The low-density-lipoprotein receptor-related protein (LRP) is processed by furin *in vivo* and *in vitro*

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The low-density-lipoprotein receptor-related protein (LRP) is a multifunctional receptor involved in the clearance of a large number of diverse ligands, including proteases, protease-inhibitor complexes and lipoproteins. The mature receptor is composed of a 515 kDa and a 85 kDa subunit generated by proteolytic cleavage from a 600 kDa precursor polypeptide in a *trans*-Golgi compartment. Proteolytic processing occurs C-terminal to the tetrabasic amino acid sequence RHRR, a consensus recognition site for precursor processing endoproteases or convertases. In this study we have identified furin, a subtilisin-type protease, to be necessary for efficient processing of LRP in

cells. Furin-deficient RPE.40 cells exhibited an impaired processing of endogenous LRP and of a recombinant soluble form of the receptor containing the processing site. The processing defect in RPE.40 cells could be complemented by expression of furin from a transfected cDNA in cultured cells and by purified furin *in vitro*. The impaired maturation of LRP in RPE.40 cells did not affect its intracellular transport, and correlated with a slight but consistent reduction in the endocytosis of LRP-specific ligands. These data suggest that proteolytic processing of LRP by furin is not necessary for intracellular trafficking but might be required for normal receptor activity.

INTRODUCTION

The low-density-lipoprotein (LDL) receptor-related protein (LRP) is a multifunctional receptor expressed in a variety of cell types [1]. It is a member of the LDL receptor gene family, which also includes the LDL receptor [2], the very-low-density-lipoprotein receptor [3], the vitellogenin receptor [4] and gp330/megalin [5]. All members of the gene family are involved in the clearance of ligands from the extracellular space, and LRP in particular has been shown to bind and internalize a multitude of diverse ligands (reviewed in [6]). These ligands include the active proteases tissue-type and urokinase-type plasminogen activator (tPA and uPA) [7–9] and complexes of these proteases with the protease inhibitor plasminogen activator inhibitor-1 (PAI-1) [10,11]. Furthermore, LRP mediates the cellular uptake of proteases complexed with α_2 -macroglobulin [12,13], of tissue factor pathway inhibitor [14] and of neutrophil elastase bound to α_1 -antitrypsin [15]. Besides its role in the regulation of protease homeostasis, the receptor has been implicated in the hepatic clearance of chylomicron remnants, the carriers of dietary cholesterol. LRP was shown to take up apolipoprotein (apo) E-enriched lipoproteins [16–19] and inhibition of LRP activity *in vivo* results in accumulation of chylomicron remnants in the plasma of mice [20]. Recently, *Pseudomonas aeruginosa* exotoxin A (PEA) was found to enter cells through LRP-mediated endocytosis [21] and murine embryonic fibroblasts homozygous for a disruption of the LRP gene consequently are resistant to PEA [22].

Structurally, LRP exhibits features which are common to all receptors of the LDL receptor gene family. Its extracellular domain is composed of cysteine-rich epidermal growth factor-type repeats and complement or ligand binding-type repeats. The

latter make up the ligand-binding domain in the LDL receptor with which apoE and apoB-100 interact. In addition, LRP contains YWTD motifs, which may be responsible for pH-dependent release of ligands in endosomal compartments. The receptor is anchored in the plasma membrane by a single transmembrane domain and its cytoplasmic tail includes two copies of the internalization signal NPXY. Within the gene family LRP is unique, inasmuch as the receptor is a heterodimer composed of a 515 kDa extracellular and an 85 kDa membrane-anchored subunit. Both subunits are generated by proteolytic cleavage from a 600 kDa precursor in a *trans*-Golgi compartment and both remain non-covalently associated. Processing occurs at amino acid position 3925 C-terminal to the 4-amino-acid sequence RHRR [23]. This sequence is homologous to a tetrabasic consensus motif RX(K/R)R found in a number of proproteins of the constitutive secretory pathway. Although proteolytic processing is common for prohormones, growth factors, bacterial toxins and viral glycoproteins, cleavage of cell-surface receptors is unusual. Besides LRP, the receptors for insulin and insulin-like growth factor and the receptor for the hepatocyte growth factor/scatter factor are proteolytically processed [24–26].

Recently, an endoprotease-deficient Chinese hamster ovary (CHO) cell line, RPE.40, has been reported that is defective in processing of viral glycoproteins and bacterial toxins [27]. The cell line was subsequently shown to be deficient in the protease furin and transfection of the mouse furin cDNA into RPE.40 cells could complement the processing defect [28,29]. Furin is the mammalian homologue of the yeast protease KEX2 and structurally related to the prokaryotic serine proteases of the subtilisin type (reviewed in [30]). The protease contains a transmembrane domain and resides in the *trans*-Golgi network and in recycling vesicles of the endocytic pathway [31,32]. Furin has been shown

Abbreviations used: apo, apolipoprotein; CHO, Chinese hamster ovary; D-MEM, Dulbecco's modified Eagle medium; LDL, low-density lipoprotein; LRP, LDL receptor-related protein; PAI-1, plasminogen activator inhibitor-1; PEA, *Pseudomonas aeruginosa* exotoxin A; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; IIIs and IVs, soluble forms of regions III and IV; YPACK, tyrosyl-prolyl-arginyl-chloromethyl ketone.

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to cleave several target proteins at a multibasic site *in vivo* or *in vitro*. These include the precursors of the insulin receptor, anthrax toxin-protective antigen, PEA, von Willebrand factor, influenza virus haemagglutinin and gp160 of the human immunodeficiency virus (HIV) [29,33–38].

In this study we have investigated the role of furin in the proteolytic processing of LRP. Furin-deficient RPE.40 cells did not process a transiently expressed truncated LRP minireceptor containing the cleavage site, and exhibited impaired processing of endogenous LRP. Normal processing was restored *in vivo* by transfection of a cDNA encoding mouse furin into the cells. Furthermore, purified furin efficiently cleaved the LRP minireceptor *in vitro*, demonstrating the specificity of LRP processing by furin.

EXPERIMENTAL

Materials

The generation of cell strains RPE.40 and 40.fur13d has been described previously [28,39]. Cells were grown in Dulbecco's modified Eagle medium (D-MEM)/F-12, supplemented with 10% fetal-calf serum. Human α_2 -macroglobulin activated with methylamine was a gift from Dudley Strickland (American Red Cross, Rockville, MD, U.S.A.). Purified recombinant human furin [40] was the gift of Gary Thomas (Vollum Institute, The Oregon Health Sciences University, Portland, OR, U.S.A.). Complexes of ^{125}I -tPA/PAI-1, ^{125}I -uPA/PAI-1 and ^{125}I -tPA/YPACK (tyrosyl-prolyl-arginyl-chloromethyl ketone) were kindly provided by Kim Orth (UT Southwestern Medical Center, Dallas, TX, U.S.A.) and had been prepared as described previously [10]. PEA was obtained from Sigma. All proteins were radiolabelled by the IODOGEN method [41]. Protein A-Sepharose CL-4B was purchased from Pharmacia; Trans ^{35}S -label was from ICN Radiochemicals.

Ligand and immunoblot analysis, recombinant DNA techniques and protein determinations

Ligand and immunoblot analyses of partially purified membrane preparations from cells have been reported previously [17]. DNA manipulations were performed essentially as described in [42]. The protein content of cell lysates was determined using the Coomassie Plus Protein Assay Reagent (Pierce).

Cellular degradation of ^{125}I -labelled ligands

CHO cells (2×10^5 /well) were seeded into 12-well plates and grown for 24 h. The medium was replaced by D-MEM (without glutamine) containing 0.2% (w/v) BSA and the indicated iodinated ligands. Cellular degradation of ^{125}I -labelled proteins was measured as previously described [43] and is expressed as nanograms of ^{125}I -labelled trichloroacetic acid-soluble (non-iodide) material released into the culture medium per mg of total cell protein.

Metabolic labelling and immunoprecipitation of LRP

CHO-K1 and RPE.40 cells (5×10^5) were grown in 60-mm-diam. dishes for 48 h before the medium was replaced by 2 ml of D-MEM (without cysteine and methionine), containing 20 $\mu\text{g}/\text{ml}$ proline and 200 $\mu\text{Ci}/\text{ml}$ Trans ^{35}S -label. After incubation for 1 h at 37 °C the medium was replaced by 2 ml of complete medium and incubations were continued for the indicated time (chase). After the chase period, the medium was removed, the cells were recovered from the dish by scraping and lysed in 100 μl of buffer

A (20 mM Tris/HCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 150 mM NaCl, 1% Triton X-100, pH 7.5). Nuclei were removed by centrifugation at 12000 g for 2 min and the supernatants were pre-cleared by incubation with 100 μg of rabbit non-immune IgG for 30 min at 4 °C and subsequent addition of 100 μl of a 1:1 slurry of Protein A-Sepharose in buffer B (20 mM Tris/HCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 150 mM NaCl, 0.5% Triton X-100, pH 7.5) for 30 min at 4 °C. LRP was then precipitated using polyclonal rabbit anti-LRP IgG. The immunoprecipitates were washed three times in 500 μl of buffer B, once in buffer C (20 mM Tris/HCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 500 mM NaCl, 0.5% Triton X-100, pH 7.5) and once in buffer D (20 mM Tris/HCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 150 mM NaCl, pH 7.5). For neuraminidase treatment, the immunoprecipitates were split into two equal parts and incubated in 50 μl of 20 mM sodium citrate, 2 mM CaCl_2 , 150 mM NaCl, pH 6.0, without or with the addition of 70 m-units neuraminidase (Sigma). After incubation for 2 h at 37 °C, the proteins were subjected to reducing SDS/5–10%-PAGE and fluorography.

Expression of recombinant forms of LRP in CHO cell lines

A PCR fragment encoding the LRP leader peptide and the first 5 amino acids of the mature N-terminus (nucleotide 467–538; GenBank/EMBL accession no. for LRP is X13916) was cloned into expression plasmid pCMV8 [44] to generate plasmid pT113. Conservative nucleotide changes (nucleotide 535C to T, nucleotide 538T to C) were introduced to generate an *Sma*I restriction site at the 3' end of the fragment. To construct soluble forms of regions III (nucleotides 8036–10462) and IV (nucleotides 10466–13018) (IIIs and IVs) cDNA fragments were derived by PCR from the human LRP cDNA and cloned blunt-ended into *Sma*I-digested plasmid pT113. The following oligonucleotides were used for PCR amplification: 5'-TGCTGGTCGACTCAGCCCACACCATGCTGACCCCCCG-3' and 5'-GGGAGCGTCGATAGCCGCCGCGACC-3' to amplify the leader peptide, 5'-AAGACTTGCCGAGCACAAGATGAG-3' and 5'-CTCGAGTTAGGACACACAGGTGCGCCCATCGCTG-3' for region III and 5'-AAGACTTGACGGCTAGCCAGTTTG-3' and 5'-CTCGAGTTAAGGCACGCATGTGCCGTTGTCCAGC-3' to amplify region IV. The first cysteine in each region is preceded by the first seven amino acids of the mature LRP. This is intended to ensure proper cleavage of the signal peptide. Amino acid numbers 1 to 5 are encoded by the leader peptide, numbers 6 and 7 by the oligonucleotides used to amplify the regions. All regions are followed by a single stop codon (TAA). No other sequence alterations were introduced. To express the soluble regions of LRP 5×10^5 cells/dish were seeded into 60-mm-diam. dishes and grown for 24 h. The medium was replaced by 1.5 ml of D-MEM containing 2 $\mu\text{g}/\text{ml}$ plasmid DNA mixed with Transfectam reagent (Promega). The mixture of DNA and cationic lipopolyamine (Transfectam) had been preincubated according to the manufacturer's protocols. After incubation for 5 h at 37 °C the transfection mixture on the cells was replaced by complete medium. For isolation of ^{35}S -labelled truncated forms of LRP, transfected cells were metabolically labelled for 2 h as described above. The secreted soluble regions of LRP were isolated from the medium by immunoprecipitation.

Proteolytic cleavage of LRP region IVs *in vitro*

Secreted region IVs, produced by transiently transfected and metabolically labelled RPE.40 cells, were incubated with purified recombinant furin or cell membrane preparations [38] in a buffer containing 10 mM Hepes, 3 mM CaCl_2 and 0.1% Triton X-100

(pH 7.2). Reaction mixtures (100 μ l final volume) were incubated for 16 h at room temperature on a rocking platform. Region IVs was immunoprecipitated with polyclonal rabbit anti-LRP antibody, resolved on SDS/7.5%-polyacrylamide gels, and analysed by autoradiography.

RESULTS

Cellular resistance to PEA can be acquired by different biochemical mechanisms such as mutations that render elongation factor-2 insensitive to ADP-ribosylation by the toxin [45], mutations that affect proteolytic activation of PEA [38], or a genetic receptor defect that abolishes cellular uptake of the toxin via LRP-mediated endocytosis [22]. Recently, a furin-deficient CHO cell line (RPE.40) has been described, which is resistant to PEA [27,28]. Because LRP itself is proteolytically processed, we wanted to test whether furin activity is necessary to cleave LRP and whether furin-deficiency in RPE.40 cells results in a defect of LRP-mediated ligand uptake and PEA resistance. To do so, we compared LRP processing in the furin-deficient RPE.40 cells, the parental cell line CHO-K1 and the RPE.40 subclone 40.fur13d, which is stably transfected with an expression construct containing the mouse furin cDNA [28].

Accumulation of LRP 600 in RPE.40 cells

Membrane proteins were isolated from CHO-K1, RPE.40 and 40.fur13d cells and subjected to Western blot analysis using an antibody directed against the C-terminus of LRP present in both the 600 kDa precursor (LRP 600) and the 85 kDa subunit of the mature receptor (LRP 85). As shown in Figure 1, RPE.40 cells exhibited a significant increase in the ratio of LRP 600 to LRP 85 (lane 5) when compared with parental CHO-K1 cells (lane 4), indicating impaired conversion of the precursor into the mature form of the receptor. Stable expression of the mouse furin cDNA restored normal LRP processing efficiency in RPE.40 cells (40.fur13d, lane 6). The total amount of receptor protein expressed in all three cell lines was similar, as demonstrated with a polyclonal antibody directed against both LRP 600 and LRP 515 (lanes 1–3), which migrate with the same apparent mobility

in the gel system. Receptors expressed in RPE.40 cells were able to bind PEA as were receptors of CHO-K1 and 40.fur13d cells. This was shown by ligand blot analysis using 125 I-labelled PEA (Figure 1, lanes 7–9).

LRP-mediated ligand uptake by RPE.40 cells

To determine if RPE.40 cells were impaired in LRP-mediated endocytosis we analysed the efficiency of cellular uptake and lysosomal degradation of 125 I-tPA (Figure 2a), 125 I-tPA complexed with PAI-1 (Figure 2b), 125 I-uPA complexed with PAI-1 (Figure 2c) and methylamine-activated α_2 -macroglobulin (Figure 2d). Although RPE.40 cells were able to internalize and degrade these ligands, they did so at a consistently lower rate than CHO-K1 cells or RPE.40 cells in which furin activity had been restored by transfection with a furin expression construct (40.fur13d).

Defect of LRP cleavage in RPE.40 cells

The accumulation of LRP 600 in RPE.40 cells is due to reduced efficiency of proteolytic processing and not to a defect in transport of the receptor from the endoplasmic reticulum to the *trans*-Golgi compartment where processing takes place. This was shown by a pulse-chase experiment in which we monitored the processing of N-linked carbohydrates on LRP from the neuraminidase-resistant to the neuraminidase-sensitive form in the *trans*-Golgi. CHO-K1 and RPE.40 cells were metabolically labelled for 1 h using [35 S]methionine and [35 S]cysteine and then chased with unlabelled amino acids for up to 2 h. Immediately after labelling and after chase for up to 30 min, LRP was present as a neuraminidase-resistant 600 kDa precursor in both CHO-K1 and RPE.40 cells (Figure 3, lanes 1–8). The amount of non-sialylated precursor produced was similar in both cell types. After a 2 h chase, the protein band in CHO-K1 cells increased its mobility in the gel after neuraminidase treatment (lane 10), indicating that it consisted of LRP 515, carrying N-linked carbohydrates in the neuraminidase-sensitive form. A faint higher-molecular-mass band still visible after neuraminidase treatment may represent either non-sialylated LRP 600, or LRP 515 which had not been desialylated completely. As shown in Figure 1 and results published previously [23], both forms of the receptor migrate with the same mobility in the gel. In contrast, in RPE.40 cells, LRP was present in two forms after the 2 h chase, one being of slower mobility than N-glycosylated LRP 515 or non-sialylated LRP 600 and one of the same mobility (lane 11). Both bands migrated faster after neuraminidase treatment, indicating that they represent LRP 600 and LRP 515, both with neuraminidase-sensitive carbohydrates attached (lane 12). As sensitivity to neuraminidase treatment is acquired in the *trans*-Golgi compartment, this implies that transport and carbohydrate modification of the receptors is normal in RPE.40 cells, but proteolytic processing is inefficient compared with CHO-K1 cells. A considerable amount of LRP 600 apparently traverses the Golgi compartments without undergoing proteolytic processing.

Figure 1 Expression of LRP in CHO-K1, RPE.40 and 40.fur13d cells

Partially purified membrane proteins from cell lines CHO-K1, RPE.40 and 40.fur13d were subjected to non-reducing 4–15% (lanes 1–6, 20 μ g of protein per lane) or 5–10% (lanes 7–9, 100 μ g of protein per lane) SDS/PAGE and transferred to nitrocellulose. The position of migration of the precursor (LRP 600) and the two subunits of mature LRP (LRP 515 and 85) as well as marker proteins (rainbow marker, Amersham) in the gel is indicated. The nitrocellulose membranes were subjected to immunoblot analysis with 5 μ g/ml of polyclonal anti-LRP antibodies that are directed against the whole protein (IgG 483, lanes 1–3) or the cytoplasmic tail of the receptor only (IgG 377, lanes 4–6). Binding of the antibodies was detected using the enhanced chemiluminescence detection system (ECL, Amersham). The nitrocellulose strips in lanes 7–9 were subjected to ligand blot analysis using 5×10^6 c.p.m./ml of 125 I-labelled *Pseudomonas aeruginosa* exotoxin A (125 I-PEA, specific radioactivity: 1.3×10^5 c.p.m./ng). The blot was exposed to Kodak XAR film for 48 h.

Processing of LRP is not necessary for transport through the secretory pathway

To assess further the significance of the proteolytic processing event for transport of LRP to the cell surface, we constructed soluble truncated forms of the receptor and studied their intracellular transport in RPE.40 cells. As shown in Figure 4, the extracellular domain of LRP is organized in four distinct segments (regions I to IV), each of which is characterized by N-terminal ligand binding-type repeats, followed by one or more

Figure 2 Degradation of ^{125}I -labelled ligands in CHO-K1, RPE.40 and 40.fur13d cells

Replicate monolayers of CHO cell lines CHO-K1, RPE.40 and 40.fur13d received 1 ml of D-MEM (without glutamine) containing 0.2% (w/v) BSA, and either ^{125}I -tPA/YPACK (**a**; 100 ng/ml ^{125}I -tPA, specific radioactivity: 2662 c.p.m./ng), ^{125}I -tPA/PAI-1 (**b**; 100 ng/ml of ^{125}I -tPA, specific radioactivity: 2662 c.p.m./ng), ^{125}I -uPA/PAI-1 (**c**; 100 ng/ml ^{125}I -uPA, specific radioactivity: 9156 c.p.m./ng) or $5 \mu\text{g/ml}$ ^{125}I - α_2 -macroglobulin (**d**; specific radioactivity: 688 c.p.m./ng). Prior to addition to the cell medium, ^{125}I -tPA had been inactivated by incubation with the peptide inhibitor tyrosyl-prolyl-arginyl-chloromethane ketone (YPACK) to prevent it from binding to PAI-1 secreted by the cells (**a**). After incubation at 37°C for the indicated periods of time, the amount of ^{125}I -labelled degradation products secreted into the medium was determined. Each value represents the mean of duplicate incubations of 1 (**a**, **b** and **c**) or 3 (**d**) individual experiments. The error bars represent the standard error of the mean. Where not shown, the error bar is smaller than the symbol used.

Figure 3 Biosynthesis and processing of ^{35}S -labelled LRP in CHO-K1 and RPE.40 cells

CHO-K1 and RPE.40 cells were pulse-labelled for 1 h with Trans ^{35}S -label as described in the Experimental section. The cells were then chased with complete medium for the indicated periods of time, after which detergent-solubilized cell extracts were immunoprecipitated with polyclonal anti-LRP serum. The immunoprecipitates were divided into two equal parts that were either not treated (—) or treated with neuraminidase (+). The samples were run on an SDS/5%-polyacrylamide gel and subjected to fluorography. The gel was exposed for 5 days. The positions of the precursor (LRP 600) and the large subunit of the mature receptor (LRP 515) are indicated.

epidermal growth factor-precursor homologous domains. Using a PCR-approach we generated expression constructs for soluble regions of LRP containing amino acids 2501 to 3313 (region IIIs)

or amino acids 3315 to 4163 (region IVs) fused to the authentic leader peptide of the receptor. Region IVs contains the tetrabasic processing site of LRP. In previous experiments, we had shown that region IVs, when fused to the membrane anchor and the cytoplasmic tail of LRP, is fully functional as a minireceptor in binding and uptake of ligands into cells [46].

To study the intracellular transport of regions IIIs and IVs, we transiently transfected CHO-K1, RPE.40 and 40.fur13d cells with constructs containing the different cDNA fragments driven by the strong cytomegalovirus promoter. Transport of the truncated LRPs through the constitutive secretory pathway should result in secretion of the proteins into the medium, from where they could be recovered by immunoprecipitation. If processing was necessary for transport to the cell surface, only cleaved region IVs should be recovered from the extracellular space. All three cell lines efficiently produced and secreted the truncated LRPs into the medium (Figure 5), and no difference in the expression pattern of region IIIs could be observed (lanes 2, 5 and 8). Region IVs was predominantly recovered as a 80 kDa processed form (IVs*) from the medium of CHO-K1 (lane 3) and 40.fur13d cells (lane 9). In contrast, RPE.40 cells secreted region IVs exclusively in the unprocessed 120 kDa form (IVs, lane 6).

Figure 4 Structure of wild-type and truncated LRPs

A comparison of the structural organization of the wild-type protein and truncated versions of the receptor are shown. The extension of the individual extracellular regions of LRP (I–IV) are indicated by brackets. The constructed soluble forms of LRP (IIIs and IVs) contain the leader peptide of the wild-type receptor followed by regions III and IV. The arrow indicates the position of the proteolytic processing site.

Figure 5 Transient expression of regions IIIs and IVs in CHO-K1, RPE.40 and 40.fur13d cells

Replicate monolayers of CHO-K1 (lanes 1–3), RPE.40 (lanes 4–6) and 40.fur13d cells (lanes 7–9) were transfected with 1.3 μ g/ml of plasmid DNA encoding the LRP leader peptide only (Mock; lanes 1, 4 and 7) or regions IIIs (lanes 2, 5 and 8) and IVs (lanes 3, 6 and 9). After incubation for 48 h at 37 °C the cells were labelled metabolically and 700 μ l of the medium was subjected to immunoprecipitation using a polyclonal anti-LRP antibody (IgG 758). The samples were run on a reducing SDS/5–10%-polyacrylamide gel and subjected to fluorography for 3 days. The position of migration of region IIIs, unprocessed (IVs) and processed region IVs (IVs*) as well as phosphorylase b (97 kDa) and BSA (69 kDa) in the gel is indicated.

Proteolytic cleavage of region IVs by furin *in vitro*

The specificity of LRP processing by furin was further investigated by studying the cleavage of LRP by furin *in vitro*. Region IVs was transiently expressed in CHO-K1 and RPE.40 cells, recovered by immunoprecipitation from the medium of trans-

Figure 6 Processing of LRP region IVs by furin *in vitro*

CHO-K1 and RPE.40 cells were transfected transiently with the region IVs coding construct and labelled metabolically. The expressed region IVs was recovered from the medium by immunoprecipitation and incubated with either purified recombinant furin or membrane preparations from the various cell strains. Lane 1, mock-transfected control cells; lane 2, region IVs produced in CHO-K1 cells; lane 3, region IVs produced in RPE.40 cells; lanes 4–7, region IVs, produced in RPE.40 cells, treated *in vitro* with 750 μ g of CHO-K1 membranes (lane 4), 1 mg of RPE.40 membranes (lane 5), 100 μ g of 40.fur13d membranes (lane 6), or 0.5 ng of purified furin (lane 7). After separation on SDS/polyacrylamide gels, region IVs was analysed by autoradiography. The position of migration of unprocessed (IVs) and processed region IVs (IVs*) in the gel is indicated. The asterisk denotes an unknown protein present in all samples, including the mock-transfected control cells.

ected and metabolically labelled cells and processed *in vitro* by purified recombinant furin or cell membrane fractions (Figure 6). As shown before in Figure 5, CHO-K1 cells secreted region IVs predominantly in the processed form (IVs*, lane 2), whereas RPE.40 cells exclusively produced the higher-molecular-mass precursor (IVs, lane 3). Incubation of region IVs precursor from RPE.40 cells with either 0.5 ng of purified furin (lane 7) or with furin-containing membrane extracts from 40.fur13d cells (lane 6) resulted in efficient conversion into the processed form IVs*. In contrast, extracts from CHO-K1 cells (lane 4) or from RPE.40 cells (lane 5) did not process the LRP region IVs precursor. This was due to either the absence of furin (RPE.40) or low amounts of furin present in these cell extracts (CHO-K1) which were insufficient to detect processing in this *in vitro* assay. Cell extracts from furin-overproducing 40.fur13d cells contain 75-fold higher furin activity than the parental CHO-K1 cells [38].

DISCUSSION

In this study we have demonstrated that the endoprotease furin processes the 600 kDa precursor of LRP into the 515 kDa and 85 kDa subunits that make up the mature receptor. Furin-deficient RPE.40 cells did not efficiently process endogenous LRP or a truncated soluble form of the receptor containing the processing site. The processing defect could be corrected by overexpression of a mouse furin cDNA in cultured cells and by purified furin *in vitro*.

Furin-mediated processing is an important regulatory step for activation of proproteins such as viral membrane glycoproteins or bacterial toxins. The significance of proteolytic cleavage for the function of cell-surface receptors is less clear at present. Uncleaved insulin receptor binds significantly less insulin than the mature receptor [29], whereas the precursor of the hepatocyte growth factor/scatter factor is fully functional in signal transduction [24]. We were unable to unambiguously demonstrate whether processing is necessary for activation of LRP, because the furin-deficient RPE.40 cells produced residual amounts of mature heterodimeric LRP (Figures 1 and 5). However, reduced processing activity in these cells correlated with a slight but consistent reduction in LRP-mediated endocytosis, and normal

LRP activity was restored by overexpression of furin in these cells. Taken together with the fact that a fraction of the LRP transported in RPE.40 cells to the cell surface is in the precursor rather than the mature form (Figure 5), these findings raise the possibility that uncleaved LRP 600 does not bind certain ligands. An alternative, albeit unlikely, explanation would be that furin itself participates directly or indirectly in ligand degradation.

Not only LRP but also its ligand PEA is proteolytically processed by furin. Therefore, toxin resistance in RPE.40 cells most likely results from their inability to activate PEA and not from a defect in toxin uptake. Receptors expressed in these cells were able to bind PEA *in vitro* and efficiently internalized ligands in culture. Support for this hypothesis comes from the observation that membrane extracts from RPE.40 cells were unable to cleave PEA *in vitro*, whereas CHO-K1 extracts did so efficiently [38].

RPE.40 cells are unable to process endogenous insulin receptor, PEA or a truncated soluble form of LRP, region IVs. Endogenous full-length LRP, on the other hand, is processed, albeit at low efficiency. This might be explained by the fact that other proteases exist in RPE.40 cells that can partially compensate for furin in the processing of endogenous LRP. Furin is a member of a family of related precursor processing endoproteases or convertases which act either in the constitutive or the endocrine secretory pathway [30]. In particular, the endoproteases PACE4 and PC6 have been identified, whose substrate spectrum overlaps with that of furin. These, or other uncharacterized proteases, might form a backup system for the maturation of LRP.

Although a fraction of endogenously synthesized LRP was processed in untransfected RPE.40 cells, RPE.40 cells that had been transiently transfected with a construct expressing region IVs did not process this truncated receptor. One possible explanation might be that the residual, inefficient LRP-processing mechanism in RPE.40 cells becomes saturated and is unable to process a significant fraction of the overexpressed region IVs. Alternatively, as maturation of LRP 600 is not necessary for transport to the cell surface, membrane-bound precursors may repeatedly enter the endocytic pathway which contains furin and perhaps other related proteases [31,32]. There, the full-length receptor, through repeated exposure to the protease, might be processed by this less efficient maturation mechanism that is not accessible to secreted proproteins or soluble region IVs.

In conclusion, analysis of LRP processing in RPE.40 cells has revealed furin as the primary protease responsible for the cleavage of LRP 600. Our results have also shown that maturation is not required for intracellular transport of the receptor. These findings will aid in future research into the mechanism and regulatory role of this maturation process, which is unusual for cell-surface receptors and unique within the LDL receptor superfamily.

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