F (not shown). These findings further support the idea that furin is involved in the generation of the 180-kDa intermediate and that cleavage occurs at the -Arg-Ala-Ser- site.

Which Proteases Could Be Involved in the Generation of Mature LPH?—The bond split for this process in the 180-kDa intermediate, or in human pro-LPH, differs from that which is cleaved in the generation of the 180-kDa intermediate from rabbit pro-LPH and which is discussed in the previous paragraphs. The site which is split in the generation of mature LPH is (K/R)XRX− (i.e. KTR− in the rabbit, KVR− in the human, and RAR− in the rat enzyme, respectively). To our knowledge, the only other protein which is cleaved at a similar site is the meprin A α-subunit precursor (PRTR−), which is also expressed and processed in enterocytes (51, 52).

Furin and the other subtilisin-like PCs tested in the present work do not cleave this site leading to mature LPH in rabbit. In some experiments, we have detected a band of approximately 150 kDa, i.e with a size similar to mature LPH (cf. Fig. 2). This band, however, is also present in COS 7 cells which are transfected with rabbit or human pre-pro-LPH only, suggesting that this polypeptide is generated by an endogenous protease of COS 7 cells (see also Refs. 23, 39, 53). Both rabbit and human pro-LPH have a RXRX− motif approximately 28 amino acids upstream of the mature cleavage site. This site might be used by an endogenous protease as well as by furin (at least in the case of human pro-LPH). We do not know why rabbit pro-LPH is not further processed at this site by furin or any other PC used in the experiments. Further sequence constraints, however, which are different between the human and rabbit enzyme may play an important role. Indeed, as shown recently, replacement of amino acids close to the cleavage site in stromelysin-3 strongly influences the furin-dependent processing of this protease (53).

Pancreatic proteases play a minor role, if at all, in the processing of pro-LPH (9 and references therein). Granzyme A, a potential candidate (54), shows trypsin-like activity and, at least in vitro, cleaves synthetic substrates with basic amino acids in positions −1 and −3. However, by coexpression of rabbit or human prepro-LPH together with a cDNA coding for mouse granzyme A in COS 7 cells, and also by an in vitro assay with pure pro-LPH and pure granzyme A, we did not find any processing of pro-LPH. Other candidates could comprise duodenase, a serine protease with trypsin- and chymotrypsin-like activities that has been isolated recently from bovine duodenal mucosa (55). Further candidates are homologues of the arginine-selective yeast aspartic protease (56), or an arginine-selective endoprotease that was isolated from rat intestinal mucosa (57, 58). Involvement of enzymes from the latter groups would, however, imply that final processing of rabbit pro-LPH occurs in the intestinal lumen by a protease of the enterocytes.

What Is the Role of the Pro-region in Pro-LPH?—Removal of the prosequence is not required to activate the enzyme, since human pro-LPH is fully active (39). Nevertheless, the large prodomain may be needed for proper folding of the enzyme and/or transport to the brush-border membrane. Indeed, deletion of the prosequence does not allow the protein to appear at the plasma membrane (14, 59). The fact that rabbit pro-LPH, in contrast to the human enzyme, is cleaved twice is difficult to explain. Due to slight structural differences, removal of a first part may be required for accessibility of a second protease at the second site. For the same reason, removal of the first part in rabbit may be needed for the enzyme to dimerize. Interestingly, Danielsen (60) could show that pig LPH dimerizes before, or simultaneously with, the proteolytic cleavage(s) which gen-

4 J. Tschopp, personal communication.
erate(s) the mature enzyme. Also glycosylation may play a role. Human and rabbit pro-LPH contain several consensus sequences for N-linked glycosylation, but not all of them are at identical positions (1). However, at least in human LPH, the proregion does not seem to be glycosylated, whereas mature LPH is (59). Site-specific mutations in the rabbit pro-LPH sequence should allow us now to address these issues.

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The proteolytic processing of pro-lactase-phlorizin hydrolase (LPH) is a complex process involving several proteases, including proprotein convertases (PCs), which are integral membranespanning enzymes that mediate the proteolytic cleavage of precursor proteins. The study of LPH has been crucial in understanding the mechanisms of protein processing and the role of glycosylation in these processes.

The protein sequence analysis of LPH reveals several consensus sequences for N-linked glycosylation, suggesting that these modifications might play a role in the maturation of the enzyme. However, the data indicate that the proregion of human LPH is not glycosylated, whereas mature LPH is.

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The study highlights the importance of protein processing in the context of human and rabbit LPH sequences. The authors extend their gratitude to all those who kindly provided them with the cDNAs of proprotein convertases, particularly Dr. Jürg Tschopp for his contribution.