Limited Redundancy of the Proprotein Convertase Furin in Mouse Liver*

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Furin is an endoprotease of the family of mammalian proprotein convertases and is involved in the activation of a large variety of regulatory proteins by cleavage at basic motifs. A large number of substrates have been attributed to furin on the basis of in vitro and ex vivo data. However, no physiological substrates have been confirmed directly in a mammalian model system, and early embryonic lethality of a furin knock-out mouse model has precluded in vivo verification of most candidate substrates. Here, we report the generation and characterization of an interferon inducible Mx-Cre/loxP furin knock-out mouse model. Induction resulted in near-complete ablation of the floxed fur exon in liver.

In sharp contrast with the general furin knock-out mouse model, no obvious adverse effects were observed in the transgenic mice after induction. Histological analysis of the liver did not reveal any overt deviations from normal morphology. Analysis of candidate substrates in liver revealed complete redundancy for the processing of the insulin receptor. Variable degrees of redundancy were observed for the processing of albumin, α2 integrin, lipoprotein receptor-related protein, vitronectin and α1-microglobulin/bikunin. None of the tested substrates displayed a complete block of processing. The absence of a severe phenotype raises the possibility of using furin as a local therapeutic target in the treatment of pathologies like cancer and viral infections, although the observed redundancy may require combination therapy or the development of a more broad spectrum convertase inhibitor.

A large number of secretory proteins, including neuropeptides, peptide hormones, growth and differentiation factors, enzymes, adhesion molecules, receptors, and plasma proteins are activated by proprotein convertase cleavage at basic amino acid motifs by proprotein convertases (PCs). PCs are a family of seven closely related subtilisin-like serine proteases (furin, PC1 (also known as PC3), PC2, PC4, PC5 (also known as PC6), PACE4 and LPC (also known as PC7 and PC8)) (1–3) and two more distantly related members (SKI-1/S1P and NARC-1/PCSK9) (2, 4), with different, albeit partially overlapping, expression patterns and subcellular localization. Since the former seven PCs have similar specificities (basic amino acid motifs) it is impossible to predict physiological enzyme-substrate pairs, and most attempts at identification have relied on in vitro studies. However, co-overexpression studies of substrates with a PC are prone to false positive results, due to non-physiologically high enzyme levels, often in a heterologous cellular context. Direct evidence for enzyme-substrate pairs in vivo has been obtained from knock-out mouse models and human patients (recently reviewed in Ref. 5). Most informative have been the knock-out mouse models for PC1, PC2, and PC4, which have a restricted expression pattern. Inactivation of PC1 and PC2, which are restricted to neuroendocrine tissues, causes multiple endocrine peptide-processing defects that result in severe but viable phenotypes (6, 7). The differences between PC1 deficiency in mouse and humans are striking, the former is characterized by dwarfism, the latter by obesity (8, 9). Inactivation of germ cell-specific PC4 results in reduced fertility, at least in part due to blocked processing of propituitary adenylate cyclase-activating polypeptide (PACAP) (10, 11). Altogether, these studies have indicated that some substrates can be cleaved only by one PC, whereas other substrates are cleaved by multiple PCs. Depending on the co-expression of other PCs, redundancy may be cell type-specific.

The knock-out mouse model of the ubiquitously expressed convertase LPC has not reported phenotype suggesting complete redundancy (12). Of course, it is possible that a more specialized function of LPC has resulted in a phenotype too subtle to detect by standard analysis. The null phenotypes of the other broadly expressed enzymes furin, PACE4 and PC6, feature early embryonic lethality, indicating vital, non-redundant functions of these enzymes at early developmental stages (5, 13, 14). However, direct analysis of potential substrates involved in embryogenesis is difficult because of their low expression levels. Only BMP-4 cleavage has been examined directly and was shown to occur normally even in furin/PACE4 double knock-out embryos (15). Indirect evidence was given for other substrates such as Nodal. The embryonic lethality precludes the analysis of many substrates expressed only in adult life or well differentiated tissues.

This study describes the first conditional knock-out mouse
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model available for PCs. The inducible nature of the model has allowed us to investigate adult mice. We have focused on processing of substrates in liver for three reasons. First of all, the inducible knock-out system used here results in near-complete inactivation of the fur gene in liver but is less efficient in other tissues. Second, this biosynthetically very active organ produces a variety of potential substrates. Finally, the expression of high levels of furin in the presence of other PCs such as PACE4, PC6, and LPC provides an excellent opportunity to study whether specific enzyme-substrate relations exist in the presence of an extensive processing back-up potential.

**MATERIALS AND METHODS**

**Derivation of fur<sup>lox/lox</sup> Mice**—The same 6-kb NotI fragment comprising exon 1A to intron 6 murine fur sequences as used for the construction of a targeting vector for the general fur knock-out mouse (14) was used for the construction of a floxed targeting vector to generate a conditional fur knock-out mouse. A 2-kb BamHI fragment encoding the hygromycin B phosphotransferase (HygB) gene, fused to the phosphoglycerate kinase promoter and flanked by loxP sites, was cloned into a BamHI site upstream of exon 2. A third loxP site was introduced in a BamHI fragment of exon 2 (Fig. 2B). A 2.5-kb PCR fragment was conserved out of a central 34-bp loxP recognition sequence flanked by additional sequences, which were used to generate, by means of PCR, fragments with suitable restriction enzyme recognition sites at their ends. The total size of each loxP site insertion was 92 bp. The NotI insert of the targeting construct was excised and electroporated into ES cell clones were injected into C57Bl6/J blastocysts using a needle and a microinjection system (kindly provided by W. Müller, University of Cologne). To obtain ES cell lines with the desired deletion of the HygB selection marker cassette resulting from partial recombination by Cre recombinase (Fig. 1B), the protocol as described by Torres and Kuhn (17) was applied. Correct, partial Cre recombination was confirmed by PCR amplification, and these ES cell clones were injected into C57Bl6/J blastocysts using standard procedures. The resulting chimeras were mated with C57Bl6/J mice. Southern blotting and PCR analysis of tail DNA samples were used to confirm transmission of the fur<sup>lox</sup> allele to the offspring. Heterozygous offspring were intercrossed to obtain homozygous fur<sup>lox/lox</sup> mice.

**Breeding with Cre Recombinase Transgenic Mice**—To obtain in vivo inactivation of the fur<sup>lox</sup> allele by Cre recombinase homozygous fur<sup>lox/lox</sup> mice were crossed with two Cre transgenic mice. The pGK-Cre.NLS.pHpa plasmid (kindly provided by W. Müller, University of Cologne). To obtain ES cell lines with the desired deletion of the HygB selection marker cassette resulting from partial recombination by Cre recombinase (Fig. 1B), the protocol as described by Torres and Kuhn (17) was applied. Correct, partial Cre recombination was confirmed by PCR amplification, and these ES cell clones were injected into C57Bl6/J blastocysts using standard procedures. The resulting chimeras were mated with C57Bl6/J mice. Southern blotting and PCR analysis of tail DNA samples were used to confirm transmission of the fur<sup>lox</sup> allele to the offspring. Heterozygous offspring were intercrossed to obtain homozygous fur<sup>lox/lox</sup> mice.

**Southern Blot Analysis and Quantitative Reverse Transcription PCR**—Total RNA was extracted from liver tissue from induced wildtype and transgenic mice. For Northern blot analysis 15 μg of RNA was size-fractionated on a 1% agarose gel, blotted and hybridized according to standard procedures as described (20). For fur an exon 2-specific cDNA probe of 380 bp was used and a 2.8-kb cDNA probe spanning the entire coding sequence was also used. In addition, a 1.7-kb cDNA probe for PACE4, a 3-kb PC5 cDNA probe (containing the sequences common in PC5A and PC5B), a 2.5-kb LPC cDNA probe, and a 350-bp actin reverse primer (containing sequences from the actin transgene) was used. PCR amplification was performed using TaqMan real-time PCR. cDNA was prepared using the SuperScript first-strand synthesis system for reverse transcription PCR (Invitrogen). Real-time PCR was performed using the qPCR core kit (Eurogentec) and was carried out using the ABI Prism 7700 sequence detection system (Applied Biosystems). Probes and primers for fur and GAPDH (glyceraldehyde-3-phosphate dehydrogenase gene), the reference gene, were designed by Primer Express 2.0 (Applied Biosystems). The probes were labeled on the 5′-end by 6-carboxyfluorescein and the 3′-end by 6-carboxytetramethylrhodamine. The following probes were used: fur exon 2 forward primer, 5′-CAGAAGACAGCTGCTCCC-ACAC-3′, fur exon 3 reverse primer, 5′-TGTACTGCTCTGTCGCA-GAAA-3′; fur exon 2–3 probe, 5′-TGGCCGACATCTGCTGATATA-TCCA-3′; GAPDH forward primer, 5′-ATGACCCTCTCCGTGTC-3′; and GAPDH reverse primer, 5′-CCCCAATGTTGCCTGCT-3′. The relative gene expression was quantified using the comparative threshold cycle method (Applied Biosystems).

**Histology**—For histological analysis, liver tissues from both induced and non-induced wild-type and transgenic mice were processed in parallel to provide appropriate controls also for the induction procedure itself. In H&E stained sections there were no apparent differences in liver architecture or in the number and size of the bile ducts.

**Western Blotting**—Processing of candidate substrates was analyzed by Western blotting. Postnecrotic supernatant of liver tissue was centrifuged for 1 h at 100,000 × g to obtain soluble and membrane fractions. SDS-PAGE and isotopic focusing were performed as described previously (21); equal amounts of protein from liver extracts from mice of the indicated genotype were loaded. Immunodetection of the insulin receptor was performed by a combination of immunoprecipitation using rabbit antibody CO-10 followed by Western blotting using monoclonal antibody CO-10. Immunodetection of the fur receptor was performed by a combination of immunoprecipitation using rabbit antibody CO-10 followed by Western blotting using monoclonal antibody CO-10.
antibody CT-1 as described (22). Both antibodies were kindly provided by Ken Siddle (Cambridge, UK). Anti-α1-microglobulin antibody was a generous gift of Cecilia Falkenberg (Lund, Sweden). Anti-α3 integrin antibody was purchased from Chemicon, anti-mouse albumin was from Biotrend, and anti-vitronectin was from Santa Cruz Biotechnology. Anti-CD300 receptor-related protein (LRP) antibody, directed against the C terminus of the LRP precursor and the β-subunit, has been described before (23).

RESULTS

Derivation of ES Cells with a FURfloxed Allele—The targeting construct for the generation of a floxed fur (furfloxed) allele, enabling conditional inactivation, contained the hygromycin B phosphotransferase (HygB) gene, flanked by loxP sites, and was cloned upstream of exon 2 (Fig. 1A). A third loxP site was introduced downstream of exon 2. Deletion of exon 2 sequences, encoding the translation initiation site, the signal peptide, and part of the prodomain of furin, was expected to inactivate the fur gene. Following electroporation into E14 ES cells individual drug-resistant colonies were analyzed by Southern blotting using external cDNA probe 2 (corresponding to exons 7 and 8). Of 180 drug-resistant colonies analyzed, 24 clones appeared to using external cDNA probe 2 (corresponding to exons 7 and 8).

The construct for the generation of a floxed furflox allele, flanked by loxP sites, and was inserted upstream of exon 2 (Fig. 1A). A third loxP site was introduced downstream of exon 2. Deletion of exon 2 sequences, encoding the translation initiation site, the signal peptide, and part of the prodomain of furin, was expected to inactivate the fur gene. Following electroporation into E14 ES cells individual drug-resistant colonies were analyzed by Southern blotting using external cDNA probe 2 (corresponding to exons 7 and 8). Of 180 drug-resistant colonies analyzed, 24 clones appeared to result from homologous recombination. PCR analysis showed that in 6 out of 24 clones, recombination at the 3’-end of the construct had occurred downstream of the third loxP site. Southern blot analysis of these six clones confirmed that the targeting had indeed occurred correctly (data not shown).

Since the presence of the floxed drug-resistance gene in intron 1 (furfloxed-HygB allele, Fig. 1, A and B) could have some deleterious effect on the expression of the floxed fur gene, this cassette was removed in vitro by partial loxP recombination. To achieve this, a Cre recombinase plasmid was transiently expressed in one of the ES cell lines. To obtain ES cell lines in which Cre recombinase resulted in only partial deletion, the protocol as described (17) was applied. After choosing 100 individual clones and splitting into two pools, one of the pools was analyzed for HygB sensitivity. Out of 40 clones, which had regained HygB sensitivity, three showed the proper partial deletion of the HygB cassette (furfloxed allele), whereas the others resulted from complete deletion (furfloxed allele) (Fig. 1B). Fig. 2A shows a Southern blot analysis confirming the partial or complete deletion, which was further substantiated by PCR (data not shown).

Generation of furfloxed/flox Mice and Conversion of the Floxed fur Allele (furfloxed) into a fur Null Allele (furnull) by Cre Recombinase—All three furfloxed ES cell lines gave rise to germ line transmission after mating of chimeras with C57Bl/6J mice. Homozygous (C57Bl/6J × 129) F1 offspring were intercrossed, and viable offspring were genotyped by PCR. Of a progeny of 94 pups, 24 (26%) were wild type (furnull/furnull), 43 (46%) were heterozygotes (furfloxed/furlnull), and 27 (29%) were homozygous for the floxed allele (furfloxed/furfloxed). The homozygous furfloxed/furfloxed mice were, as expected, normal, viable, and fertile. Southern blot analysis was used to confirm the genotyping results as obtained by PCR analysis (Fig. 2B). Northern blot analysis of liver samples of littermates revealed similar fur mRNA expression levels for all three genotypes (data not shown).

To demonstrate that both the floxed fur allele (furfloxed) could be converted into a fur null allele (furnull) via inactivation by Cre recombinase in vivo, homozygous furfloxed/flox mice were crossed with a general deleter pGK-Cre transgenic mouse (18). All heterozygote furfloxed/furlnull offspring carrying the pGK-Cre transgene showed the expected general deletion of fur exon 2 sequences as monitored by PCR and Southern blot analysis (Fig. 2B). These heterozygotes (furfloxed/furlnull) were intercrossed and viable offspring was genotyped by PCR. Only heterozygote (furfloxed/furlnull) (42 pups, 65%) and wild type (furnull/furnull) (23 pups, 35%) offspring was identified. This ratio of about 2 to 1 is in accordance with the expectation, that the furfloxed/flox genotype is embryonically lethal like the general fur knock-out mouse (14). It should be noted that these mice carried zero, one, or two copies of the pGK-Cre allele, as both the parental mice carried one copy of the pGK-Cre allele. However, since the null allele (furfloxed) is germ line-transmitted, it is independent of continued expression of Cre recombinase. To exclude any interference of the presence of the pGK-Cre transgene, these heterozygotes (furfloxed/furlnull) were crossed with wild type C57Bl/6J mice. Heterozygotes (furfloxed/furlnull) without the pGK-Cre transgene were identified by PCR analysis and intercrossed. Again no viable homozygous furfloxed/flox mice were born. To determine the timing of the developmental arrest, embryos from heterozygous intercrosses were analyzed macroscopically and genotyped by PCR. As described in detail for the classical fur knock-out embryos (14), no homozygous furfloxed/flox mutant embryos were recovered from 11.5 days postcoitus onwards, although at this stage some deciduas contained remnants of resorbed tissue. At 9.5 and 10.5 days postcoitus about 25% of the embryos analyzed were homozygous furfloxed/flox mutant. Macroscopically these mutant embryos showed a similar phenotype as the classical fur knock-out embryos: defects in ventral closure and axial rotation (data not shown). The furfloxed allele (deletion of exon 2 sequences) and the classical fur knock-out allele (targeted disruption of exon 4 sequences by a selection gene) can both be considered genuine fur null alleles.

Conditional Inactivation of the furfloxed Allele in the Liver—To inactivate the furfloxed allele conditionally, the furfloxed/flox mice were crossed with Mx-Cre transgenic mice. In this transgenic line, Cre expression can be induced by pI-pC. This has been shown to result in complete deletion of reporter genes in liver; near-complete deletion in spleen; partial deletion in duodenum, heart, lung, uterus, thymus, and kidney; and inefficient deletion in muscle, tail, and brain (19). Offspring heterozygous for the floxed fur allele and the Mx-Cre transgene were subsequently intercrossed to obtain homozygous furfloxed/flox mice carrying in addition one or two Mx-Cre alleles. After induction, Cre recombination of the floxed fur allele was analyzed in the liver and in brain tissue. In brain very little recombination could be detected, whereas near-complete inactivation of the floxed fur allele was observed in liver, consistent with previous results obtained with Mx-Cre mice (8 and 100%, respectively (19)). However, besides the inactivated furfloxed allele and the non-recombined furfloxed allele, a third kind of fur allele could be detected, albeit at very low amounts. Analysis with an exon 2 specific probe revealed that this third fur allele had arisen from integration of the excised fragment into the other allele in the same cell (data not shown). Obviously, as long as the excised circular DNA fragment remains present, it can participate in a second Cre recombination event. To avoid this problem of reintegration resulting in a third kind of fur allele with unknown impact, a different crossing scheme was designed. The Mx-Cre allele was crossed into mice heterozygous for the furfloxed allele obtained initially by crossing with a pGK-Cre transgene (see above). Subsequently, mice heterozygous for both the furfloxed allele and the Mx-Cre transgene were crossed with homozygous furfloxed/flox mice (Fig. 3). Since one of the fur alleles is already inactivated, reintegration will just restore one of the two inactivated alleles into a furfloxed allele with a zero net effect. The resulting offspring was used to inactivate the furfloxed allele conditionally in the liver. As shown in Fig. 4, in furfloxed/flox and furfloxed/furlnull mice, heterozygous for the Mx-Cre allele, the furfloxed allele was recombined into a furfloxed allele in the liver with high efficiency, whereas in brain hardly any Cre recombination could be detected. From these analyses it can be concluded that in furfloxed/flox mice, heterozygous for the Mx-Cre allele, the fur...
gene can be efficiently inactivated in liver upon induction of Cre expression. This was further substantiated by Northern blot analysis and quantitative PCR using RNA isolated after pl-pC induction from livers of fur<sup>lox<sup>-<sup>/flo</sup>x</sup>/flo<sup>x</sup> mice, heterozygous for the Mx-Cre allele (Fig. 5 and Table I). This results demonstrated that ~1% of normal levels of wild type fur mRNA was

**Fig. 1.** Strategy used for the generation of a conditional fur knock-out mouse model. A, homologous recombination was used to flank exon 2 with loxP sites and to insert a HygB selection marker cassette. B, after the generation of recombined ES cells, the HygB cassette was removed by transiently transfected Cre recombinase. Inactivation of the fur gene was accomplished by induction of the Mx-Cre transgene by pl-pC. Genomic analysis after homologous recombination and Cre recombination was performed using PCR and Southern blotting as described under “Materials and Methods.” The position of the PCR primers and cDNA and genomic probes are indicated. Exons are indicated as black boxes and loxP sites as arrowheads. ATG indicates the start codon in exon 2. N = NotI; K = KpnI; B = BamHI; E = EcoRI; H = HinDIII; hygB = hygromycin B selection marker cassette.
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On routine stains, we occasionally observed a mild congestion of the liver as evidenced by distended blood vessels filled with packed erythrocytes. This inconstant effect was, however, linked to the pI-pC induction procedure and was independent of genotype.

Differences in liver lobe architecture or hepatocyte morphology were neither observed by routine hematoxylin and eosin stains on paraflin material nor by toluidine blue stains on Karnovsky-fixed 1-μm resin sections. The architecture of blood vessels and bile ducts was analyzed by PNA and Dolichos bifloris lectin histochemistry and was found to be unaffected.

Likewise, a screen for leukocytes and macrophages by CD45 and F4/80 immunohistochemistry did not reveal any changes in distribution, density, or morphology of these cells. Finally, no increase of RCA-1 stainability of macrophages, which is an early marker of phagocyte activation upon pathologic stimuli, was recorded. In summary, no evidence was found for pathologic changes of the liver.

Biochemical Analysis of Potential Furin Substrates in Liver—To obtain insight into physiological furin substrates and potential redundancy, mice carrying zero or one functional fur allele in the liver, generated as described above, were analyzed. Three membrane-anchored and three soluble proteins, with expression levels ranging from low to high, were selected (Fig. 7). All candidate substrates have been suggested to be furin substrates on the basis of in vitro or ex vivo experiments.

The insulin receptor is a heterotetrameric cell surface protein composed of two α and two β chains of 135 and 90 kDa, respectively (22). Both chains are derived from a single precursor by cleavage at a R-P-S-R-K-R-R S-L sequence. Endoproteolytic cleavage of the proinsulin receptor was studied using antibodies directed against epitopes in the α chain. As is shown in Fig. 7, processing of the proinsulin receptor was unaffected by functional inactivation of furin, suggesting complete redundancy or that furin is not the physiological convertase.

Integrins are cell surface transmembrane glycoproteins that function as adhesion receptors linking the extracellular matrix proteins to the cytoskeleton. All integrins are heterodimers consisting of 1 of 18 α-subunits and 1 of 8 β-subunits. Of the 18 α-subunits, 9 of the 18 α-subunits are internally cleaved into a membrane-bound light chain and an amino-terminal heavy chain that remain associated. The α5 integrin precursor is cleaved at a H-H-L-Q-E-A sequence and is expressed at high levels in liver (24). Under steady state conditions, virtually all α5 integrin is in its processed form as demonstrated with an antibody directed against a carboxyl-terminal epitope, which detects mainly the processed 35-kDa light chain and only trace amounts of the 140-kDa precursor. However, in the absence of furin the majority remains unprocessed, indicating an important role for furin in processing of α5 integrin. On the other hand, the presence of (sub)normal amounts of processed α5 integrin indicate that at least one other enzyme can cleave pro-α5 integrin in liver.

The low density LRP is a multifunctional receptor with multiple ligands. The mature receptor is derived from a 600-kDa precursor by cleavage at a S-N-R-H-R-R Q-I site to generate 515- and 85-kDa fragments that remain associated non-covalently (25). Using an antibody directed against the 85-kDa fragment, the processing of LRP was studied in the conditional furin knock-out mouse. Under steady state conditions, virtually all immunodetectable LRP in all samples was in the processed form indicating redundancy of furin. However, longer exposure (upper panel) revealed some high molecular weight precursor. Moreover, additional bands were observed in the furin-deficient liver membranes with slightly different electrophoretic mobilities (indicated with an asterisk). Although the nature of

still present. The residual presence of the furfl ox allele points toward the presence of a small number of cells in the liver that do not respond to the induction. Northern blot analysis of PACE4, PC5 (A and B), and LPC did not reveal any major compensatory up-regulation in the fur-deficient livers (Fig. 5).

Histological Analysis of Furin-deficient Mouse Liver—Liver architecture was analyzed in four sets of mice (wild type induced and not induced, transgenic mice induced and not induced) by both routine stains of paraffin- and plastic-embedded tissues as well as by immunohistochemical and lectin histochemical analysis (Fig. 6). The latter was employed to screen for abnormalities in vascular and bile duct architecture and macrophage or leukocyte reactions that could point toward less overt pathological changes of liver tissue.

FIG. 2. Genomic analysis of ES cells and transgenic mice. Genomic DNA from wild type and transgenic ES cells (A) or transgenic mice (B) was digested with KpnI and analyzed by Southern blotting using cDNA probe 1 (A) and genomic probe 3 (B). Different alleles are indicated.
these bands was not further investigated, their persistent appearance in only the furin-deficient samples suggests that it is the consequence of a slightly altered maturation process.

The most abundant plasma protein is albumin, which is produced in the liver from its precursor proalbumin by cleavage at a R-G-V-F-R-R→E-A site (26). Since the propeptide is only

**Fig. 3.** Crossing scheme of conditional fur knock-out mice. Schematic representation of the crossing scheme used for the analysis. Mice with the correct genotype were induced with pl-pC.

**Fig. 4.** Induction with pl-pC results in efficient recombination in liver but not in brain. Mice of different genotypes were induced with pl-pC, and genomic DNA from liver and brain was digested with KpnI and analyzed by Southern blotting using genomic probe 3. Near-complete recombination of the floxed allele occurs in the liver after induction of Cre recombinase (compare middle panel with right panel). L = liver; B = brain.

**Fig. 5.** Northern blot analysis of fur, PACE4, PC5, and LPC in transgenic mice. RNA was isolated from liver after pl-pC induction and used for Northern blotting. Wild type fur mRNA was detected using an exon two specific probe, whereas a larger probe was used to detect both wildtype mRNA and mRNA lacking exon 2 sequences. After hybridization with PACE4, PC5, and LPC, the blots were stripped and reprobed with actin for normalization. Δflox/flox indicates furΔflox/flox mice without Mx-Cre allele, and Δflox/Δflox indicates furΔflox/Δflox mice containing one Cre allele, resulting in the knock-out genotype after induction.
deficient liver. Furin induction in wild type mice (shaded bars) did not modify density and distribution of macrophages (shown by F4/80 immunohistochemistry in B; see A for a nuclear counterstain). Hepatocyte morphology remained unaffected (shown on a toluidine blue-stained semithin section in B).

Table 1
Quantification of fur mRNA in liver after pl-pC induction
using real-time PCR

| Genotype         | Exons 2 and 3 |
|------------------|---------------|
| flox/flox        | 100           |
| Δflox/flox       | 38            |
| Δflox/Δflox      | 47            |
| Δflox/Δflox      | 1.2           |
| Δflox/Δflox      | 0.4           |

FIG. 6. No morphological abnormalities are found in furin-deficient liver. Furflox/flox mice with or without Mx-Cre transgene were analyzed without (data not shown) or after pl-pC induction. pl-pC induction in wild type mice (A–C) did not modify density and distribution of macrophages (shown by F4/80 immunohistochemistry in B; see A for a nuclear counterstain). Hepatocyte morphology remained unaffected (shown on a toluidine blue-stained semithin section in C). In furin-deficient liver (D–F), no changes in these parameters were noted as compared with the induced wild type mice. Co = central vein; sin = sinusoid; hep = hepatocyte.

Six amino acids, the precursor cannot be separated easily from the processed form by SDS-PAGE. The presence of these arginines in the propeptide, on the other hand, facilitates the separation by isoelectric focusing. Inactivation of both fur alleles resulted in an increase of proalbumin. It should be noted, however, that the vast majority was normally processed indicating substantial redundancy for processing of proalbumin.

Two additional abundant plasma proteins synthesized in liver are α1-microglobulin and bikunin, which are derived from the same precursor by cleavage at a I-A-R-A-R-R | A-V sequence (27). α1-Microglobulin is an immunosuppressive protein that belongs to the lipocalin superfamily and bikunin is a member of the Kunitz-type protease inhibitor superfamily. Processing of the α1-microglobulin/bikunin precursor was studied using an antibody directed against α1-microglobulin. Inactivation of both fur alleles resulted in a strong reduction in processing, although mature α1-microglobulin remained detectable in (sub)normal amounts.

Finally, the processing of vitronectin was studied. Vitronectin is an adhesive glycoprotein in the extracellular matrix and in plasma. It is composed of two chains of 65 and 10 kDa, which are generated by cleavage of the precursor at a R-R-S-S-R | S-I site (28). As is shown in Fig. 7, furin is largely redundant for the processing of vitronectin since ablation of active furin results in only a minor increase in precursor.

Taken together, these data show that in liver of adult mice there is (limited) redundancy of furin for every candidate substrate tested, although the level of redundancy for different substrates is highly variable.

Discussion
In this study we have examined the inducible inactivation of the fur gene. No adverse effect was observed from treatment with pl-pC per se, with the exception of occasional mild congestion of liver. Despite the near-complete inactivation in liver, only a mild phenotype was observed. No morphological abnormalities could be found, and processing of various proproteins appeared to be unaffected or impaired, but never completely blocked. Accumulation of substantial amounts of precursors in mice carrying one intact fur allele was never observed. Altogether, these data indicate considerable redundancy of proprotein processing activity in liver. This biosynthetically very active organ expresses high levels of furin as well as other PCs such as PACE4, PC6, and LPC (29, 30), which are likely to provide this processing redundancy. It is possible that the physiological role of furin in liver is underappreciated in this model due to overcapacity of processing activity provided by other PCs. Redundancy was not acquired by up-regulation of other PCs. Redundancy was not acquired by up-regulation of other PCs. Redundancy was not acquired by up-regulation of other PCs. Redundancy was not acquired by up-regulation of other PCs. Redundancy was not acquired by up-regulation of other PCs. Redundancy was not acquired by up-regulation of other PCs. Redundancy was not acquired by up-regulation of other PCs. Redundancy was not acquired by up-regulation of other PCs.
It also contrasts with a number of processing studies performed in furin-deficient cell lines, which have suggested a crucial role for furin for many substrates. These discrepancies can be due to a number of reasons. First, in heterologous expression experiments misleading results can arise from the co-expression of substrates with convertases, which are normally never expressed in the same cell. In addition, redundancy may be cell type-dependent, as was for instance shown for PACAP, which is cleaved exclusively by PC4 in testis (10), whereas in brain PC4 is absent, and PACAP may be cleaved by PC1 or PC2 instead (31).

Processing of the insulin receptor by furin has been suggested by several studies, based on lack of processing in furin-deficient cells (32) or biochemical studies using purified furin (33). The former study was later complemented by a study showing partial rescue by PACE4 (34). Our results show complete redundancy in the knock-out mice, although this does not exclude that in wild type mice furin is partly responsible for precursor cleavage. Normal processing was anticipated given the severe phenotype of the liver-specific insulin receptor knock-out and the requirement of processing for activation (35, 36).

Processing of α5 integrin has been studied in furin-deficient cells and by overexpression studies, which indicated involvement of furin and PC6A but not PACE4 or LPC (24). Our results are consistent with these observations, although we cannot determine which enzyme(s) is (are) responsible for the redundancy.

Processing of LRP, which does not seem to be essential for its endocytic functions (37), has been studied in furin-deficient cells that display severely impaired processing (25). These results contrast with our observation that virtually all LRP was processed under steady state conditions, and only traces of precursor could be detected. The small differences in electrophoretic mobility of the precursors in furin-deficient liver (indicated with asterisks in Fig. 7) might be the consequence of a slight changes in timing of processing relative to other post-translational modifications such as complex glycosylation.

Proalbumin processing has been well studied in both human and in animal model systems. Naturally occurring human proalbumin genetic variants have been described in which mutations in the cleavage site result in impaired cleavage without significant clinical consequences (38). To identify the physiological processing enzyme(s) many different approaches have been taken implicating furin but also other enzymes like PACE4 and LPC (26, 39, 40). Here, we demonstrate that in the absence of furin, the majority of proalbumin is normally cleaved in mouse liver. The clear increase in proalbumin, on the other hand, indicates a significant involvement of furin under physiological conditions.

Processing of the precursor of α1-microglobulin/bikunin has also been studied in furin-deficient cells without differences in degree of processing as compared with the parental cell line. This suggests processing by another PC, although furin was able to cleave the substrate in overexpression studies (27). Here we find an important role for furin in the processing of α1-microglobulin/bikunin, although partial cleavage was still occurring in its absence.

Intracellular processing of vitronectin has only recently been demonstrated (28). In that study furin was put forward as a candidate for processing on the basis of in vitro cleavage. Although furin might be involved in this cleavage step, it clearly does not play an essential role as virtually all immunoreactive vitronectin in furin-deficient liver was cleaved to Vn65.

It is difficult to understand why there is complete redundancy of furin for some but not other substrates. It is clearly not related to expression levels as (near) complete redundancy was observed for both albumin, the most abundant secretory protein in liver, and the non-abundant insulin receptor. It is also not correlated with membrane association, since both α5 integrin and α1-microglobulin/bikunin seemed, to a certain extent, to depend on furin for cleavage, whereas for LRP and albumin processing, furin is much less important. We have also analyzed the substrates using a recently described method for prediction of cleavage sites specific for furin or PCs in general, based on artificial neural networks (Table II) (41). Again, no correlation was observed. In contrast, the cleavage site of α5 integrin was predicted to be a poor furin site but a good general PC cleavage site. Albumin on the other hand was predicted to be a furin substrate but not a general PC substrate. These data show that the program is a powerful tool for predicting cleavage sites but not for predicting physiological PCs.

The consequence of the observations made in this study for therapeutic targeting of furin is 2-fold. The mild phenotype of this inducible knock-out model might be indicative for a lack of severe side effects when applying furin inhibitors in the treatment of pathologies such as cancer, viral infections, or bacterial toxins (5, 43). On the other hand, inhibition of furin should have a severe impact on processing of the target substrate(s) to exert a therapeutic effect. The feasibility of using furin as a therapeutic target has recently been demonstrated in mice injected with bacterial toxins (44, 45). Inhibition of furin with hexa-d-arginine provided protection against an otherwise lethal dose of toxin. It remains to be established, however, whether furin is a useful therapeutic target in the treatment of human pathologies.

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