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A MUTAGENESIS STUDY OF THE CHICKEN LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN

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Thesis submitted to the Department of Biochemistry, Microbiology, and Immunology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

University of Ottawa

Abstract

The low density lipoprotein receptor-related protein (LRP) is a 600 kDa endocytic plasma membrane receptor. LRP is involved in the uptake of numerous structurally and functionally unrelated ligands, including α_2-macroglobulin:protease complexes (α_2M*), apolipoprotein E-enriched remnant lipoproteins, *pseudomonas* exotoxin A (PEA), and receptor associated protein (RAP). To date, analysis of LRP ligand-binding regions has proven challenging due to its large size and repeating modular structure. Using a recombinant DNA approach, full-length somatic chicken LRP, as well as several deletion mutants, were stably expressed in a mutant CHO-derived cell line deficient in endogenous LRP expression. LRP100, LRP67 and LRP25 encode 100%, 67% and 25% of the protein respectively, with LRP67 and LRP25 encoding large internal deletions but retaining an N-terminal portion, transmembrane region, and cytoplasmic tail. These three mutants were found to possess varying degrees of ligand-binding activity toward α_2M*, RAP, and PEA. Generation of this expression system has allowed the mapping of several LRP ligands to the N-terminal portion of the receptor and should allow further study of domains within LRP responsible for its multifunctionality. It was recently postulated that eight spacer regions within LRP containing repeating YWTD motifs adopt β-propeller conformations. While developing cell lines expressing refined mutants encoding deletions of putative ligand-binding regions, disruption of the naturally occurring, ordered arrangement of β-propeller domains was found to have an effect on intracellular trafficking and plasma membrane presentation of mutant receptors. Although highly expressed, several mutants possessing crippled β-propellers were not detected at the plasma membrane by biotin labeling. These mutants also exhibited delayed or no resistance to endoglycosidase H (endo H) suggesting ER exit was delayed or impaired. Restoration of integral β-propellers and flanking EGF modules restored ER exit and plasma membrane presentation to a transport incompetent mutant. The β-propeller domains may play an important role in conferring structural stability to LRP. Caution should therefore be exercised in the mutagenesis of this enormous receptor.

Acknowledgements

A project of this magnitude can hardly be completed by a single individual. It is, therefore, a fitting tribute to devote the initial pages of this thesis to those individuals that have been instrumental to its development.

I would first like to acknowledge Dr. Yuwei Wang who was my first molecular biology teacher. Under his initial guidance, I learned the basic techniques that enabled me to carry out a large part of the work described in this thesis. I would also like to acknowledge Dr. Roger McLeod for his patient instruction and assistance in the development of ideas as well as the refinement of many experimental techniques used in these studies. Roger's input was instrumental in the evolution of several "quick and dirty" preliminary experiments into more elegant ones.

I would like to thank Dr. Guojun Bu, Dr. Dudley Strickland, Dr. Nabil Seidah and I am especially grateful to Dr. Johannes Nimpf and Dr. David FitzGerald. Their willingness to share elements of their own research and their continued collaboration in large part enabled this project to get off the ground.

I would like to acknowledge my fellow laboratory members past and present; Robert Brown, Dr. Khai Tran, Jelena Vukmirica, and Philip Links. In particular, I would like to thank Robert and Jelena for their contribution to this work.

I would also like to thank Tracey Neville for her help with instruments and equipment as well as Vivian Franklin for maintenance of the tissue culture facilities. Thanks also to Dr. Xiaohui Zha for teaching me some basic microscopy principles and techniques on which I hope to build on in the future.

I would like to acknowledge my thesis committee members Dr. Daniel Sparks and Dr. Yves Marcel for their guidance during the course of my studies at the University of Ottawa. Their willingness to listen to both problems and progress and to offer advice and guidance as to possible research directions was very much appreciated. I would like to acknowledge Dr. Ross Milne for many helpful discussions and for answering my endless questions as well as Dr. Ruth McPherson and Malcolm Robb for their help in obtaining human adipocytes.

I also would like to acknowledge Dr. Kerry Ko who shared this project with me from the start. Kerry worked alongside me on every aspect and was involved in the development of everything from cell lines to techniques to conceptual ideas. Kerry's direct or indirect contribution to this work is a sizeable one and for this I owe him a large debt of gratitude.

Finally, I am sincerely grateful to my thesis supervisor, Dr. Zemin Yao, for having the faith and patience to allow me to undertake this challenging research project. I have come to realize that Dr. Yao's most important role in the past five years was to keep me on the right course while allowing me the freedom to explore my own ideas. Although often frustrating and difficult, this has allowed me to leave the Ottawa Heart Institute a little closer to my goals.

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List of abbreviations

α_2M	alpha-2-macroglobulin
α_2M*	activated alpha-2-macroglobulin
ACAT	acyl CoA cholesterol acyltransferase
AD	Alzheimer's disease
APP	amyloid precursor protein
apoB	apolipoprotein B
apoE	apolipoprotein E
apoER2	apolipoprotein E receptor 2
BSA	bovine serum albumin
CAD	coronary artery disease
CHO	Chinese hamster ovary
C-terminus	carboxyl terminus
DMEM	Dulbecco's minimum essential medium
DTT	dithiothreitol
EDTA	ethylene-diaminetetra acetic acid
EF2	elongation factor 2
EGF	epidermal growth factor
Endo H	endoglycosidase H
ER	endoplasmic reticulum
FH	familial hypercholesterolemia
GST	glutathione-S-transferase
HRP	horseradish peroxidase

Kb	kilobase
kDa	kilo Dalton
LDL	low density lipoprotein
LRP	low density lipoprotein receptor-related protein
N-terminus	amino terminus
PBS	phosphate buffered saline
PEA	*pseudomonas* exotoxin A
PEG	polyethylene glycol
PMSF	phenylmethylsulfoxide
PNGase F	peptide: N-glycosidase F
PVDF	polyvinylidene difluoride
RAP	receptor associated protein
SDS	sodium dodecylsulfate
TCA	trichloroacetic acid
VLDL	very low density lipoprotein

1. Introduction

1.1 General background

The plasma membrane receptors are membrane proteins that have evolved as a sophisticated mechanism allowing communication between extracellular and intracellular environments. One of the functions of the plasma membrane receptors is to internalize cargo molecules required for cellular growth or maintenance.

1.1.1 LRP is a member of the LDL receptor gene family

The low density lipoprotein receptor-related protein (LRP) is one of the larger members of the low density lipoprotein (LDL) receptor gene family. In humans, this gene family includes several members including the LDL receptor itself (1), megalin (2), the very low density lipoprotein (VLDL) receptor (3), the apolipoprotein E receptor 2 (apoER2) (4), LRP (5), LRP5 (6), LRP6 (7), and LRP1B (8). These structurally related plasma membrane receptors deliver extracellular ligands to lysosomes for degradation.

LRP was identified from a mouse lymphocyte cDNA library using primers complimentary to ligand-binding regions of the LDL receptor (5). All members of the LDL receptor gene family are mosaic proteins composed of tandemly arranged, recurring protein modules depicted in figure 1 and described below. Significant differences between the members of this gene family occurs in the number and the arrangement of constituent modules and in the ligands bound and internalized by each receptor (9,10).

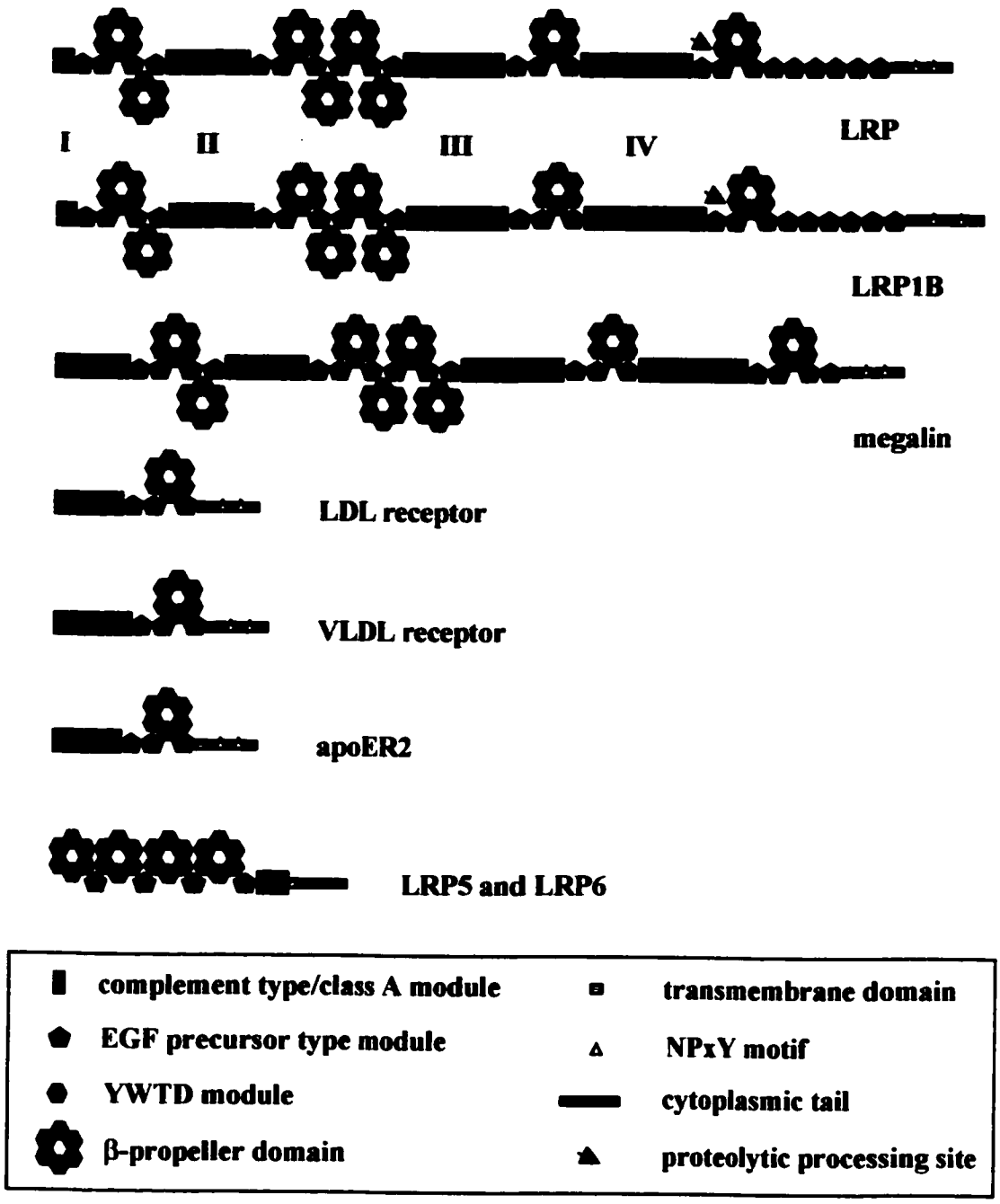

Figure 1. LRP is a member of the LDL receptor gene family.

Schematic representation of mammalian LDL receptor gene family members. Structures have been depicted to emphasize LDL receptor complement-type/class A modules and EGF precursor type regions comprised of EGF modules and spacer regions that contain repeating YWTD or YWTD-like motifs. *Roman numerals* indicate clusters of ligand-binding complement-type/class A modules. Adapted from Springer *et al.* (11).

1.1.2 Function of LDL receptor gene family members

The seminal work of Goldstein and Brown was fundamental to our understanding of LDL receptor mediated endocytosis of LDL and cellular cholesterol homeostasis (1,12). The LDL receptor contains binding sites for apolipoprotein B (apoB), the sole protein component of LDL, as well as apolipoprotein E (apoE), a protein found at the surface of several different lipoprotein classes.

Studies of the newer members of the LDL receptor family (reviewed in 10) suggest that these receptor proteins may be involved in the uptake of another lipoprotein class, the remnant lipoproteins. Remnant lipoproteins are large, triacylglycerol-rich lipoproteins implicated in the pathogenesis of atherosclerosis and believed to bind LRP via apoE (13).

In contrast to the LDL receptor, LRP binds a wide variety of structurally and functionally diverse ligands, several of which are involved in lipoprotein metabolism. These include lipoprotein lipase (14) and hepatic lipase (15), two triacylglycerol hydrolases that convert triacylglycerol into glycerol and free fatty acids. Both lipases share sequence homology, but are believed to be localized to different tissues. Lipoprotein lipase is synthesized in several tissues including adipose and muscle, but is localized to the vascular endothelium. Hepatic lipase is localized to the liver.

LRP also binds ligands involved in protease metabolism. These include both urokinase type (uPA) and tissue type plasminogen activators (tPA), either alone or bound to their inhibitor, plasminogen activator inhibitor (PA:PAI1) (16,17), as well as activated alpha-2-macroglobulin (α_2M*) (18,19), and complement C3 (20). Although α_2M* and

complement C3 share some sequence homology, they are involved in different metabolic processes.

Pathogens such as minor group rhinovirus (21,22) and *pseudomonas* exotoxin A (PEA) (23) also bind LRP. The intracellular molecular chaperone termed receptor associated protein (RAP) binds LRP with high affinity and has been shown to inhibit binding of all known LRP ligands when added to cells in a purified form (24,25). A comprehensive list of LRP ligands is shown in Table I. Ligands relevant to this work are discussed in detail below.

The VLDL receptor is expressed in adipose tissue, heart and brain (3). The apoER2 receptor is expressed in the brain and placenta (4). Both receptors are specific for apoE. There is evidence that both the VLDL receptor and apoER2 may be required in embryonic development. Mice lacking both receptors exhibit a phenotype similar to Reelin and disabled 1 knockout animals, which suffer from severe central nervous system developmental abnormalities (26).

Megalin was originally identified as the antigen causing a renal autoimmune disorder in rats known as Heymann nephritis. This receptor is highly expressed in several types of epithelial cells, particularly within the kidney. Like LRP, megalin possesses a wide ligand-binding specificity and binds several structurally and functionally diverse ligands (10). Homozygous megalin knockout mice show brain, lung, and kidney abnormalities (27).

LRP5 is highly expressed in the adrenal gland cortex and adipocytes. LRP6 appears to be expressed in many tissues including heart, brain, placenta, lung, kidney,

Protease metabolism	Lipoprotein metabolism	Other
α_2-macroglobulin:protease complexes (18,19)	apolipoprotein E-enriched lipoproteins (14,40,41)	receptor associated protein (24,25)
PAI-1 complexes with tPA (17), uPA (16), or thrombin (30)	lipoprotein lipase (14)	lactoferrin (43)
protease nexin-1 complexes with uPA or thrombin (31)	hepatic lipase (15)	*pseudomonas* exotoxin A (23)
protease nexin-2/secreted β-amyloid precursor protein (32)	Lp(a) (42)	minor group rhinovirus (21,22)
tissue factor pathway inhibitor (33)		saposin precursor (44)
tPA (17,34) or pro-uPA (35)		malaria sporozites (45)
human pregnancy zone protein (36)		calcium (46)
collagenase 3 (37)		midkine (47)
activated complement C3 (20)		
thrombospondin (38)		
factor VIII (39)		

Table 1. LRP binds a wide variety of functionally unrelated ligands.

Ligands shown to interact with LRP can be subdivided into several classes based on their function.

spleen and the adrenal glands. Both proteins share high sequence homology and both bind apoE enriched β-VLDL (6,7).

Finally, the recently characterized LRP1B has been detected in tumors and is a candidate tumor suppressor. A close similarity in the domain structures of LRP and LRP1B suggests these two receptors may share similarity in their ligand interactions (8).

1.1.3 Species and tissue distribution

Avians (28), humans (5), as well as *c. elegans* (29) share highly conserved forms of the LRP gene, suggesting a common ancestry. The essential role of LRP in development is supported by the observation that LRP knockout in mice prevents embryonic implantation and causes death *in utero* (17). There are currently no known naturally occurring LRP deficient cell types other than platelets (48).

Although LRP is widely expressed in mammalian tissue, its expression level is highest in several cell and tissue types. In humans these include neurons and astrocytes of the central nervous system, hepatocytes, smooth muscle cells, fibroblasts, kidney, lung and intestinal cells (49).

1.1.4 The LRP gene product

The human LRP gene covers approximately 92 kb with a total of 89 exons (50). Full-length human LRP contains 4525 amino acids (5), while full-length chicken LRP contains 4523 amino acids (28). LRP is synthesized as a single 600 kDa polypeptide

chain that undergoes extensive co- and post-translational modifications including N-linked glycosylation and intracellular proteolytic cleavage. Cleavage of LRP yields 515 kDa and 85 kDa subunits denoted as α-chain and β-chain, respectively. Mature LRP is presented at the plasma membrane as an α/β heterodimer of these two subunits (5). In the mature protein, these two subunits remain tightly, but non-covalently associated via an unknown mechanism and are completely separated under denaturing or reducing conditions (5). Proteolytic cleavage of LRP is inhibited in a reversible manner upon addition of brefeldin A, suggesting that cleavage occurs within the late Golgi compartment (51).

1.2 Primary structure of LRP

Examination of LRP primary structure reveals many well known sequences and motifs, some possessing known function in unrelated proteins.

1.2.1 The complement-type/class A modules

LRP is a type I membrane protein consisting of a short cytoplasmic tail, a transmembrane domain, and an unusually large extracellular domain (52). The primary amino acid sequence of LRP is highly repetitive consisting of modules that are arranged in several clusters (figure 2). LRP is composed of 31 LDL receptor complement-type/class A modules each of which is approximately 40 amino acids in length (figure 3). The 31 complement-type/class A modules contain six conserved cysteines that form three disulphide linkages. In addition, an SDE motif forming part of a calcium-binding pocket

Figure 2. Amino acid sequence of full-length chicken LRP.

The amino acid sequence of chicken LRP is shown. *Green*, clusters of LDL receptor complement-type/class A modules; *blue*, EGF modules; *red*, spacer regions containing YWTD or YWTD-like motifs; *red underlined*, YWTD and YWTD-like motifs; *black italics*, putative signal peptide; *double underlined*, furin cleavage consensus motif; *purple bold*, putative transmembrane domain; *purple italics*, cytoplasmic tail.

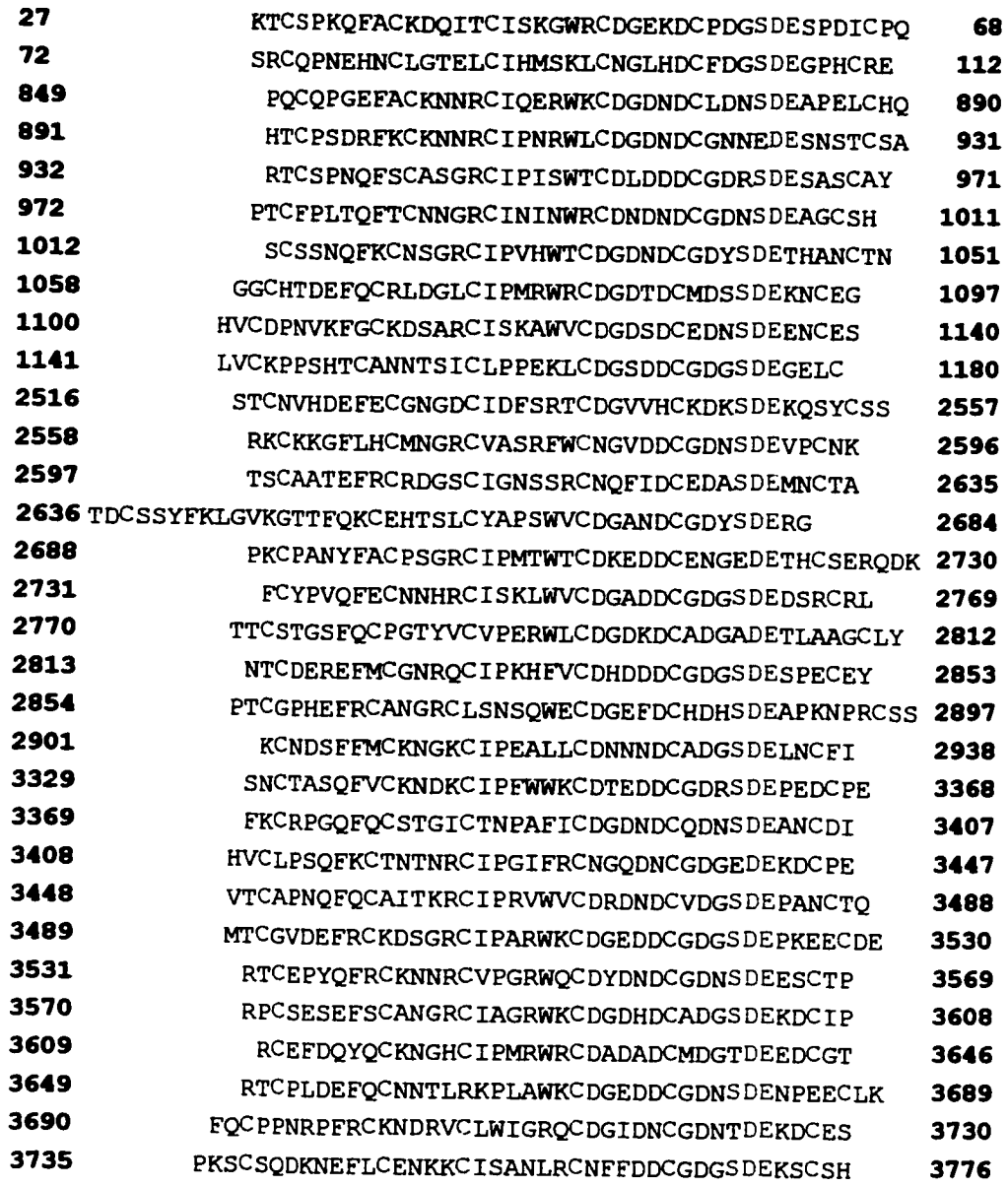

Figure 3. Alignment of the amino acid sequence of chicken LRP complement-type/class A modules.

LRP is comprised of 31 complement-type/class A modules. Conserved residues are highlighted. The six cysteine residues found in each module form three disulphide linkages. The acidic SDE motifs reside in a calcium binding pocket. *Red*, conserved cysteines; *blue*, acidic SDE motifs.

is also conserved among these modules (figure 4). The 31 complement-type/class A modules are grouped into four clusters of two, eight, ten, and eleven, beginning at the N-terminus of LRP (figure 1). Similarity between the LRP complement-type/class A modules and those comprising the ligand-binding domain of the LDL receptor has led to the belief that the ligand-binding regions within LRP may reside within one or more clusters of complement-type/class A modules. Experimental evidence thus far supports this hypothesis, although there is also evidence suggesting that in the case of certain ligands, binding may not be exclusively confined to these regions (53).

1.2.2 The EGF-precursor type modules

The four clusters of complement-type/class A modules are separated by EGF precursor-type regions comprised of EGF modules and spacer regions. Each of the 22 EGF modules is approximately 40 amino acids in length and also contains six cysteines forming three disulphide linkages. Little amino acid homology exists among these regions (figure 5).

In the LDL receptor, it is thought that the EGF modules are involved in ligand dissociation from the receptor within lysosomes (55). There is also evidence that these regions may be involved in the binding of certain LRP ligands (56).

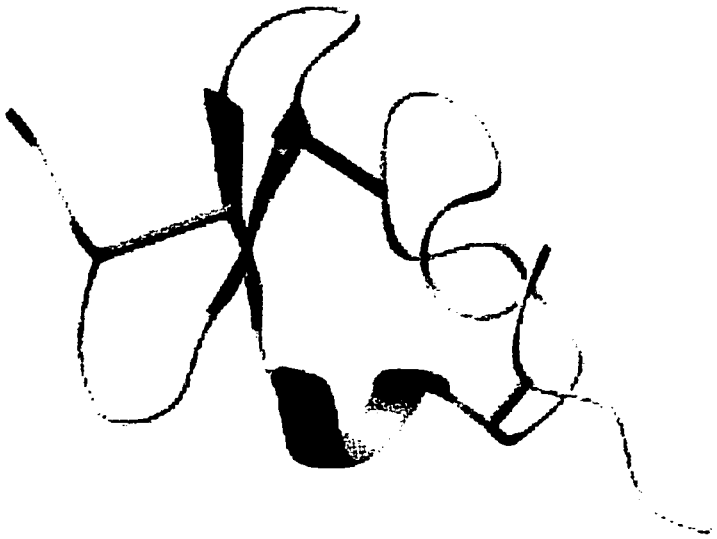

Figure 4. Tertiary structure of the complement type class A module of LRP.

Tertiary structure of the third complement-type/class A module of human LRP solved by NMR (54).

112	QLANCTALGCQHHCVPTLSGPACYCNNSFQLAEDRRSCK	**151**
152	DFDECTVYGTCSQTCTNTEGSYTCSCVEGYLLQPDNRSCK	**191**
476	RSHACEPDQFGKPGGCSDICLLGNSHKSRTCRCRSGFSLGSDGKSCK	**522**
800	GSNKCRVNNGGCSSLCLATPRGRQCACAEDQILGADSVTCE	**841**
1181	DQCSLNNGGCSHNCTVAPGEGIVCSCPLGMELGADNKTCQ	**1220**
1221	IQSYCAKHLKCSQKCEQDKYNVKCSCYEGWMLEPDGESCR	**1260**
1533	APNPCEANGGKGPCSHLCLINYNRTLSCACPHLMKLDKDNTTCY	**1577**
1842	GSNPCSVNNGDCSQLCLPTSETSRSCMCTAGYSLKSGQQSCE	**1883**
2150	GTNVCAQNNGGCQQLCLFRGGGRRTCACAHGMLSEDGVSCR	**2191**
2472	ELSPCRVNNGGCQDLCLLTPKGHVNCSCRGERVLQEDFTCK	**2512**
2938	NECLNKKLSGCSQECEDLKIGYKCRCRPGFRLKDDGKTCI	**2978**
2979	DIDECSTTYPCSQKCINTLGSYKCLCIEGYKLKPDNPTSCK	**3019**
3286	PNHPCKTNNAGCSNLCLLSPGGGHKCACPTNFYLGSDGKTCV	**3328**
3779	KSYDCMTNTTMCGDEAQCIQAQSSTYCTCRRGFQKVPDKNSCQ	**3821**
3822	DVNECLRFGTCSQLCNNTKGSHVCSCAKNFMKTDNMCK	**3859**
4146	VTNPCDRKKCEWLCLLSPSGPVCTCPNGKRLDNGTCV	**4182**
4195	TTDTCDLVCLNGGSCFLNARKQAKCRCQPRYNGERCQ	**4231**
4232	INQCSDYCQNGGLCTASPSGMPTCRCPTFTGSRCD	**4267**
4268	QQVCTNYCHNNGSCTVNQGNQPNCRCPPTFIGDRCQ	**4303**
4304	YQQCFNYCENNGVCQMSRDGVKQCRCPPQFEGAQCQ	**4339**
4340	DNKCSRCQEGKCNINRQSGDVSCICPDGKIAPSCL	**4374**
4375	TCDSYCLNGGTCSISDKTQLPECLCPLEVTGMRCE	**4409**

Figure 5. Alignment of the amino acid sequence of chicken LRP epidermal growth factor modules.

LRP is comprised of 22 EGF modules. The six cysteine residues (*red*) in each module form three disulphide linkages.

1.2.3 The spacer regions

In addition to complement-type/class A modules and EGF-precursor type modules, all the members of the LDL receptor gene family contain at least one spacer region approximately 260 amino acids in length. While the LDL receptor contains a single spacer region, LRP contains eight. Spacer regions contain few cysteines and no disulphide linkages. Each spacer region contains six repeating YWTD or YWTD-like motifs (figure 6). The function of these spacer regions within LRP is currently unknown, however, several studies show impaired ligand dissociation from LDL receptors possessing mutations within this region (55). Evidence also suggests point mutations within this region impair LDL receptor exit from the ER.

1.2.4 Endoproteolytic cleavage motif

LRP contains the consensus motif RxRR that is recognized by several members of the proprotein convertase family of endoproteases (57). These endoproteases are known to cleave several proteins including the insulin receptor (58,59). Chicken LRP contains a RNRR motif beginning at amino acid position 3939 of its extracellular domain, while human LRP contains an RHRR motif beginning at amino acid position 3940. Sequencing of the N-terminus of the human LRP β-chain has confirmed that proteolytic cleavage occurring at this site is necessary for production of the heterodimeric mature form of LRP (51).

3838 AEGSEHQILYIADDNKIRSMYPFNPNSAYEPAFQGDENVRIDAMDIYVK
GNKIYWTNWHTGRISYCELPASSAASTASNRNRRQIDGGVTHLNISGLKMPRGIAVDWV
AGNIYWTDSGRDVIEVAQMKGENRKTLISGMIDEPHAIVVDPL
RGTMYWSDWGNHPKIETAAMDGTLRETLVQDNIQWPTGLAVDYH
NERLYWADAKLSVIGSIRLNGTDPVVAIDNKKGLSHPFSIDIF
EDYIYGVTYINNRIFKIHKFGHKSVTNLTSGLNHATDVVLYHQYKQPE **4124**

Figure 6. Chicken LRP spacer regions contain six repeating YWTD or YWTD-like motifs.

Amino acid sequence of the eighth spacer region of chicken LRP. The YWTD or YWTD-like motifs are highlighted (*red*).

1.2.5 Motifs found within the cytoplasmic tail

Like other members of the LDL receptor family, LRP contains a single membrane-spanning domain predicted to be highly hydrophobic. The intracellular tail of LRP is 100 amino acids in length and contains several sites that may be functionally important.

Two NPxY motifs are found within the cytoplasmic tail of LRP. At least a single copy of this motif is found within the cytoplasmic tails of most members of the LDL receptor family. This motif is also present among many other proteins or receptors including both G protein-coupled receptors such as integrins and non G protein-coupled receptors such as the insulin receptor (60-62). Several functions have been ascribed to the NPxY motif, including receptor internalization via clathrin-coated pits (63), adhesion (64), migration, and metastasis (65). LRP also contains a di-leucine motif as well as a YxxL motif (66). Both motifs have been implicated in internalization mechanisms (67).

1.3 Secondary and tertiary structure of LRP

Little is known about secondary or tertiary structure of LRP. Existing information is either based on NMR studies obtained from small LRP fragments expressed in bacteria or on extensive computer modeling based on homologous sequences within proteins of known structure.

1.3.1 Characterization of LRP fragments expressed in bacteria

LRP complement-type/class A modules three (54) and eight (68) (first and sixth module in cluster II) have been expressed in bacteria. NMR studies of these LRP modules have revealed overall structural similarity to that previously seen in the LDL receptor complement-type/class A modules 1 (69) and 2 (70) by NMR and module 5 (71) by X-ray crystallography. The overall structures all contain a two-strand antiparallel beta-sheet and a one-turn alpha-helix (figure 4). Disulphide linkages are conserved and all the solved structures also appear to contain a high affinity calcium binding pocket containing a common conserved SDE motif. Despite an overall similarity in structure, differences exist within these conserved regions as not all modules possess similar ligand-binding capacity. This has been shown for the LDL receptor where apoE binding requires the fifth complement-type/class A module, while apoB binding requires a combination of modules three to seven with some contribution from adjacent EGF modules (53). To date, there has been no report on the structure of the EGF modules for the members of the LDL receptor gene family.

1.3.2 Theoretical β-propeller domains

Based on computational modeling studies, it has recently been suggested that the spacer regions found within all members of the LDL receptor gene family adopt a β-propeller conformation that is also found in many unrelated proteins (11). β-propellers are torroidal structures composed of between four to eight structurally similar repeats.

Each repeat is likened to a propeller blade in that it contains four antiparallel β strands that radiate from a central axis (72) (figure 7). Nidogen also contains YWTD motifs within a region thought to adopt a β-propeller conformation. By electron microscopy the β-propeller domain of nidogen is visible as a large compact globular region (73).

1.4 Co- and post-translational modification of LRP

LRP undergoes extensive co- and post-translational modifications before it can attain a mature conformation. Ongoing studies are beginning to shed some light on LRP processing and on co-factors believed to aid in LRP maturation.

1.4.1 Glycosylation

Human and chicken LRP are thought to possess 52 and 50 putative N-linked glycosylation sites, respectively. It is currently not known which sites are truly glycosylated as these have not yet been directly characterized. However, endoglycosidase H (endo H) digestions of LRP have shown large shifts in the electrophoretic mobility of the receptor confirming the presence of extensive glycosylation (51).

The precise function of N-linked glycosylation within proteins is currently not entirely clear. It is thought that N-linked glycosylation may be required for correct protein folding (75,76). It is believed that incorrectly folded, N-linked glycoproteins may act as substrate for glucosylating/deglucosylating enzymes or chaperones that can sense misfolded proteins within the ER (77). This retention mechanism forces proteins to

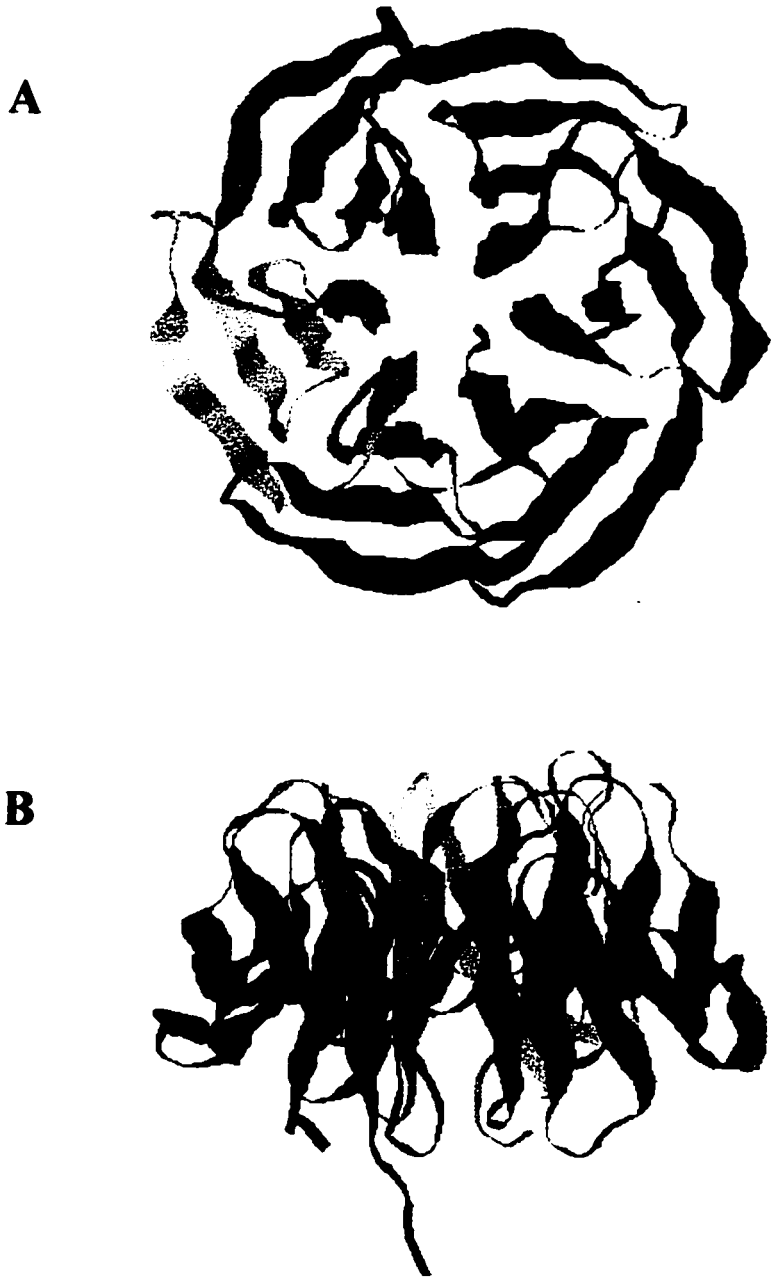

Figure 7. Chicken LRP spacer regions may adopt a β-propeller structure.

Theoretical tertiary structure of the seventh putative six-bladed β-propeller of chicken LRP. Each "blade" is comprised of four anti-parallel β-strands arranged around a central symmetry axis. Each "blade" contains one YWTD or YWTD-like motif. *A*, top view; *B*, side view (74).

remain in the ER until they can reach the correctly folded state. There is, however, conflicting data suggesting that for the v-sis gene product, N-linked glycosylation may not always be essential for plasma membrane presentation of a mature protein (78). It is currently unclear whether the extensive glycosylation present on LRP is required for efficient maturation of the receptor.

1.4.2 Disulphide bond formation

Human LRP possesses an unusually high number of cysteine residues that are involved in the formation of disulphide bonds. It is likely challenging for the cell to correctly fold a protein as large as LRP while forming and maintaining correct disulphide linkages and LRP is thought to fold with the assistance of intracellular chaperone(s) such as RAP. It is not yet known whether other intracellular chaperone molecules such as protein disulphide isomerase, a molecular chaperone known to be involved in the formation of correct protein disulphide linkages (79), is involved in the formation of disulphide linkages within LRP.

1.4.3 Role of molecular chaperones in LRP folding and expression

Maturation of LRP is dependent on RAP expression (80), and presentation of mature LRP was found to be severely impaired in cells derived from RAP deficient mice (81). RAP is a 39 kDa, ER resident protein that was first identified as a co-eluent with LRP by affinity chromatography. RAP is believed to act as a folding chaperone for LRP as well as other members of the LDL receptor gene family (82-84). Localization of RAP

to the ER suggests it may function as a folding chaperone (85). RAP contains a HNEL sequence similar to the KDEL sequence found in other ER resident proteins. This sequence is required for ER retention by the KDEL receptor or a KDEL-like receptor. Deletion of the HNEL sequence caused RAP to be secreted and decreased its intracellular half-life (85).

RAP may exert an additional novel chaperone function, which prevents the premature binding of LRP to ligands that are often synthesized within the same tissue. For example, overexpression of apoE in murine embryonic fibroblasts caused a dramatic reduction in cellular LRP expression, an effect that could be prevented by overexpression of RAP (80). Knockout of RAP in mice resulted in a 75% decrease in the formation of mature processed LRP and a concomitant 75% decrease in ^{125}I-α_2M clearance from the circulation (81). In the absence of RAP overexpression, cellular retention of individual LRP minireceptors encoding each of the complement-type/class A clusters but not the transmembrane domain or cytoplasmic tail was observed under non-reducing conditions. These aggregates were dissociated to monomers under reducing conditions suggesting that the formation of intermolecular disulphide bonds caused the aggregation. A decrease in aggregation of the same soluble LRP fragments was observed upon RAP co-expression.

Recent evidence has shown that the LDL receptor binds the molecular chaperone BiP suggesting this intracellular chaperone molecule is involved in the folding of this LDL receptor family member (86). Whether BiP also aids in LRP folding remains to be determined.

1.4.4 Role of calcium in LRP folding

Calcium is essential for binding of ligands to all members of the LDL receptor gene family. Evidence now suggests that calcium may also be involved in LRP folding. Following intracellular calcium depletion with ionomycin or thapsigargin, both endogenous HepG2 LRP as well as recombinant LRP minireceptors, showed aggregation thought to be caused by intermolecular disulphide bond formation. This effect was reversible upon addition of calcium (87). This effect also appears to be RAP-independent, as RAP remained bound to LRP mini-receptors following calcium depletion.

1.4.5 Proteolytic cleavage of LRP in the Golgi compartment

Sequencing of the N-terminus of the LRP β-chain has confirmed that cleavage of human LRP occurs following the consensus motif RHRR (5). The endoprotease responsible for LRP cleavage has been identified as furin. Using a furin deficient cell line, the proteolytic processing of endogenous LRP was shown to be virtually abolished (88). Furin was also shown to cleave purified LRP *in vitro* (88). In addition to LRP, furin cleaves several other proteins that travel through the trans-Golgi, including the insulin receptor (58). In the insulin receptor, the ligand-binding activity was compromised by lack of processing (89-90). Although LRP undergoes similar proteolytic cleavage, data suggest that this cleavage is not essential for the endocytic activity of LRP. When a soluble LRP minireceptor containing the processing site was overexpressed, the transfected cells were incapable of cleaving all of the recombinant protein. A significant portion of the recombinant protein was, nevertheless, secreted suggesting that processing

is not essential for intracellular trafficking or plasma membrane presentation to (91). In addition, a single arginine-to-serine point mutation at position 3942 within the full-length chicken LRP did not affect the receptor's ability to internalize several ligands tested (92).

1.5 LRP at the plasma membrane

Presentation of LRP at the plasma membrane can be regulated by factors such as insulin. Motifs within the LRP cytoplasmaic tail have been shown to bind adaptor proteins. These data implicate LRP in a complex signaling cascade and suggest that LRP may be more than a mere cargo receptor, as previously thought.

1.5.1 Regulation of LRP expression and plasma membrane presentation

Several studies have reported an increase in the ligand-binding activity of LRP following acute stimulation by insulin. Both 3T3-L1 adipocytes and primary adipocytes isolated from rat epididymal fat pads show enhanced binding of ^{125}I-α_2M* following insulin treatment (93). There was also an increase in uptake of [^{3}H]cholesteryl esters from apoE-enriched β-VLDL following treatment with insulin. This increase was inhibited using RAP or antibodies against LRP (94).

In neuronal cells, there was a reported increase in LRP at the plasma membrane upon the addition of nerve growth factor (95). A decrease in LRP mRNA level, as well as ^{125}I-α_2M* binding was seen upon treatment of cells with lipopolysaccharides and interferon-γ (96).

In contrast to the LDL receptor that is involved in regulating cellular cholesterol homeostasis and contains a sterol regulatory element that responds negatively to sterols, sterols do not appear to play a role in the regulation of LRP despite the presence of a sterol regulatory element consensus motif (97).

1.5.2 LRP endocytosis motifs

LRP is believed to undergo constitutive endocytosis similar to the LDL receptor. Several naturally occurring mutations within the cytoplasmic tail of the LDL receptor were initially observed to prevent its endocytosis (98). This defect in receptor internalization can lead to the development of the hereditary condition familial hypercholesterolemia (FH) where uptake of LDL is impaired (reviewed in 99). As a result, LDL accumulates in the circulation causing an early onset of coronary artery disease. Mutagenesis studies of the LDL receptor cytoplasmic tail have since revealed that the first 22 amino acids are required and the NPxY motif, in particular, is involved in clustering of the LDL receptor into clathrin coated pits at the plasma membrane prior to internalization (63).

At least one copy of the NPxY motif is found within the cytoplasmic tail of several other endocytic receptors, including the insulin receptor. Studies using other receptors have demonstrated that the NPxY motif does not serve as a universal internalization signal. In the insulin receptor, the juxtamembrane domain, including an NPxY motif is believed to link the insulin receptor to downstream signaling substrates (100). In the integrins, the phosphorylated tyrosine residue of the NPxY motif has been

implicated in the binding of intracellular Shc and the subsequent intracellular signaling cascade (64).

A recent study using an LRP minireceptor encoding only the fourth cluster of complement-type/class A modules, the transmembrane domain, and cytoplasmic tail encoding mutagenized NPxY motifs strongly suggests that the NPxY motifs are not involved in the internalization of LRP. This same study implicated the YxxL motif and two di-leucine motifs in LRP internalization (66). Di-leucine motifs have been shown to be involved in the internalization of both the insulin receptor (101) and the epidermal growth factor receptor (102) following ligand-binding and phosphorylation.

1.5.3 Ligand endocytosis and receptor recycling rate

Recycling studies using stably expressed recombinant LRP suggest that the entire plasma membrane pool of LRP could be internalized to an endosomal compartment within approximately five minutes (92). Internalized LRP labeled with thiol cleavable biotin was found to be internalized and recycled to the plasma membrane within 30 minutes as measured by the amount of biotin labeled LRP protected from thiol cleavage and, therefore still found within endosomes. This is consistent with studies using a ligand-mimicking monoclonal antibody directed towards the α-chain of LRP. Receptor-anitbody complexes were found to dissociate in the lysosomes within approximately 30 minutes following endocytosis (103). When an ^{125}I-labeled antibody was bound at 4° C, it was found to dissociate when the cells were washed at a pH of 5.2, again suggesting that receptor:ligand dissociation occurs within an acidic environment similar to that of

lysosomes. These experiments were performed using fibroblasts from both normal and LDL receptor defective WHHL rabbits, suggesting the observed effects were likely specific to LRP.

1.5.4 Binding of LRP to adapter proteins

Several adapter proteins interact with the cytoplasmic tail of LRP. Both the neuronal adaptor protein FE65 and mammalian Disabled were found to bind to the cytoplasmic tail of LRP using a yeast two-hybrid system as well as in co-precipitation experiments (104). It is postulated that these proteins can potentially serve as scaffolds for the assembly of cytoplasmic multiprotein complexes. FE65 possesses two protein interaction sites and could theoretically link two proteins such as LRP and amyloid precursor protein (APP). mDab contains a single protein interaction site, but can bind nonreceptor tyrosine kinases upon phosphorylation. Both proteins are almost exclusively expressed in neurons.

There is additional evidence that the other members of the LDL receptor gene family may bind adaptor proteins. Yeast two-hybrid and co-precipitation analysis revealed that megalin binds a mammalian form of disabled protein, also implicating it in downstream signaling (105).

1.5.5 Cellular signaling by LRP

Although members of the LDL receptor gene family are generally thought as endocytic receptors, growing evidence suggests several of them may be involved in directing extracellular signals across the plasma membrane. One such pathway involved coupling of LRP to stimulatory G-proteins (106). This coupling increased intracellular cyclic adenosine monophosphate and activated protein kinase A. Stimulation of LRP also occurred within several neuronal cell lines upon incubation with nerve growth factor (95). This resulted in a two-fold increase in the total incorporation of ^{32}P [orthophosphate] into the β-chain of endogenous LRP.

1.5.6 Soluble LRP

Evidence suggests that a soluble form of LRP exists in the circulation (107). The plasma form of LRP appears to be comprised solely of the receptor α-chain. Several other plasma membrane proteins are known to possess soluble counterparts in the circulation. These include the transferrin receptor (108), heparin-binding EGF (109), and the amyloid precursor protein (110). Mechanisms producing soluble counterparts to plasma membrane proteins may involve alternative splicing, proteolysis, usually at the plasma membrane, or GPI-hydrolysis (111). The role of circulatory receptor fragments is currently unclear. Current thinking suggests that these may be involved in regulation of membrane bound receptor ligands by acting as soluble inhibitors (112).

1.6 LRP function

LRP is an endocytic plasma membrane receptor that functions as a scavenger receptor by possessing unusually broad ligand-binding specificity. The wide range of structurally and functionally diverse ligands that are internalized via LRP can be roughly divided into three categories that include; 1) proteins involved in protease metabolism, 2) lipoproteins or proteins involved in lipoprotein metabolism, and 3) miscellaneous proteins including several pathogens.

1.6.1 Protease metabolism

Proteases are required for a diverse number of functions including embryonic development (113), cell migration (114,115), and conversion of zymogen to active form. It was realized, following its identification, that LRP is identical to the α_2M receptor (18,19). The widely acknowledged primary function of LRP is uptake of the protease inhibitor α_2M bound to proteases (18,19). α_2M is a large 720 kDa homotetramer. It is a broad spectrum endopeptidase inhibitor that is capable of clearing all four classes of proteases from the circulation (116). Upon binding to protease, α_2M undergoes cleavage of a thiol ester bond resulting in a conformational change. This conformational change makes the complex competent for receptor binding (117). α_2M may also attain a receptor binding conformation by treatment in vitro with small nucleophiles such as methylamine (118).

LRP is also the hepatic receptor for plasminogen activators. These are proteases involved in the generation of plasmin from plasminogen. Plasmin derived from urokinase plasminogen activator (uPA) is primarily involved in events surrounding pericellular proteolysis such as tissue remodeling (119). Tissue-type plasminogen activator (tPA) generates mostly plasmin involved in fibrinolysis. The activated plasmin is involved in degradation of the fibrin network associated with blood clots (120). Both uPA and tPA have been shown to bind LRP either alone or complexed with one isoform of the plasminogen activator inhibitor (PAI-1) (16,17,34,35).

1.6.2 Lipoprotein metabolism

Lipoproteins have evolved as a means of transporting hydrophobic lipids within the aqueous environment of the circulatory system following the absorption of dietary lipids by the intestine. Lipoprotein particles are composed of a hydrophobic neutral lipid core composed of varying ratios of triacylglycerol, cholesterol and cholesteryl ester. The neutral lipid core is surrounded by a phospholipid monolayer decorated with apoprotein and cholesterol. Lipoproteins are transported in the circulation to the appropriate target tissue where they are taken up by mechanisms such as receptor-mediated endocytosis. Several different classes of lipoproteins have been characterized. Each class of lipoprotein is defined by its lipid composition and by the apoproteins found on the phospholipid monolayer and each class serves a specialized function (reviewed in 121).

Of particular interest in the context of LRP are the remnant lipoproteins, chylomicron remnants and β-VLDL. These are defined as large lipoproteins with buoyant

density of less than 1.006 mg/ml. The protein component of interest on these particles is apoE which has been shown to bind all the members of the LDL receptor gene family including both LRP and the LDL receptor (38,39).

1.6.3 Receptor-mediated internalization of chylomicron remnants

Early clinical evidence supported an LDL receptor-independent mechanism of remnant clearance. Chylomicron remnant metabolism was found to be relatively unimpaired in LDL receptor defective Watanabe rabbits (122). These animals are unable to take up LDL particles and suffer from coronary artery disease as a result (123). Similarly, in a study using human subjects with defective LDL metabolism caused by defects in LDL receptor, chylomicron remnant metabolism was shown to be relatively normal (124). Experimental evidence has now demonstrated that LRP plays a role in chylomicron remnants metabolism. ApoE-containing liposomes were shown to bind LRP isolated from HepG2 cells in ligand blots (38). ApoE-enriched β-VLDL particles increased cholesterol esterification activity in LDL receptor deficient fibroblasts, indicating that cholesterol from an extracellular lipoprotein source was being internalized and metabolized. This effect could be ablated by incubating the cells with an antibody against LRP, but not against the LDL receptor. This effect was also blocked by chloroquine, suggesting the mechanism by which the β-VLDL particles were being internalized involved receptor-mediated endocytosis followed by lysosomal hydrolysis of whole lipoprotein particles (39). ^{125}I-β-VLDL was also shown to bind specifically to

LDL receptor negative fibroblasts. Internalization of DiI-labeled β-VLDL enriched with apoE was also shown using the same cell model (125).

The initial step in binding of apoE-enriched β-VLDL and chylomicron remnants is thought to be sequestration within the space of Disse in the liver. Unlike the direct ligand interaction of LDL with its receptor, apoE-enriched β-VLDL particles and chylomicron remnants are thought to be endocytosed via a mechanism involving proteoglycans at the plasma membrane. These proteoglycans, most likely heparan sulphate proteoglycans and syndecans are involved in tethering lipoproteins to the vicinity of the receptor prior to internalization (126). This model was based on the observation that lipoproteins appear to be captured by plasma membrane proteoglycans before they can be metabolized (127). ApoE containing a point mutation within its sequence causing hyperlipoproteinemia bound to heparan sulphate proteoglycans more poorly than normal apoE3 in both HepG2 and rat hepatoma McA-RH7777 cells (127). Furthermore, the binding of normal apoE-enriched β-VLDL was almost completely abolished by treatment of cells with heparinase (128). Intravenous heparinase injection was found to inhibit remnant clearance from the plasma and uptake by the liver in mice (129). What remains unclear, is why the remnant particles needed to be enriched with apoE in order to be metabolized and whether this process occurs in vivo.

Recent studies using animal models have provided conclusive evidence that LRP is involved in remnant lipoprotein uptake. RAP overexpression using an adenoviral vector in LDL receptor deficient as well as in normal mice resulted in secretion of RAP into the plasma compartment. This secreted RAP could act as an antagonist blocking the uptake of LRP ligands. The result was an accumulation of chylomicron remnants in the

LDL receptor deficient mice and a lesser degree of accumulation in the normal mice (130). The conclusion from this important study was that in mice, both the LDL receptor and LRP were involved in remnant clearance mechanisms. The final important piece of conclusive evidence implicating LRP in remnant clearance was the recent generation of liver specific LRP knockout mice using both normal and LDL receptor deficient animals. The knockout of LRP in the livers of the LDL receptor deficient animals resulted in the accumulation of chylomicron remnants in the circulation, while an upregulation of the LDL receptor mRNA was seen in the normal animals (131).

Several proteins involved in the metabolism of lipoproteins have also been shown to bind LRP. This has been shown for both hepatic lipase (15) and lipoprotein lipase (132,133). In the case of lipoprotein lipase, the receptor binding region was localized to the C-terminal of the enzyme using recombinant fragments of human lipoprotein lipase (134). Inhibition of lipoprotein lipase activity in the plasma by injection of a monoclonal antibody also impaired clearance of chylomicron remnants by the liver (135). Enhanced uptake of remnant lipoproteins was shown upon addition of hepatic lipase to rat hepatoma McA-RH7777 cells (136).

It is believed that both hepatic lipase and lipoprotein lipase can facilitate lipoprotein binding to LRP by forming a bridge between LRP and lipoprotein particles. This occurs when the lipase either binds directly to the receptor, or is concentrated locally upon binding heparan sulphate proteoglycans at the plasma membrane in the vicinity of the receptor (10).

1.6.4 Other LRP ligands

Among other LRP ligands, of particular interest is the exotoxin from the bacteria *Pseudomonas aeruginosa* (23). This toxin enters cells opportunistically and specifically via LRP mediated endocytosis. Once in the endosomal compartment, PEA is cleaved by a cellular protease and an enzymatically active fragment undergoes retrograde transport to the ER (137). The PEA can either undergo retrograde transport to the ER where it is translocated to the cytosol, or can be directly translocated to the cytosol in an ATP-dependent fashion via an unknown mechanism (138). The catalytically active fragment of PEA, once in the cytosol, ADP-ribosylates elongation factor 2 (EF2), thus shutting down protein synthesis and causing cell death (139). A lack of cell toxicity is, therefore, seen in LRP deficient cells. This property has been exploited in order to select a chemically mutagenized cell line, denoted 13-5-1, which does not express endogenous LRP (140).

PEA is thought a candidate for cancer therapy. Research is currently under way to characterize catalytically active sites, to generate more active toxin fragments, and to find more specific means of delivery into cancer cells (141). Although PEA has been shown to enter cells via LRP, the exact nature of the receptor-ligand interaction and the specific binding site are currently unknown.

The minor group rhinovirus that is responsible for causing influenza has also been found to bind members of the LDL receptor gene family (21). An antibody fragment known to bind LRP has been found to prevent infection by the virus (22). Lactoferrin binds to LRP, but the physiological reason is not clear.

The β-amyloid precursor protein (APP) also binds LRP and has been implicated in the pathogenesis of Alzheimer's disease (142). Once internalized, APP can undergo

proteolytic cleavage within an endosomal compartment to generate the β-amyloid peptide. This peptide is a major component of the senile plaques that are characteristic of Alzheimer's disease.

1.7 Studies of ligand-binding regions within LRP

Over a decade following the identification of LRP, little is known about the ligand-binding regions within this enormous receptor protein. Studies have been complicated by the large size of LRP as well as by its multifunctional nature. Studies carried out to date agree that a region that includes cluster II of complement-type/class A modules is involved in binding of several ligands including RAP and PA:PAI-1 complexes. Known ligand-binding regions of LRP are depicted in figure 8.

RAP, rat α_1-macroglobulin, and uPA:PAI-1 complexes were shown to bind to the region of LRP containing complement-type/class A cluster II plus one N-terminal and two C-terminal EGF modules and the first YWTD repeat following these (figure 8A) (56). The second ligand-binding cluster of complement-type/class A modules plus the N-terminal flanking EGF module, was shown to contain three distinct calcium dependent binding sites (143).

Studies with surface plasmon resonance of recombinant fragments of complement-type/class A module cluster II showed that RAP binds a fragment containing the third to the fifth complement-type/class A module within cluster II (figure 8B). The same studies showed that PAI-1 and tPA:PAI-1 bind a fragment containing the fourth EGF module plus the first to the fifth complement-type/class A modules of cluster II (figure 8C) (143).

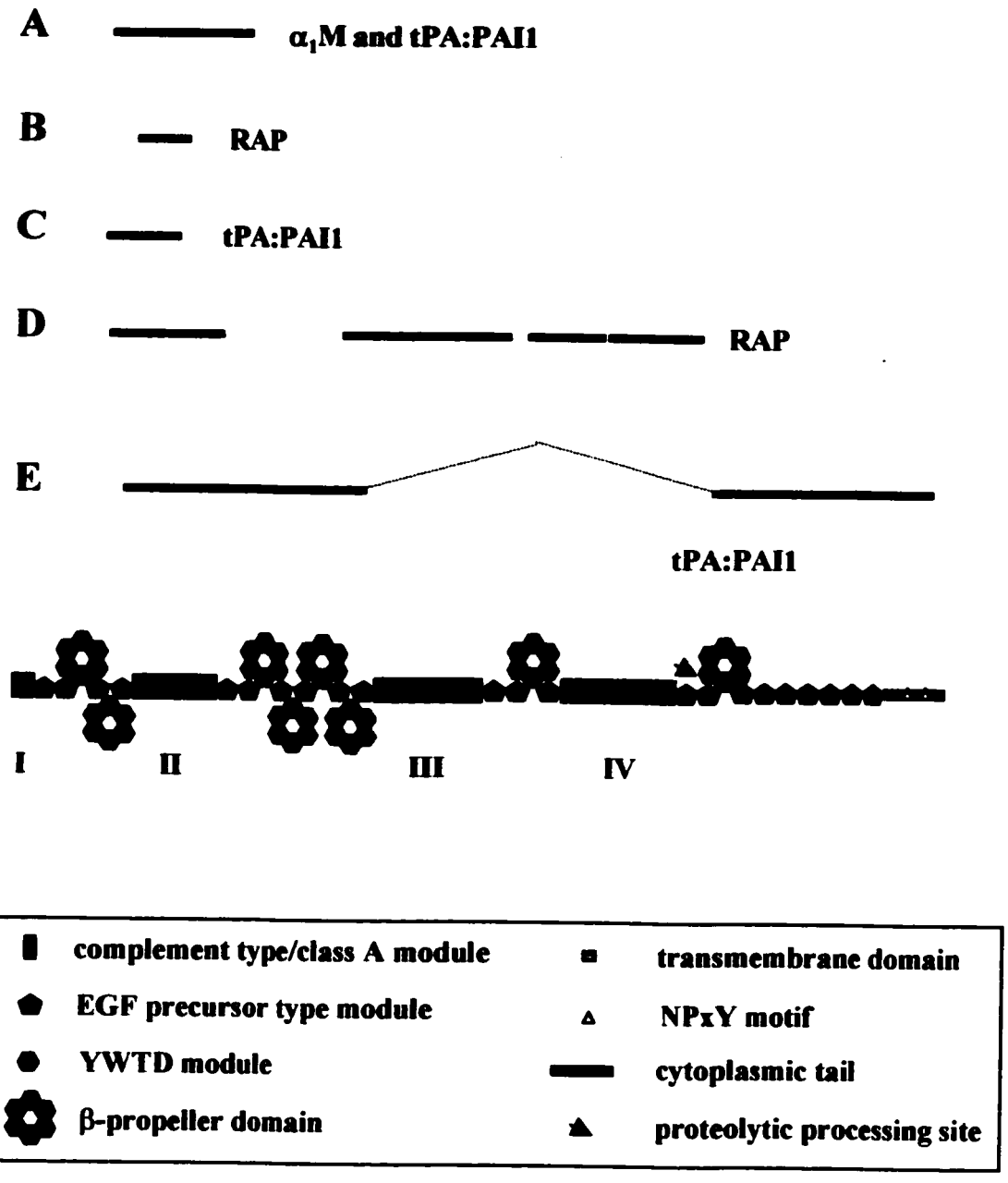

Figure 8. Known ligand-binding regions within LRP.

Schematic diagram summarizing LRP ligand-binding studies. LRP regions exhibiting ligand-binding activity are represented by *solid lines*. Deleted regions are represented by *dashed lines*.

Soluble fragments of each LRP complement-type/class A cluster were expressed. Each was capable of binding RAP (figure 8D) (144). If several RAP molecules bind to multiple sites within the receptor, and if different ligands possess affinity for different sites within LRP, this could explain the unique ability of RAP to inhibit binding of all known LRP ligands.

Two LRP mini-receptors containing cluster II or cluster IV of LRP plus flanking regions were also used for ligand-binding studies. While ^{125}I-RAP-GST bound and was degraded by cells expressing both of these mini-receptors, ^{125}I-tPA:PAI-1 and ^{125}I-uPA:PAI-1 only bound and were degraded by cells that expressed the mini-receptor containing LRP cluster II (figure 8E) (91). This evidence demonstrates that cluster II is involved in the binding of several LRP ligands, but does not exclude flanking regions from being involved in ligand binding.

2. Rationale and objective of the thesis study

LRP has been linked to the pathogenesis of both coronary artery disease (CAD) and Alzheimer's disease (AD). Although considerable circumstantial evidence implicates LRP in the development of these two diseases, the molecular mechanism by which LRP is involved is far from being understood. Some early studies have suggested that apoE may play a common role in the pathogenesis of both diseases.

Almost a decade ago, it was discovered that a variant apoE allele, apoε4 was associated with the pathogenesis of AD (145). More recently, the apoε4 allele was shown to be overrepresented in AD patients (146). The exact molecular mechanism linking the expression of apoE4 to the pathogenesis of AD is currently not understood. In addition to its important role in plasma lipoprotein metabolism, apoE also serves to transports lipid in the nervous system (147). One hypothesis suggests that only normal apoE allele, apoε3 can effectively remodel and repair neuronal damage (148). There is also contradictory evidence suggesting that non-lethal polymorphisms in LRP itself may be involved in the formation of the plaque deposits characteristic of AD (149, 150).

Evidence also strongly implicates LRP in the pathogenesis of atherosclerosis. There is now convincing evidence for the involvement of VLDL in the pathogenesis of cardiovascular disease (151). It has been known for some time that LDL particles as well as VLDL or chylomicron remnant particles may taken up by two discrete mechanisms. Both the LDL receptor defective Watanabe rabbits as well as individuals with LDL receptor defects fail to accumulate chylomicron remnants in their plasma (122, 124). Individuals affected by chylomicronemia, a disease where chylomicron remnants do

accumulate in the plasma, often exhibit defects of apolipoprotein E strengthening the belief that a discrete mechanism is responsible for remnant endocytosis and this mechanism is mediated by apoE on remnant particles (152). Accumulation of these particles in the circulation is associated with familial combined hyperlipidemia, familial dysbetalipoproteinemia, hypertension, obesity and insulin resistance (153). The mechanisms by which chylomicron remnants are efficiently removed from the circulation by the liver and lipid is delivered to adipocytes for storage or usage are poorly understood. However, there is now direct evidence showing that LRP knockout in the liver results in the accumulation of chylomicron remnants in the plasma of mice (131).

The understanding of these disease processes will require an understanding of LRP at the molecular level. Few studies to date have been successful in understanding the nature of ligand binding to LRP. The immediate objective of this study was to generate a model allowing the systematic study of ligand binding regions within LRP. The approach taken was to use molecular biology to generate deletion mutants and to overexpress these in a mammalian cell system deficient in endogenous LRP expression. This work describes the generation of such a system and its subsequent use in conducting structure-function studies of this enormous receptor molecule.

3. Materials and methods

Reagents – Cell culture medium and reagents were purchased from Life Technologies. Plasticware was purchased from Corning or Falcon. Fine chemicals were from Fisher or Life Technologies. Restriction enzymes, endoglycosidase H and PNGase F were purchased from New England Biolabs. Biotinylation reagents, streptavidin-agarose beads, D-Salt Excellulose™ columns and Iodobeads™ were from Pierce. ProMix™ (a mix of [^{35}S]methionine and [^{35}S]cysteine; 1000 Ci/mmol), carrier-free Na^{125}I and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Amersham. Enhanced chemiluminescence reagents and neuraminidase were from Boehringer Mannheim. Glutathione-sepharose was from Pharmacia. LRP antibodies and a RAP construct were obtained from Dr. Johannes Nimpf. *Pseudomonas* exotoxin A and LRP-deficient 13-5-1 cells were obtained from Dr. David FitzGerald. pCMV5 was obtained from Dr. David Russell. The human RAP-GST construct was a gift from Dr. Dudley Strickland. Human furin cDNA was a gift from Dr. Nabil Seidah.

Cell culture – Cos-7 cells and HepG2 cells were cultured in Dulbecco's Minimum Essential Medium with high glucose, supplemented with 10% fetal bovine serum. CHO-K1 and 13-5-1 cells were grown in 100-mm dishes or T75 flasks using Ham's F-12 medium supplemented with 10% fetal bovine serum. Stable cell lines generated were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum and 500 μM G418. Cell lines were allowed to grow up to 80% confluency before being subcultured 1:6. Medium was routinely changed every two days for all cell lines.

Preparation of expression plasmids encoding LRP25, LRP67, and LRP100 – The expression plasmids pcLRP25, pcLRP67, and pcLRP100 were constructed by combining 12 chicken LRP cDNA fragments that span the total length of 15598 base pairs (figure 9). Plasmids pcLRP25 and pcLRP67 encode the N-terminal 22% and 64% of the full-length LRP, respectively, plus the transmembrane and intracellular domains. Inserts were cloned into the polylinker region of the pCMV5 vector (154) positioned between the cytomegalovirus promoter and enhancer sequences and the human growth hormone transcription termination and polyadenylation signals (figure 9). A *Bam*HI-*Eag*I fragment (nucleotides 1229 - 4222), prepared from clones A5, E4, and N3 was ligated together with an *Eag*I-*Bgl*II fragment (nucleotides 14524-15248, obtained from clone L-1) into the pCMV5 vector that had been digested with *Xba*I and *Bam*HI to create pcLRP25. Next, an *Eag*I-*Eag*I fragment (nucleotides 4222-9865) was prepared from clones N1, Z19, and 18, and inserted into pcLRP25 that had been digested with *Eag*I to produce pcLRP67. Finally, an *Xho*I-*Xho*I fragment that encoded the C-terminal portion of LRP (nucleotides 9112-15248) plus the hGH region of the pCMV5 vector was prepared by ligation of clones 28, N2, 2B1-2, 112, 2B4, and L-1, and inserted into pcLRP67 to create pcLRP100 encoding the full-length chicken LRP.

Preparation of expression plasmids encoding LRPΔII, LRPΔIII, LRPΔIII–IV, and LRPΔIII–IVβ – For preparation of pcLRPΔII, the plasmid pcLRP100 was digested with *BamH*I to obtain the 12.9-kb vector fragment containing nucleotides 6957 through 15248 of the avian LRP cDNA. The vector fragment was ligated with the *BamH*I–*BamH*I fragment containing nucleotides 1229 through 3075 of the LRP cDNA to create

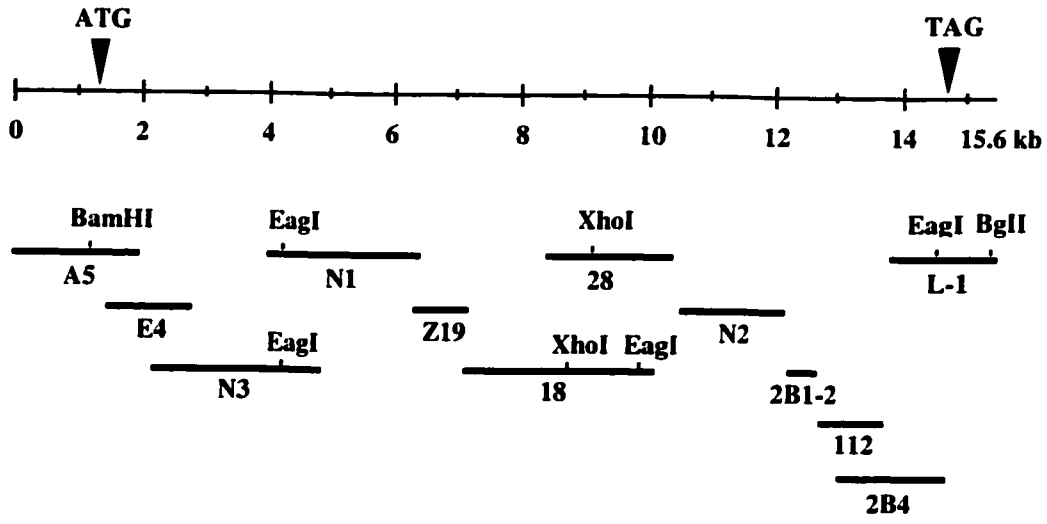

Figure 9. Chicken LRP was assembled from 12 overlapping cDNA fragments

Structures of the full-length and 12 fragments of chicken cDNA spanning the total length of 15598 base pairs. Restriction sites used in generating the full-length construct are indicated. The translation start (*ATG*) at position 1270 of the LRP cDNA and stop (*TAG*) at position 14899 of the LRP cDNA are indicated.

pcLRPΔII. The resulting pcLRPΔII encoded deletion from nucleotides 3075 through 6957 of the LRP cDNA. The plasmids pcLRPΔIII and pcLRPΔIII–IV were similarly prepared, and the resulting constructs encoded deletion of a *SalI–SalI* segment (nucleotides 7433–10811 of the LRP cDNA) and a *BssH*II–*BssH*II segment (nucleotides 7707–14274), respectively. For construction of plasmid pcLRPΔIII–IVβ, a fragment encoding nucleotides 7707 (*BssH*II) through 8798 (*Not*I) was prepared by polymerase chain reaction (PCR) using a pair of primers 5'GAACTCGTCGTGCACGTTGCGGCCGCTGTTCAGAGCTTTGCAGG3' (nucleotides 8841–8798) and 5'GTCTTCGAGGACTACATTTAC3' (nucleotides 7564–7584). The 3' of the fragment contained an engineered *Not*I site. The PCR amplified *BssH*II–*Not*I fragment was ligated with pcLRP100 that had been digested with *BssH*II (at nucleotide 7707) and *Not*I (at nucleotide 14524) to create pcLRPΔIII–IVβ.

Generation of transfection grade DNA – Transfection grade supercoiled cDNA was prepared by cesium chloride gradient centrifugation. DH5α E. coli were transformed immediately prior to DNA isolation with the DNA of interest by incubating 1 hour on ice, followed by a 5 minutes heat shock at 37 °C. Bacteria were plated and grown for 18 hours on agar plates containing 10 mg/ml ampicillin. A single colony was cultured in 5 ml of LB broth with 10 mg/ml ampicillin for 10 hours and then transferred to 500 ml of LB broth and cultured for and additional 18 hours for cDNA isolation.

Transient transfections and co-transfections – Transfection of pcLRP into Cos-7, CHO-K1, and 13-5-1 cells was achieved by calcium phosphate precipitation as described

previously (155). Cos-7 cells were subcultured one day prior to transfection. The cells were transfected when 40-50% confluent. Transfection of LRP cDNA was achieved by calcium phosphate precipitation using 10, 20, and 25 μg of LRP25, LRP67, and LRP100 respectively. For co-transfection experiments, cells were transfected with the indicated concentrations of both LRP and human RAP or furin.

Generation of stable LRP transformants – LRP-null, 13-5-1 cells were subcultured one day prior to transfection. The cells were transfected when 40-50% confluent. Stable transformants were generated by transfecting 10 μg of LRP25, 20 μg of LRP67, or 15 μg of LRP100 with 0.15 μg of pSV2neo. The optimal concentration of plasmid DNA used for transfections was determined experimentally. Transfected cells were allowed to grow for 10-14 days in growth medium containing 700 μM G418 until discrete colonies were visible. Colonies were picked using 5 μl of trypsin (0.25% trypsin in phosphate buffered saline with 5 mM EDTA) and maintained with culture medium containing 500 μM G418.

Preparation of cell extract and immunoblot analysis – Transfected cells were washed with cold phosphate buffered saline, scraped, and suspended in lysis buffer A (200 mM Tris-maleate, pH 6.0, 0.5 mM PMSF, 2.5 μM leupeptin, and 1.4% Triton X-100) and incubated on ice for 30 minutes. Insoluble protein was removed by cetrifugation (14000 rpm, 30 minutes, 4 °C) in an Eppendorf microfuge. Triton X-100 soluble proteins were recovered from the supernatant and mixed with an equal volume of sample buffer B (8 M urea, 2% SDS, 10% glycerol, 10 mM Tris-HCl, pH 8.3 and 5% β-mercaptoethanol) at 70 °C for 15 minutes and resolved by electrophoresis on 3-8% gradient polyacrylamide gels

containing 0.1% SDS. The resolved proteins were then electrophoretically transferred for 6 hours onto nitrocellulose or PVDF membranes for immunoblot analysis. Rabbit antiserum against the C-terminal 17 amino acids of the β-chain of chicken LRP (anti-β-chain antibody) or the C-terminal 15 amino acids of the α-chain of LRP (anti-α-chain antibody) were used at 1:250 dilution as primary antibody for ECL detection.

Endoglycosidase H digestions of transiently transfected cells – For transiently transfected cells, extracts were prepared in lysis buffer A. Proteins were denatured by adjusting to a final concentration of 1% SDS and 0.5% β-mercaptoethanol and heating to 100 °C for 5 minutes. The pH of the samples was adjusted to 5.5 and the samples were incubated for 3-5 hours with 500 units of endoglycosidase H. The samples were resolved on SDS-PAGE and transferred to nitrocellulose or PVDF membranes for immunoblot analysis as described above.

Neuraminidase digestions of transiently transfected cells – For transiently transfected cells, extracts were prepared in lysis buffer. Samples were incubated for 5 hours with 0.75 units of neuraminidase. The samples were resolved on SDS-PAGE and transferred to nitrocellulose or PVDF membranes for immunoblot analysis as described above.

Fractionation of subcellular membranes – Transfected cells (ten 100-mm dishes) were harvested into PBS. Cells were collected by low speed centrifugation and resuspended in 2 ml of buffer C containing 20 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM leupeptin, 40 μg/ml acetyl-leucyl-leucyl-norleucinal, and 10

kalikrein-inactivating units/ml aprotinin. The sample was homogenized by 20 passes through a ball bearing homogenizer (H and Y Enterprise, Redwood City, CA). Following centrifugation of the homogenate (16 000 x *g*, 20 minutes), the supernatant was subjected to another centrifugation (250 000 x *g*, 1.5 hours) in a TLA100.4 rotor to isolate microsomal membranes. The 16 000 x *g* pellet was resuspended in 1 ml of buffer D (20 mM HEPES, pH 7.4, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM leupeptin, 40 mg/ml acetyl-leucyl-leucyl-norleucinal, and 10 kalikrein-inactivating units /ml aprotinin). The sample was homogenized using a glass homogenizer with 10 strokes of a Teflon pestle, layered on a 1.12 M sucrose cushion, and centrifuged (100 000 x *g*, 1 hour) in an SW41 rotor. The plasma membrane fraction was collected from the interface of the sucrose cushion and pelleted by centrifugation (30 000 x *g*, 30 minutes) in a TLA100.4 rotor (155).

Pulse/chase and endoglycosidase digestions of stable transformants – Stable transformants were grown to a confluent monolayer on 60-mm dishes. The cell monolayers were depleted of methionine and cysteine by incubating with a methionine- and cysteine-free medium for 20 minutes at 37 °C. The cells were then pulse-labeled for 1 hour with 200 μCi/ml [^{35}S] methionine/cysteine in methionine- and cysteine-free medium. Following the pulse, the cells were rinsed with warm PBS, and fresh maintenance medium was added to start the chase. Cells were harvested into lysis buffer A at the indicated chase time points. For endoglycosidase digestion studies, the cells were harvested at the indicated chase times and lysed with lysis buffer A. LRP proteins were immunoprecipitated from Triton X-100 soluble fractions using the anti-β-chain antibody and protein A sepharose beads as described above. The proteins were stripped from the

beads and denatured by adjusting to a final concentration of 1 % SDS and 0.5 % β-mercaptoethanol and heating to 100 °C for 10 minutes. Each sample was then divided into three aliquots of equal volume. One aliquot was left untreated. A second and third aliquot were acidified to pH 5.5 by the addition of sodium citrate to a final concentration of 50 mM. The samples were incubated with either endo H or PNGase F for 3 hours at 37 °C and resolved by SDS-PAGE for immunoblot analysis as described above.

Fluorescence microscopy – Cells were grown to 60-70% confluency on 20-mm glass bottom dishes. The cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.1% saponin for 30 minutes. Milk in blotto buffer was used for blocking non-specific binding and primary antibody to the C-terminal of LRP was used at 1:750 dilution.

α_2-macroglobulin purification – The α_2M was purified by Zn^{2+}-chelate affinity chromatography as described previously (156). Human plasma was obtained from the blood bank at the Ottawa Hospital, Civic Campus. Briefly, 500 ml of plasma were mixed with 8 000 molecular weight polyethylene glycol (PEG-8000) to a final concentration of 5.5% for 30 minutes at 22 °C. The plasma was centrifuged at 10 000 rpm for 15 minutes. The supernatant was adjusted to a final concentration of 12.5% PEG-8000, mixed at room temperature for 30 minutes and centrifuged at 10 000 rpm for 15 minutes. The pellet was dissolved in 250 ml of 20 mM $NaPO_4$, pH 6.4 and dialyzed against 20 mM $NaPO_4$ containing 0.15 M NaCl. The sample was centrifuged at 10 000 rpm for 15 minutes at 4 °C, applied to a Zn-sepharose column equilibrated with 20 mM $NaPO_4$, pH

6.0 containing 0.5 M NaCl and eluted with 0.01 M CH_3COONa containing 0.15 M NaCl, pH 5.0. Preparation of activated α_2M was achieved by incubating the purified protein with methylamine for 15 hours at 4 °C followed by extensive dialysis according to the previously described method (157).

Preparation of Zn-sepharose matrix – Twenty ml of Matrix Sepharose 6B Fast Flow resin was incubated with 100 ml of 50 mM $ZnCl_2$ with gentle rocking at 22 °C for 18 hours. The resin was washed with 20 mM $NaPO_4$, pH 6.0 containing 0.5 M NaCl (158).

RAP purification – The RAP-GST fusion protein was purified as previously described (24). The expression plasmid pGEX-39-kDa was grown in DH5α at 37 °C to an optical density of 0.5 at 600 nm. Isopropythio-β-D-galactoside (0.01%) was added to induce expression of RAP-GST under the β-galactose promoter and the cultures were grown for an additional 4-5 hours at 37 °C. The bacteria were collected by centrifugation at 7 000 rpm for 15 minutes at 4 °C. The bacterial pellet was then resuspended in 1% of the original volume of sucrose buffer E (15% sucrose (w/v), 50 mM Tris-HCl, and 50 mM EDTA, pH 8.0 and 1% lysozyme) and incubated on ice for 30 minutes. Two percent of the original volume of a solution consisting of 0.2% (v/v) Triton X-100 and 0.5 mM PMSF in water was added, and the bacterial lysate was mixed by vigorous vortexing. The lysate was then sequentially passed through 18-, 22-, and 25-gauge needles. Dithiothreitol (DTT) was added to a final concentration of 1 mM and the sample was mixed with 5 ml of glutathione-sepharose beads equilibrated in sucrose buffer and incubated overnight at 4 °C with rotating. The glutathione-sepharose beads were washed three times with

sucrose buffer, packed into a 10 ml column, and washed with 10 column volumes of sucrose buffer. Finally, RAP-GST was eluted from the column with 25 mM glutathione in sucrose buffer. One ml fractions were collected and the protein peak was determined at optical density 280 nm. RAP-GST was dialyzed against PBS, pH 7.3 or 20 mM Hepes, 50 mM NaCl, pH 7.4. For RAP-GST cleavage, 2.5 mg of RAP-GST was incubated with 3 units of bovine thrombin in 2 ml of buffer containing 20 mM Hepes, 150 mM NaCl, and 2.5 mM $CaCl_2$ at 37 °C for 15 hours.

Iodination of proteins – Purified α_2M or RAP were iodinated using the iodobead (Pierce Chemical Company) method following the manufacturer's instructions. Briefly, 100 μg of either protein was incubated with one washed iodobead and 1 mCi carrier-free $Na^{125}I$ in 250 μl PBS for 15 minutes at room temperature. The iodobead was then removed and the reaction was quenched by incubation at room temperature for 20-30 minutes in the absence of the iodobead. The iodinated protein was then separated from free iodine by running over a D-Salt Excellulose (Pierce Chemical Company) size exclusion column equilibrated with PBS containing 0.5% BSA, pH 7.3. One ml fractions were collected, and a 10 μl aliquot of each was counted for radiation. The void volume fraction corresponding to the iodinated protein was pooled.

Pseudomonas exotoxin A cytotoxicity assay – PEA cytotoxicity assay was adapted from the previously described method (140). Cells were seeded at a concentration of 1 x 10^5 cells/well in 24 well dishes and allowed to adhere for 18-24 hours. PEA was added to the cells at the indicated concentrations for 18 hours. Following incubation with PEA, the

cells were washed with warm PBS and cell protein was labeled using 200 μCi/ml of [^{35}S] methionine/cysteine in methionine- and cysteine-free medium for 1 hour at 37 °C in a 7% CO_2 chamber. Cells were then washed with cold PBS and collected in 250 μl of lysis buffer F containing 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 1% SDS, 1 mM DTT, 0.015% PMSF, 50 mM Tris-HCl, pH 8.0. The samples were completely solubilized by heating to 75 °C for 15 minutes. Each cell lysate was diluted 10-fold in water and an aliquot was mixed with an equal volume of fetal bovine serum. The samples were spotted under gentle vacuum onto 35-mm nitrocellulose disc wet with ice cold 10% TCA. The membranes were washed with 2 volumes (5 ml each) of ice cold 10% TCA and 1 volume of water. The membranes were dried, placed in scintillation cocktail, and the radioactivity associated with total protein/TCA-insoluble material was quantified.

Ligand binding assay – Cells were seeded into 6-well or 12-well dishes 2 days prior to performing the experiment. The binding experiment was carried out when the cells had grown to a confluent monolayer. Prior to incubation with medium containing labeled ligand, monolayers were washed twice in warm medium containing no additions (2 ml per well per wash). The cells were then placed on ice and F12 medium with 6 mg/ml BSA and 1 mM Ca^{2+} containing ^{125}I labeled protein at the appropriate concentrations was added. For time course experiments, the cells were incubated between 0.5 and 4 hours. For competition experiments, unlabeled competing ligand was added simultaneously to the labeled ligand. Following incubation, the medium was removed and the cells were washed twice with PBS containing 6 mg/ml BSA (2 ml per wash) and then twice with cold PBS (2 ml per wash). The cells were incubated for a minimum of 30 minutes at

room temperature in 0.1 M NaOH. Radioactivity associated with cells was quantified by gamma counting.

Ligand degradation assay – The assay was performed in essentially the same manner as the binding assay except that the cells were incubated at 37 °C. Medium was collected in order to quantify TCA insoluble material as a measure of uptake and degradation. The medium was collected into tubes containing ice-cold TCA to a final concentration of 20% and incubated for 30 minutes on ice. After 30 minutes, TCA-insoluble material was pelleted by centrifugation, and the supernatant was removed for determination of TCA-soluble, non free-iodine radioactivity as described previously (159).

Plasma membrane biotinylation – Confluent cell monolayers were incubated on ice or at 37 °C with either Krebs-Ringers buffer or PBS for 30 minutes. The buffer was then removed and replaced with cold Krebs-Ringers buffer or PBS containing 1 mg/ml NHS-sulfo-biotin for 1 hour. Following biotin labeling, the cells were scraped into lysis buffer containing 200 mM Tris-maleate, pH 6.0, 0.5 mM PMSF, 2.5 μM leupeptin, and 1.4% Triton X-100 and resolved by non-reducing 3-8% SDS-PAGE. The proteins were transferred onto nitrocellulose or PVDF membranes, probed with streptavidin conjugated to horseradish peroxidase (HRP), and visualized by enhanced chemiluminescence detection method.

Protein determination – Proteins were determined using the bicinchoninic acid method following the manufacturer's instructions or by modified Lowry method (160).

4. Results

4.1 LRP deletion mutants

Preparation of pcLRP25, pcLRP67 and pcLRP100 constructs and structure of recombinant chicken LRP variants – The expression plasmids encoding chicken LRP variants were constructed from the 12 overlapping cDNA fragments shown in figure 9. The two intermediate plasmids, pcLRP25 and pcLRP67, were generated during the preparation of the full-length receptor, LRP100 (figure 10). Figure 11 shows the domain structure of the three LRP variants. pcLRP25 encodes the N-terminal 984 amino acids plus the 125 amino acid transmembrane and intracellular domain of LRP. This includes the cluster I and the first three modules of cluster II of full-length LRP. pcLRP67 encodes the N-terminal 2865 amino acids plus the 125 amino acid transmembrane and intracellular domain of LRP. This includes clusters I and II and the first eight modules of cluster III. LRP100 encodes the full-length protein. pcLRP100 retains the endoproteolytic furin cleavage site, while this site has been deleted in both pcLRP67 and pcLRP25.

Transient expression of LRP25, LRP67, and LRP100 in Cos-7 cells – All three LRP variants exhibited high expression level at the expected molecular mass when transiently transfected in Cos-7 cells. Absence of the β-chain and predominance of the full-length LRP 600 kDa band was detected upon probing with an antibody raised against the C-terminal 17 amino acids of chicken LRP β-chain (anti-β-chain antibody). This indicated

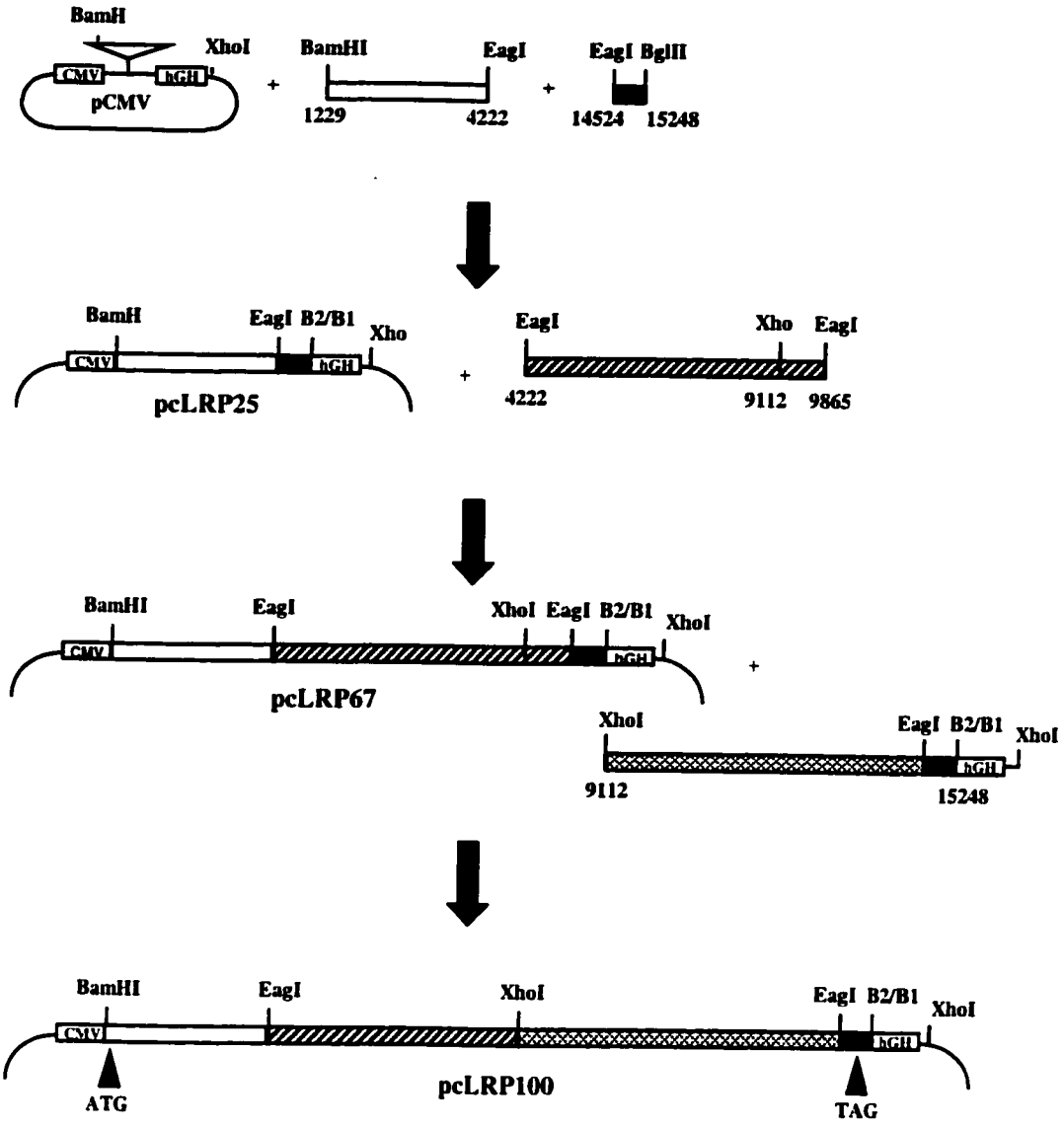

Figure 10. Construction of LRP expression plasmids encoding internal deletions.

Strategy used in cloning the full-length pcLRP100 and two deletion mutant minireceptors pcLRP67 and pcLRP25. *Cross-hatched bars* denote coding regions derived from corresponding cDNA fragments. *B2/B1* denotes the junction between BglII and BamHI sites. *CMV*, cytomegalovirus promoter-enhancer sequences; *hGH*, human growth hormone transcription termination and polyadenylation signals.

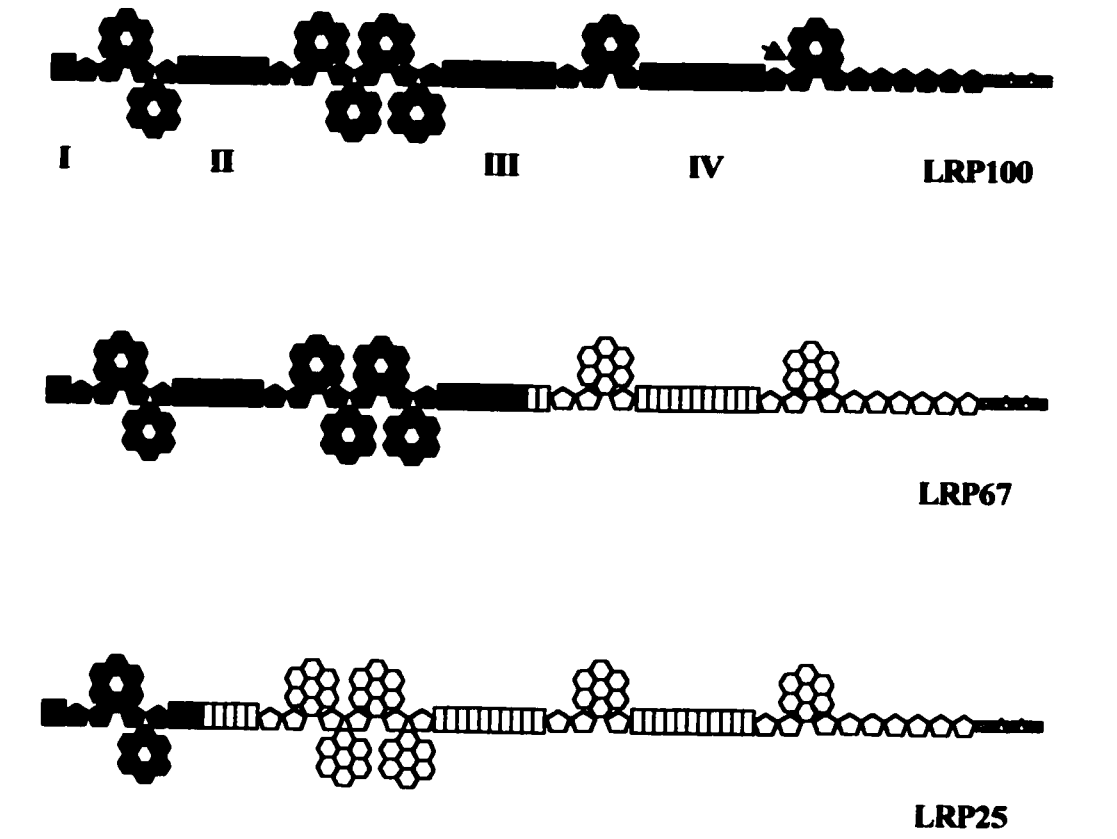

Figure 11. LRP25 and LRP67 encode internal deletions of the full-length LRP100.

Schematic representation of LRP deletion mutants. Structures have been depicted to emphasize ligand binding complement-type/class A modules and EGF precursor type regions. *Roman numerals* indicate clusters of complement-type/class A modules. *Open* shapes indicate region deleted.

that the full-length LRP proreceptor was not processed into α- and β-chains (figure 12). Since neither LRP25 nor LRP67 contain the furin consensus motif, proteolytic processing was not expected to occur in these variants.

Recombinant LRP does not reach the plasma membrane in transiently transfected Cos-7 cells – Despite a high expression level of recombinant LRP100 in transiently transfected Cos-7 cells, a large proportion of the 600 kDa proreceptor did not undergo proteolytic processing to yield the mature protein. This suggests LRP was unable to exit the ER compartment and reach the late Golgi compartment or the plasma membrane. Because LRP25 and LRP67 lack the proteolytic processing site, cleavage of these LRP variants could not be used as an indicator of subcellular localization. A previous study reported a portion of soluble LRP minireceptors encoding the furin recognition site but lacking the transmembrane domain and cytoplasmic tail were detected in the medium as a single polypeptide chain when overexpressed (94). This was postulated to occur due to the saturation of the cellular processing machinery when protein expression within the cell was excessively elevated. In order to determine whether LRP100 could be presented at the plasma membrane in proteolytically unprocessed form, endoH and neuraminidase digestion of detergent soluble fractions from transiently transfected Cos-7 cells were performed. Endo H exclusively cleaves the ER form of N-linked oligosaccharide chains found on glycoproteins. This results in a shift in electrophoretic mobility corresponding to the loss of the oligosaccharide moiety. Similarly, neuraminidase cleaves terminal syalic acid from oligosaccharide chains only when glycoproteins are localized to the late Golgi compartment. Treatment with these enzymes is, therefore, a convenient method of

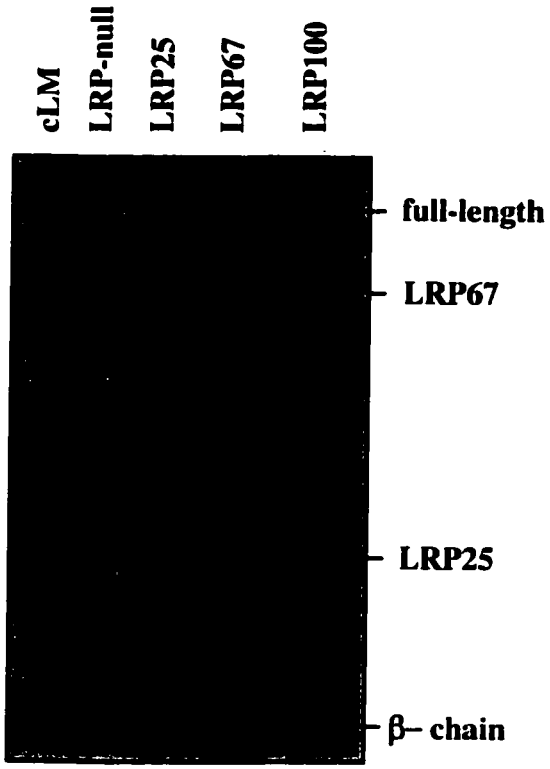

Figure 12. Transiently transfected LRP100 does not undergo efficient proteolytic processing in Cos-7 cells.

Cell extracts were prepared 48 h following transient transfection of Cos-7 cells with pcLRP25, pcLRP67, or pcLRP100. Samples were resolved by SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes and the expressed LRP proteins were detected by immunoblotting using the anti-β chain antibody. *cLM*, chicken liver membrane extract.

localizing glycoproteins to different subcellular compartments. All three LRP variants expressed, including the LRP100 proreceptor detected at 600 kDa, were sensitive to endo H (figure 13A) and resistant to neuraminidase (figure 13B) treatment. A small amount of β-chain, that was resistant to endo H digestion, was detected in the lanes corresponding to LRP100. In contrast to recombinant LRP100, both the α- and β-chains of endogenous chicken liver membrane LRP were detected when probed with the indicated antibodies. Endogenous chicken liver membrane LRP α-chain was endo H resistant and neuraminidase sensitive. Taken together, these data suggest that the recombinant LRP variants are impaired in their ER to Golgi transport and do not reach the distal Golgi compartment or the plasma membrane when expressed transiently in Cos-7 cells. Endogenous LRP prepared from chicken liver membrane, however, exists primarily in the mature heterodimeric form which is probably localized to the plasma membrane.

Endogenous LRP in HepG2 cells, Cos-7 cells, and human adipose tissue is neuraminidase sensitive – In addition to chicken liver membrane, the predominant endogenous form of LRP from several sources was compared with the recombinant form of the receptor. Immunoblot analysis showed the presence of the mature heterodimeric form of endogenous LRP in HepG2 cells, Cos-7 cells, and adipocytes isolated from human adipose tissue (figure 14). The immunoblot was probed using a polyclonal antibody raised against purified human LRP isolated from placenta (anti-human LRP antibody). This antibody is known to cross-react with endogenous LRP from the different species. Endogenous LRP extracted from each cell type was neuraminidase sensitive,

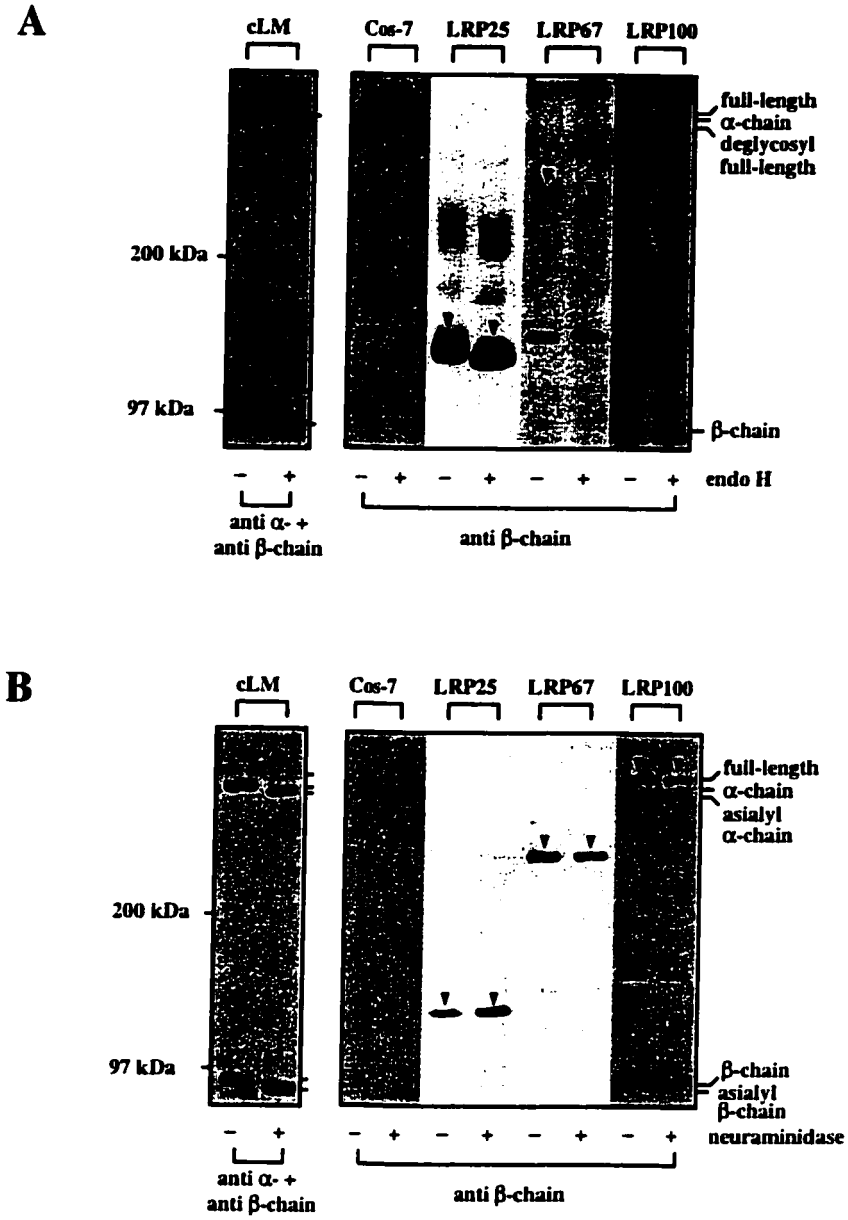

Figure 13. Recombinant LRP variants expressed transiently in Cos-7 cells are EndoH sensitive and neuraminidase resistant.

Cos-7 cells were transiently transfected with pcLRP25, pcLRP67 and pcLRP100 cDNA. Cell extracts were prepared 48 hours following transfection. In both experiments, the samples were resolved by SDS-PAGE under reducing conditions and the proteins transferred onto nitrocellulose membrane and detected by immunoblotting using the anti-β-chain antibody. *A*, cell extracts were denatured followed by incubation in the presence (+) or absence (−) of endo H; *B*, cell extracts were incubated in the presence (+) or absence (−) of neuraminidase. *cLM*, chicken liver membrane.

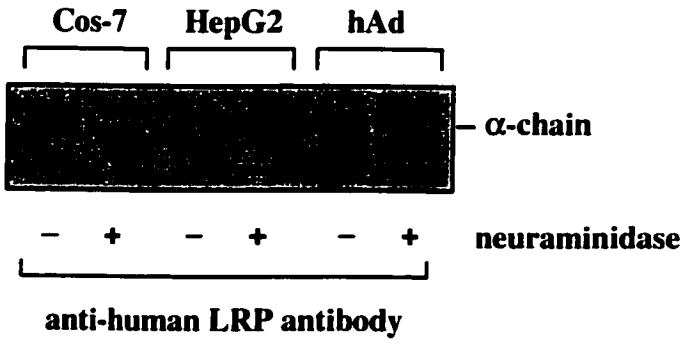

Figure 14. Endogenous LRP in HepG2 cells, Cos-7 cells, and human adipose tissue is proteolytically processed and is neuraminidase sensitive.

Cell extracts were prepared from collagenase treated human adipose tissue, HepG2 or Cos-7 cell monolayers. The cell extracts were incubated in the presence (+) or absence (–) of neuraminidase and resolved by SDS-PAGE under reducing conditions. Proteins were transferred onto nitrocellulose membranes and detected by immunoblotting using the anti-human-LRP antibody. *HepG2*, HepG2 cell extract, *hAd*, human adipose tissue extract, *Cos-7*, Cos-7 cell extract.

indicating that the major portion of the endogenous receptor was able to cross the trans Golgi compartment and was presented at the plasma membrane. The expression level of endogenous LRP was semi-quantified by immunoblot analysis. Human adipose tissue was found to express approximately the same amount of LRP as the human hepatocarcinoma cell line, HepG2. HepG2 cells are routinely used as a hepatic cell model, and are thought to possess a relatively high level of LRP expression (figure 15). A high expression level of hepatic LRP makes teleological sense since the liver is the primary target in the uptake of α_2M:protease complexes from the circulation. However, the function of a high expression level of LRP within adipose tissue is unclear, as this tissue is not believed to be a primary target for either chylomicron metabolism or protease metabolism.

Co-transfection with RAP or furin cDNA – In order to determine whether the chaperone protein RAP or the endoprotease furin might be limiting in Cos-7 cells overexpressing recombinant LRP, transient co-transfections with constructs encoding either of these two proteins were performed. Transient co-expression of LRP with RAP shows that overexpression of RAP did not enhance the proteolytic processing of LRP100 in Cos-7 cells (figure 16A). This was despite an increase in RAP protein expression in cells that were transiently co-transfected with this construct (figure 16B). Furin was also unable to enhance proteolytic processing of LRP under the transfection conditions tested (figure 17). In both experiments, the total quantity of DNA transfected was not always comparable, which might contribute to a decrease in the total transfection efficiency. However, the β-chain to α-chain ratio of LRP was not increased as would

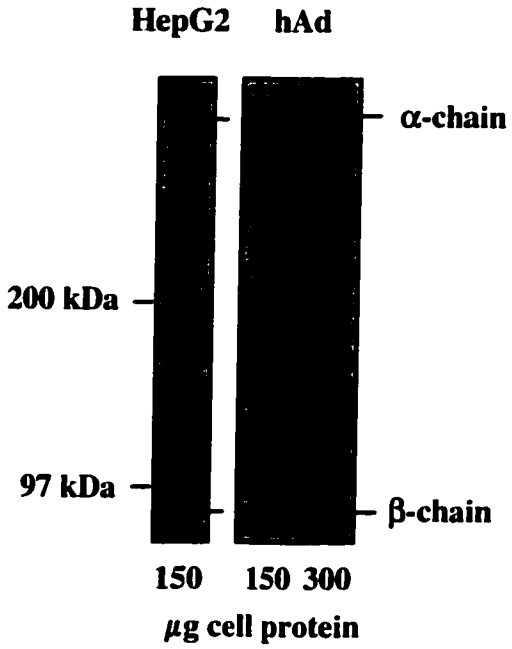

Figure 15. Level of endogenous LRP expression in human adipose tissue and hepatoma HepG2 cells is comparable.

Cell extracts were prepared from HepG2 cell monolayers or collagenase treated human adipose tissue. The indicated amount of protein was loaded in each lane and the samples were resolved by SDS-PAGE under reducing conditions followed by transfer onto nitrocellulose membrane. Proteins were detected by immunoblotting using the anti-human-LRP antibody. *HepG2*, HepG2 cell extract, *hAd*, human adipose tissue extract.

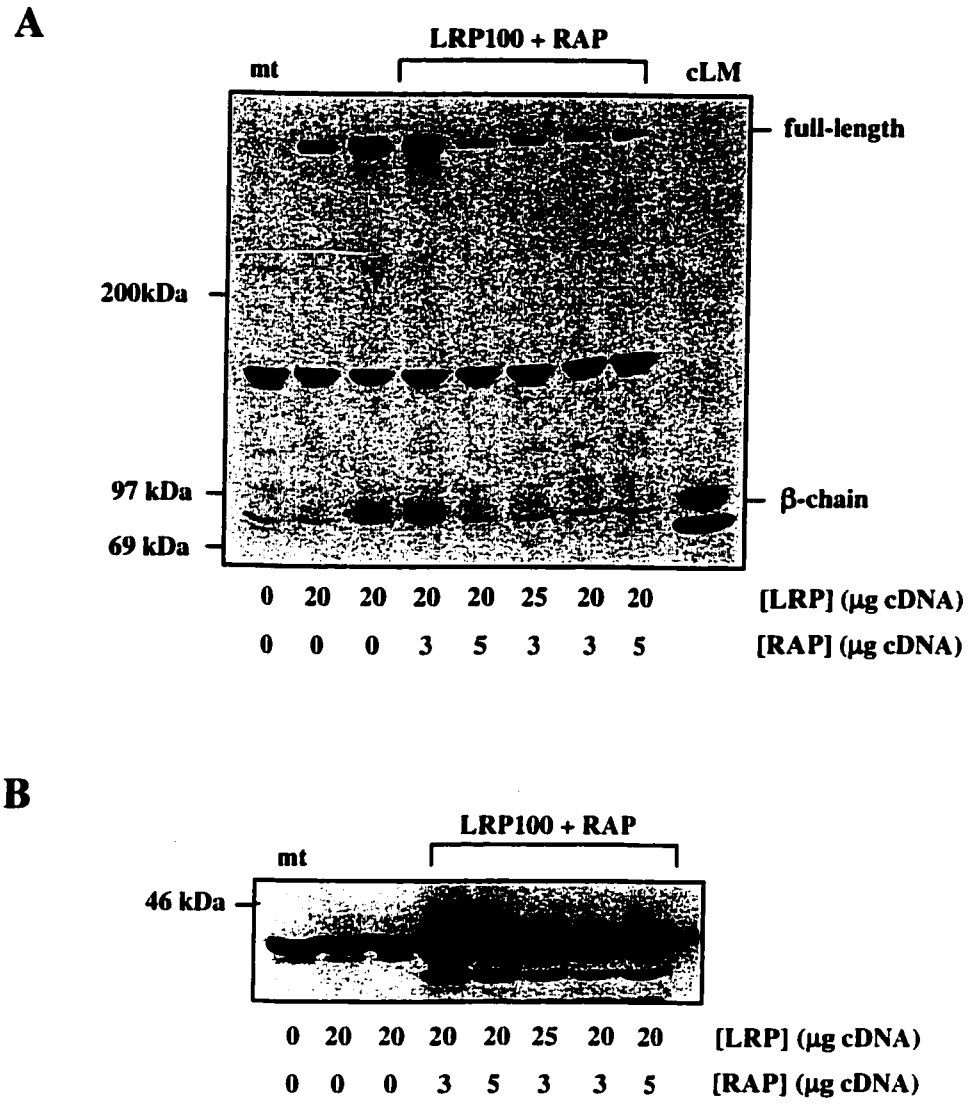

Figure 16. Transient co-transfection of LRP100 with RAP does not enhance proteolytic processing of recombinant LRP in Cos-7 cells.

Cos-7 cells were transiently co-transfected with the indicated concentrations of cDNA encoding chicken LRP100 and human RAP. Cell extracts were prepared 48 hours following transfection, proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblot analyses were performed. *A*, LRP proteins were detected using the anti-β-chain antibody. *B*, RAP was detected using a polyclonal antibody against human RAP. *cLM*, chicken liver membrane; *mt*, mock transfected.

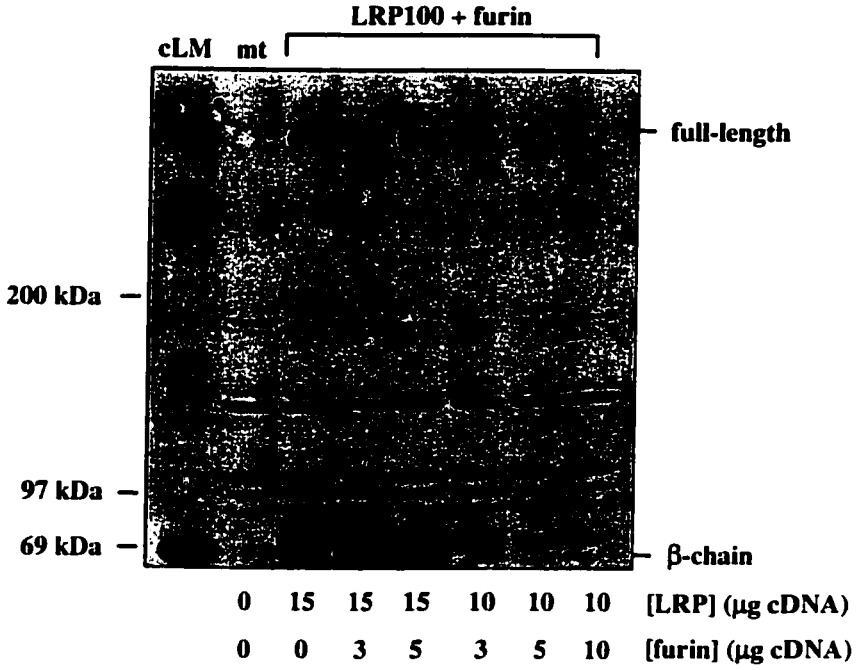

Figure 17. Transient co-transfection of LRP with furin does not enhance proteolytic processing of recombinant LRP in Cos-7 cells.

Cos-7 cells were transiently co-transfected with the indicated concentrations of cDNA encoding chicken LRP100 and human furin. Cell extracts were prepared 48 hours following transfection. LRP proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and detected by immunoblotting using an anti-β-chain antibody. *cLM*, chicken liver membrane; *mt*, mock transfected.

have been expected if either RAP or furin co-transfections enhanced LRP proteolytic processing.

Transient transfection of LRP deletion mutants – An alternate approach was taken in order to find a suitable model for LRP expression. Several cell lines were tested for their ability to express the mature form of LRP100. A CHO-K1 derived chemically mutagenized cell line, denoted 13-5-1 (146) was among the cell lines transfected. This cell line was selected for its resistance to PEA and its inability to internalize α_2M*. Unlike the parental cell line that expressed LRP (figure 18A, left lane), this cell line was deficient in endogenous LRP protein expression when probed using the anti human LRP antibody (figure 18A, right lane). When transiently transfected, 13-5-1 cells exhibited a high expression level of mature recombinant LRP as indicated by a prominent band corresponding to the LRP β-chain (figure 18B, right lane). The anti-β-chain antibody failed to recognize endogenous CHO-K1 LRP.

Generation of an expression system – Stable transformants were generated by transfecting the LRP-null cell line, 13-5-1 with the three LRP constructs, pcLRP25, pcLRP67 and pcLRP100. LRP variant expression was confirmed by screening G418 resistant clones by immunoblot using the LRP anti-β-chain antibody. Fluorescence microscopy using permeabilized LRP100 cell lines and the chicken-specific anti-β-chain antibody show a high expression level of the recombinant protein (figure 19C and D) when compared to untransfected LRP-null cells (figure 19A and B). All subsequent experiments were carried out using the stable transformants indicated.

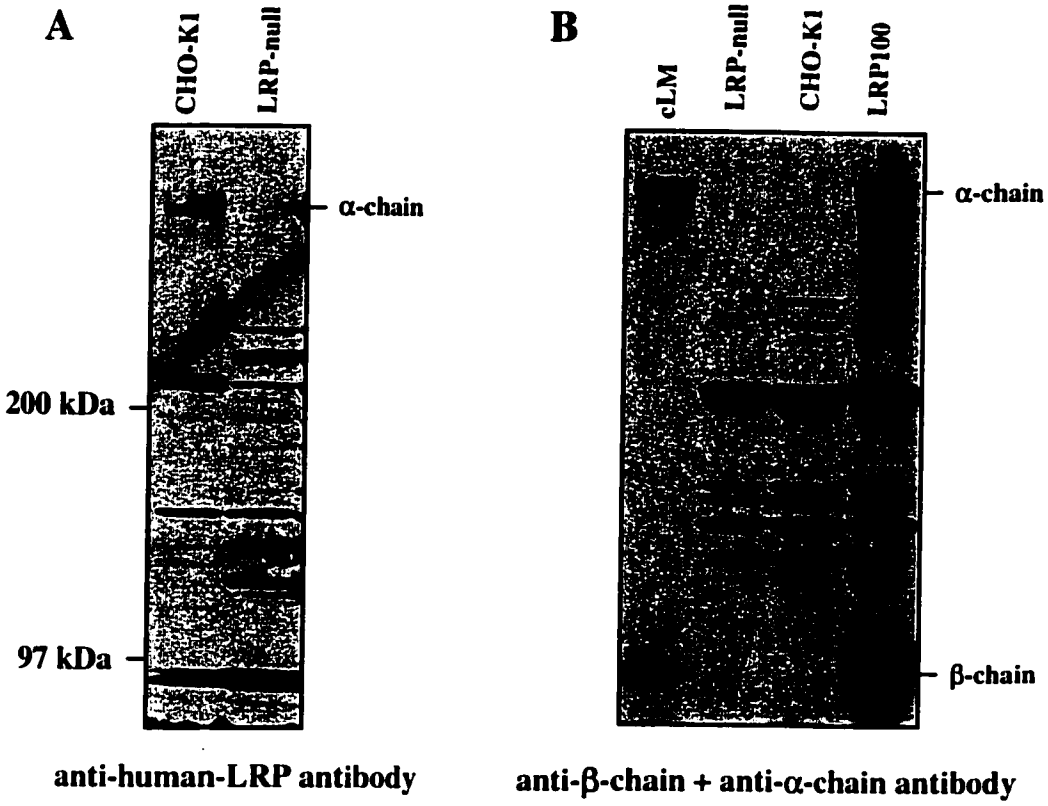

Figure 18. CHO-K1 derived 13-5-1 cells do not express endogenous LRP.

Cell extracts were prepared from the transfected or control cell lines indicated. Samples were resolved on SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes, and LRP was detected by immunoblotting using *A*, the anti-human-LRP antibody or *B*, the anti-β-chain and anti-α-chain antibodies. *cLM*, chicken liver membrane.

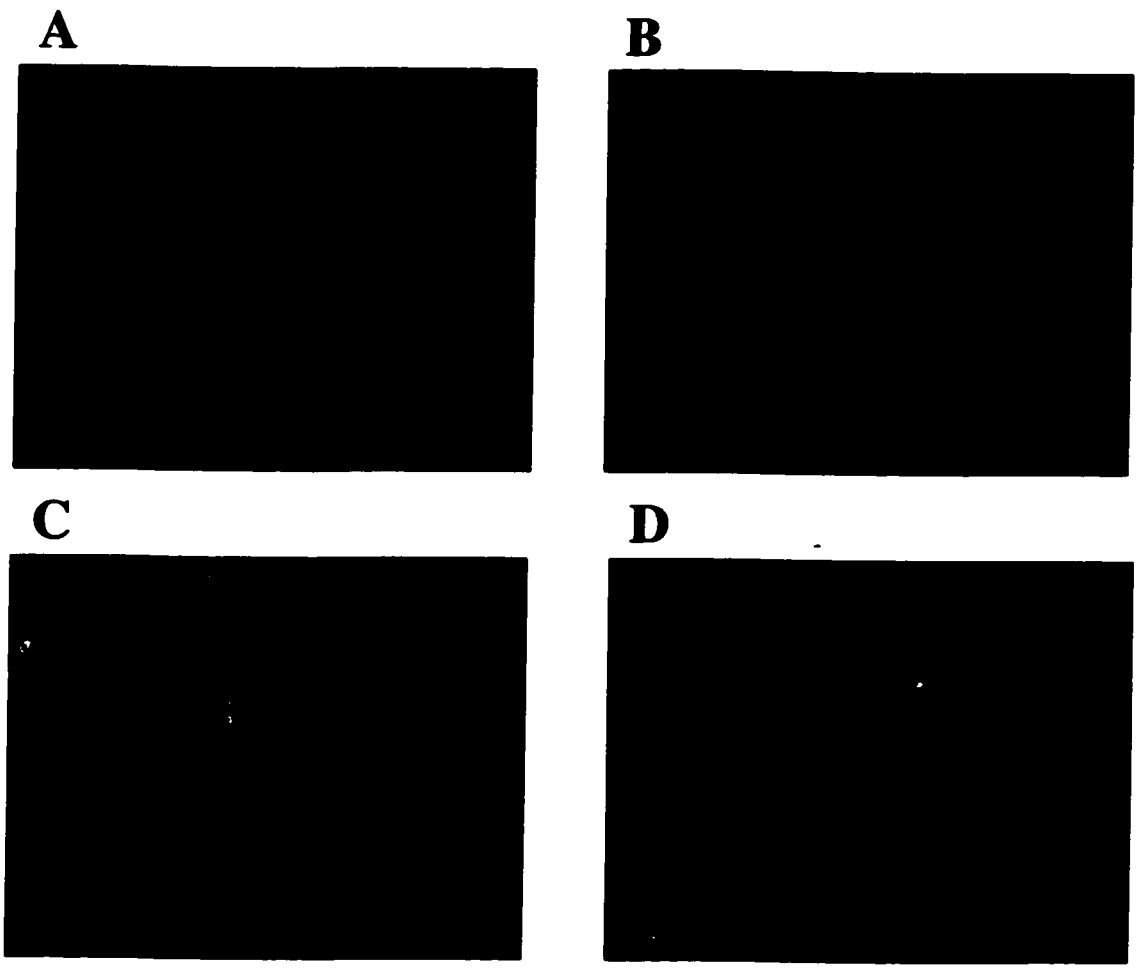

Figure 19. Stably transfected LRP100 is highly expressed in an LRP-null background.

Stable transformants expressing LRP100 and LRP-null cells were grown on 20-mm glass bottom dishes. Cells at approximately 70% confluency were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin. LRP was detected using the anti-β-chain antibody and visualized using rhodamine conjugated goat anti-rabbit secondary antibody. *A and B,* LRP-null cells; *C and D,* stable transformants expressing LRP100.

Stable LRP transformants generated in 13-5-1 cells are biotinylated at the plasma membrane – In order to verify that recombinant LRP variants were expressed at the plasma membrane, proteins were labeled with biotin at 4 °C, immunoprecipitated with anti-β-chain antibody and detected with streptavidin- HRP (figure 20). During the immunoprecipitation, the α and β-chain of LRP remain associated but can be separated by SDS-PAGE. Using this technique, all three LRP variants were detected indicating each was able to reach the plasma membrane. Alternately, biotin labeled plasma membrane proteins were also affinity purified using streptavidin-agarose beads prior to immunoblotting with LRP polyclonal antibody. Figure 21A shows a blot probed with antibody against the β-chain of LRP following adsorption of biotin-labeled material onto streptavidin-agarose beads. As expected, only the β-chain of full-length LRP is detectable. As well, LRP25 and LRP67 are detectable as full length proproteins. The α-chain is biotinylated, but not isolated under the experimental conditions. The biotinylated α-chain can be seen in the non-adsorbed component (figure 21B) with non-adsorbed LRP25, LRP67 and a small amount of β-chain. Only a small portion of LRP67 was affinity purified (figure 21A) compared to non-adsorbed material (figure 21B). It is possible that only a small proportion of the LRP67 variant may be presented at the plasma membrane despite a high total expression level.

Membrane fractionation and neuraminidase digestion shows stable LRP transformants are correctly targeted to the plasma membrane – Neuraminidase digestions of recombinant LRP variants from cells separated into plasma membrane and microsomal fractions were performed (figure 22). LRP100 in the microsomal fraction, a mixture of

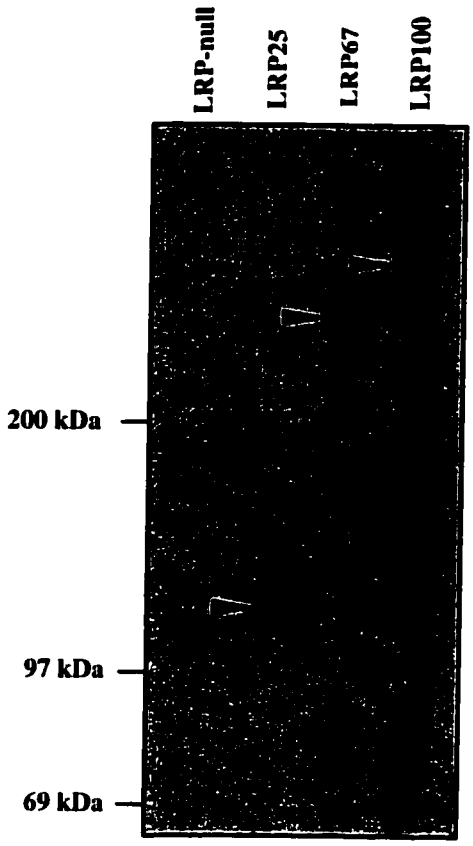

Figure 20. LRP25, LRP67, and LRP100 are biotinylated and detectable at the plasma membrane.

Confluent cell monolayers (60-mm dishes) were treated with biotin for 1 hour at 4 °C. Cell extracts were prepared and the LRP proteins were immunoprecipitated using the anti-β chain antibody. Samples were resolved by SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane. Biotin-labeled proteins were detected using streptavidin conjugated HRP and visualized by enhanced chemiluminescence detection. *cLM*, chicken liver membrane extracts.

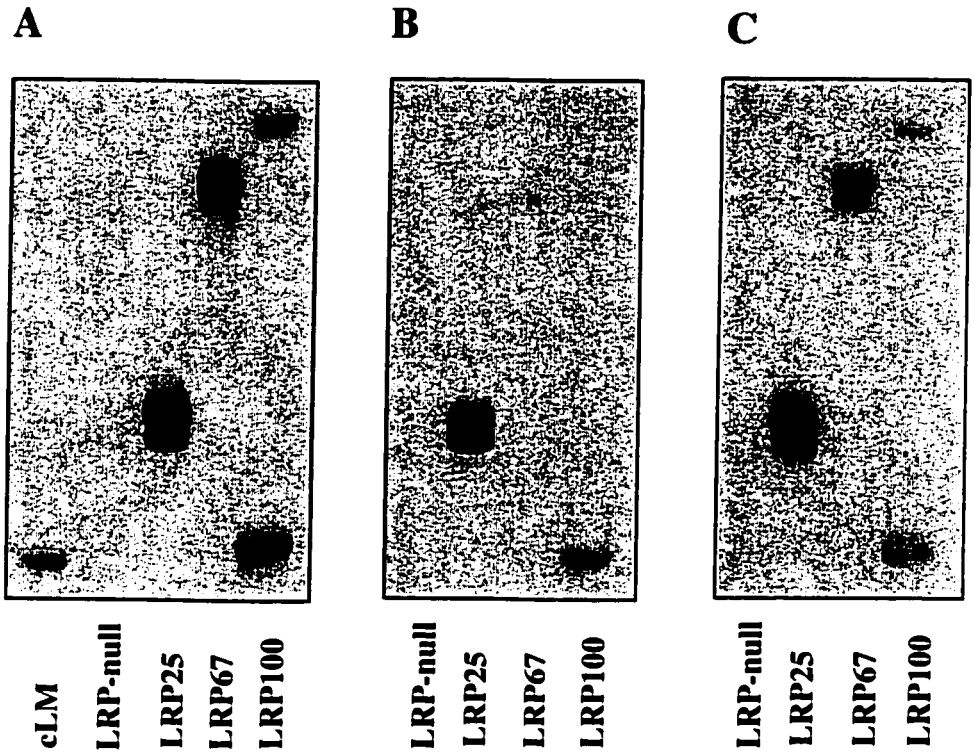

Figure 21. Streptavidin-agarose affinity purification of biotinylated LRP25, LRP67, and LRP100 suggests mature variants are presented at the plasma membrane.

Confluent cell monolayers (60-mm dishes) were treated with biotin for 2 hours at 4 °C. *A*, Whole cell extracts were resolved by SDS-PAGE under reducing conditions, transferred onto PVDF membrane and probed using the anti-β-chain antibody; *B*, cell extracts were incubated with immobilized streptavidin-agarose for 3 hours at 4 °C, resolved by SDS-PAGE under reducing conditions, transferred onto PVDF membrane and probed using the anti-β-chain antibody; *C*, non-adsorbed material in the supernatant following streptavidin-agarose adsorption was resolved by SDS-PAGE under reducing conditions, transferred onto PVDF membrane, and probed using the anti-β-chain antibody.

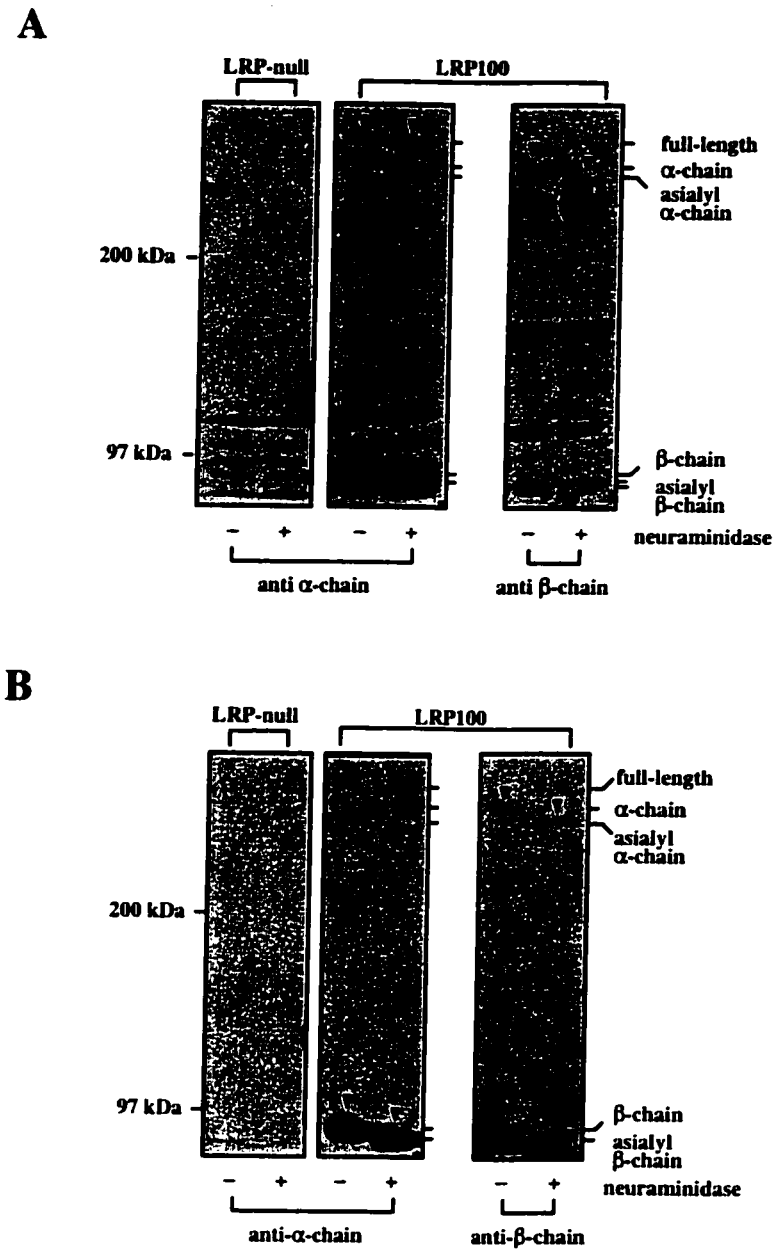

Figure 22. Neuraminidase digestion of LRP100 stably transfected in LRP-null CHO cells (13-5-1) suggests the receptor is presented at the plasma membrane.

Confluent cell monolayers (100-mm dishes) were separated into microsomal and plasma membrane fractions. Soluble cell extracts of *A*, microsomal or *B*, plasma membrane fractions were prepared from cells stably expressing LRP100. Samples were treated with neuraminidase and LRP proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes and probed using the anti-α-chain or the anti-β-chain antibody.

both ER and Golgi membranes, is expected to contain both mature and immature forms of LRP. In this case, the LRP protein was probed using both the anti-α-chain and the anti-β-chain antibodies. In LRP100 transfected cells, the proreceptor was processed into its mature form, where the α- and β-chain are detectable in both the microsomal (figure 22A) and plasma (figure 22B) membrane fractions. Immature, LRP proreceptor was also detected in the microsomal fraction, but, as expected, could not be detected in the plasma membrane fraction. This suggests that the major form of plasma membrane LRP was the mature heterodimeric form. As expected, the LRP α-chain was neuraminidase sensitive (figure 22A and B, right panels). The LRP β-chain was also neuraminidase sensitive suggesting that this subunit is sialylated (figure 22A and B, middle panels). The immature microsomal proprotein was neuraminidase resistant (figure 22A, middle panel).

LRP100 remains stable over eight hours – To determine the half-life of recombinant LRP, pulse-chase experiments were performed using [^{35}S] methionine/cysteine to metabolically label LRP variants (figure 23). As seen previously, the band corresponding to LRP100 was stable over the course of 8 hours and began to decrease by approximately 20% at the 12 hour time point. There appeared to be an increase in label incorporation at the 0.5 and 1 hour time points. This may correspond to a continued incorporation of label from an intracellular pool that is still present at these early time points despite the removal of labeling medium.

LRP100 gains endo H sensitivity two hours after labeling – To follow the intracellular trafficking itinerary of LRP100, endoglycosidase digestions were performed

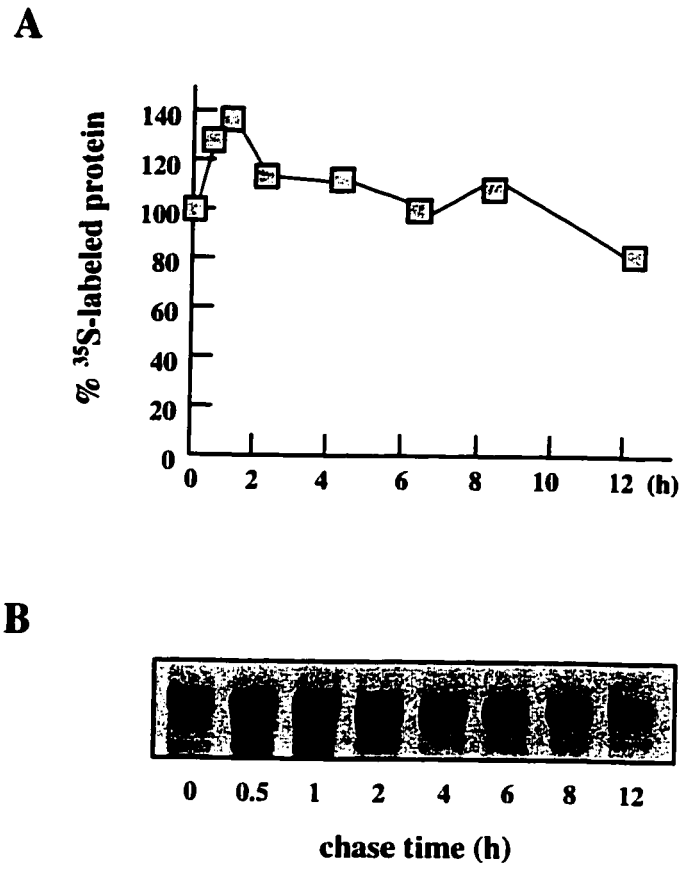

Figure 23. LRP100 remains stable over 8 hours.

Confluent cell monolayers (30-mm dishes) were metabolically labeled with [^{35}S]methionine/cysteine (200 μCi/ml) for 1 hour and chased for the indicated times. Cells were solubilized and LRP proteins were immunoprecipitated using the anti-β-chain antibody. Samples were resolved by SDS-PAGE under reducing conditions and LRP proteins detected by fluorography.

over the course of 12 hours (figure 24). At 0, 0.5, and 1 hour, LRP100 was endoH sensitive (lanes labeled H) relative to the untreated control samples (lanes labeled C). When the LRP protein was treated with PNGase F (lanes labeled F), an enzyme that non-specifically removes all N-linked oligosaccharide moieties, a single band could be seen, suggesting the immature proreceptor had not yet undergone proteolytic processing. At the 2 hour time point, a doublet was detected upon PNGase F treatment suggesting that the protein had begun to undergo proteolytic processing, a portion of it having matured to reach the late Golgi compartment. At the 2 hour time point, although the endo H treated sample appeared as a single band, there were two components within this band. One was the processed α-chain component that is endo H resistant, and the other was the unprocessed proprotein component that remains endo H sensitive and co-migrates with the α-chain. At the 4 and 6 hour time hour time points, however, the doublet had almost entirely disappeared in the PNGase F treated sample, and the majority of the protein was in endo H resistant form indicating the protein had reached a mature heterodimeric state.

Stably expressed LRP100 restores PEA sensitivity to LRP-null cells – Since PEA enters cells opportunistically via LRP, receptor function could be assessed by determining PEA sensitivity of cell lines stably expressing LRP mutants (figure 25). The deletion mutants could also be useful in delineating the region of LRP involved in mediating this process. Preliminary dose effect experiments indicated that at 200 ng/ml, PEA exhibited maximal inhibitory effect on protein synthesis following a 12 hour incubation. PEA dose effect was assessed in cells treated with the toxin for 18 hours. The dose of PEA required to reduce protein synthesis by 50% of untreated cells (IC_{50}) was approximately 25 ng/ml for

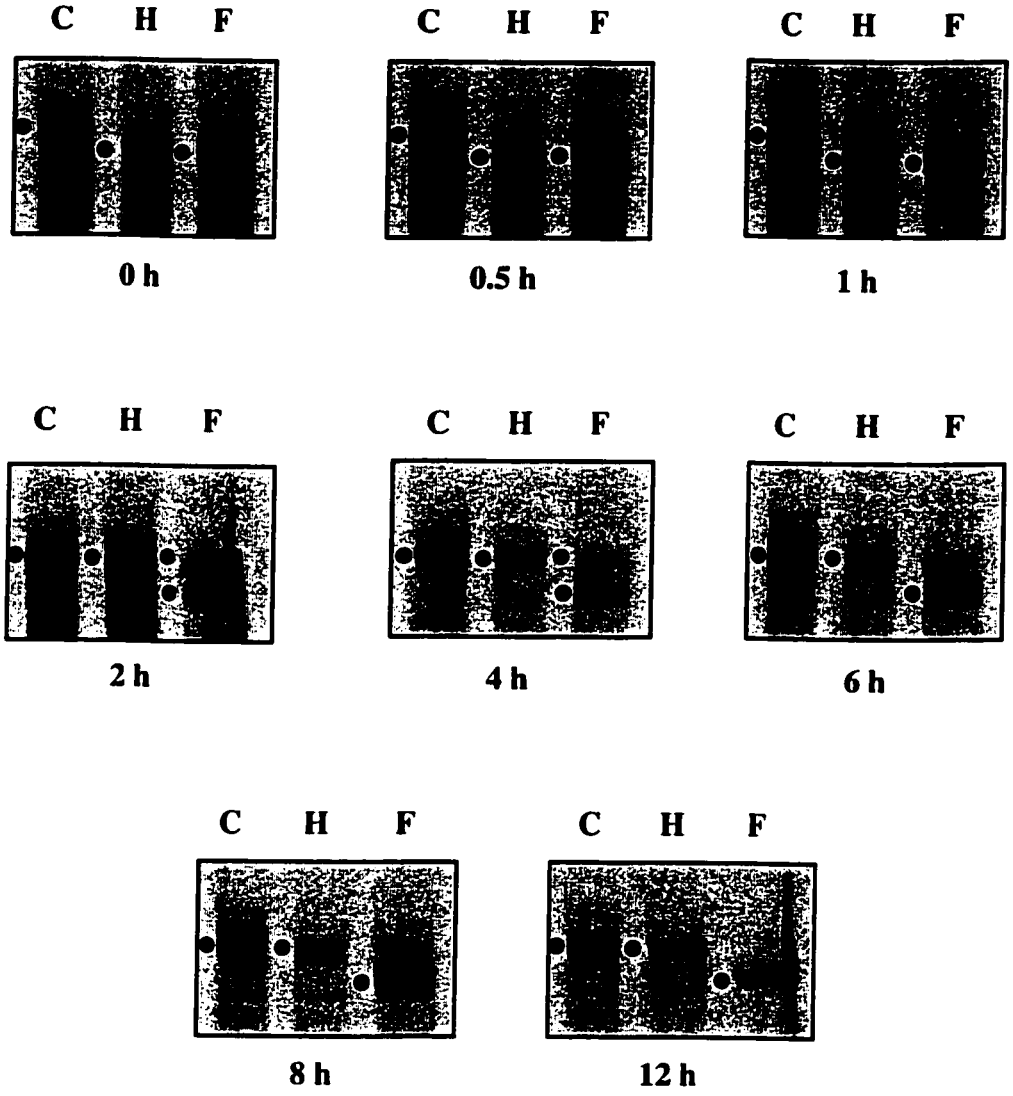

Figure 24. Endoglycosidase digestion indicates recombinant chicken LRP100 matures within 2 hours.

Confluent cell monolayers (30-mm dishes) were metabolically labeled with [^{35}S]methionine/cysteine (200 μCi/ml) for 1 hour and chased for the indicated times. Cells were solubilized and LRP proteins were immunoprecipitated using the anti-β-chain antibody. Immunoprecipitated LRP proteins were treated with endo H (*H*), PNGase F (*F*) or buffer alone (*C*). Samples were resolved by SDS-PAGE under reducing conditions and LRP proteins detected by fluorography.

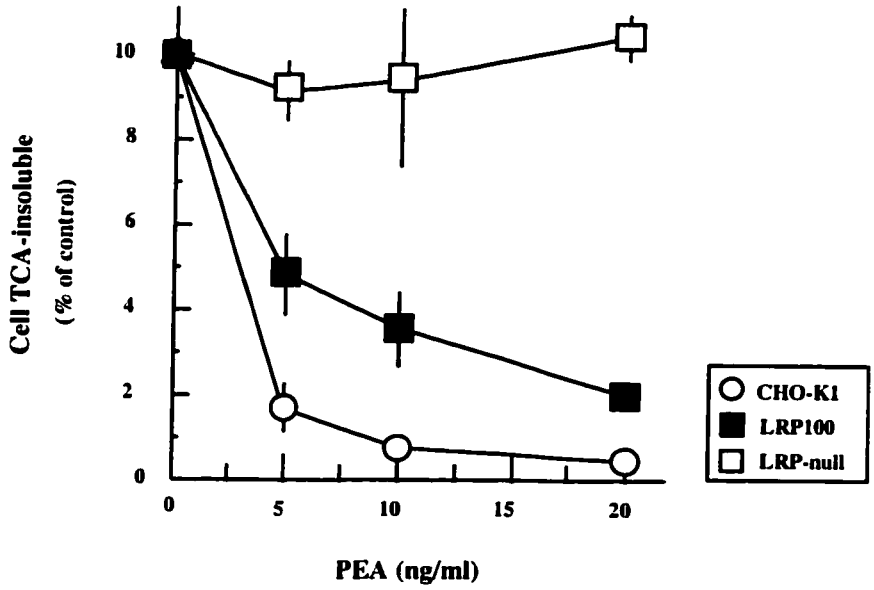

Figure 25. Stable expression of LRP100 in an LRP deficient cell line restores sensitivity to PEA.

Cells were seeded at 10^5 cell/well in 24 well dishes and allowed to adhere for 24 hours. The cells were incubated an additional 18 hours in the presence of the indicated concentrations of PEA prior to metabolic labeling for 1 hour with [^{35}S] methionine/cysteine (100 μCi/ml). Incorporation of [^{35}S] methionine/cysteine into total cellular protein was determined by quantifying the TCA-insoluble fraction by scintillation counting.

CHO-K1 cells, while the LRP-null cells remained insensitive to the toxin at the concentrations tested. Expression of full-length recombinant LRP restored PEA toxicity in LRP-null cells to an IC_{50} of 50 ng/ml.

Stable expression of LRP 100 restores ^{125}I–α_2-macroglobulin binding and degradation to an LRP-null cell line – The ability of LRP100 to bind, internalize, and degrade ^{125}I-α_2M^* at 37 °C was tested to assess the function of LRP100. A time dependant increase in ^{125}I-α_2M^* degradation was seen over four hours for the parental cell line, CHO-K1, as well as stable cells expressing LRP100 (figure 26A). The LRP-null cells did not internalize ^{125}I-α_2M^*. As expected for the cell lines expressing functional receptor, the uptake and degradation of α_2M^* was inhibited in a concentration dependent manner by the addition of RAP (figure 26B).

Membrane fractionation and neuraminidase digestion confirms stable LRP67 transformants are correctly targeted to the plasma membrane – For LRP67, three clonal cell lines exhibiting different expression levels (figure 27A) were selected for each experiment. For all three cell lines tested, the recombinant LRP67 was resolved as a doublet by SDS-PAGE suggesting that the protein exists in two different glycosylation states. Cell extracts were separated into microsomal and plasma membrane fractions and treated with Endo H. LRP 67 in the microsomal membrane fraction can be seen in both neuraminidase resistant sialylated and neuraminidase sensitive asialylated forms for the two clonal cell lines that were tested (figure 27B, bottom panel). As expected, the plasma membrane component is comprised of a single band representing the mature protein

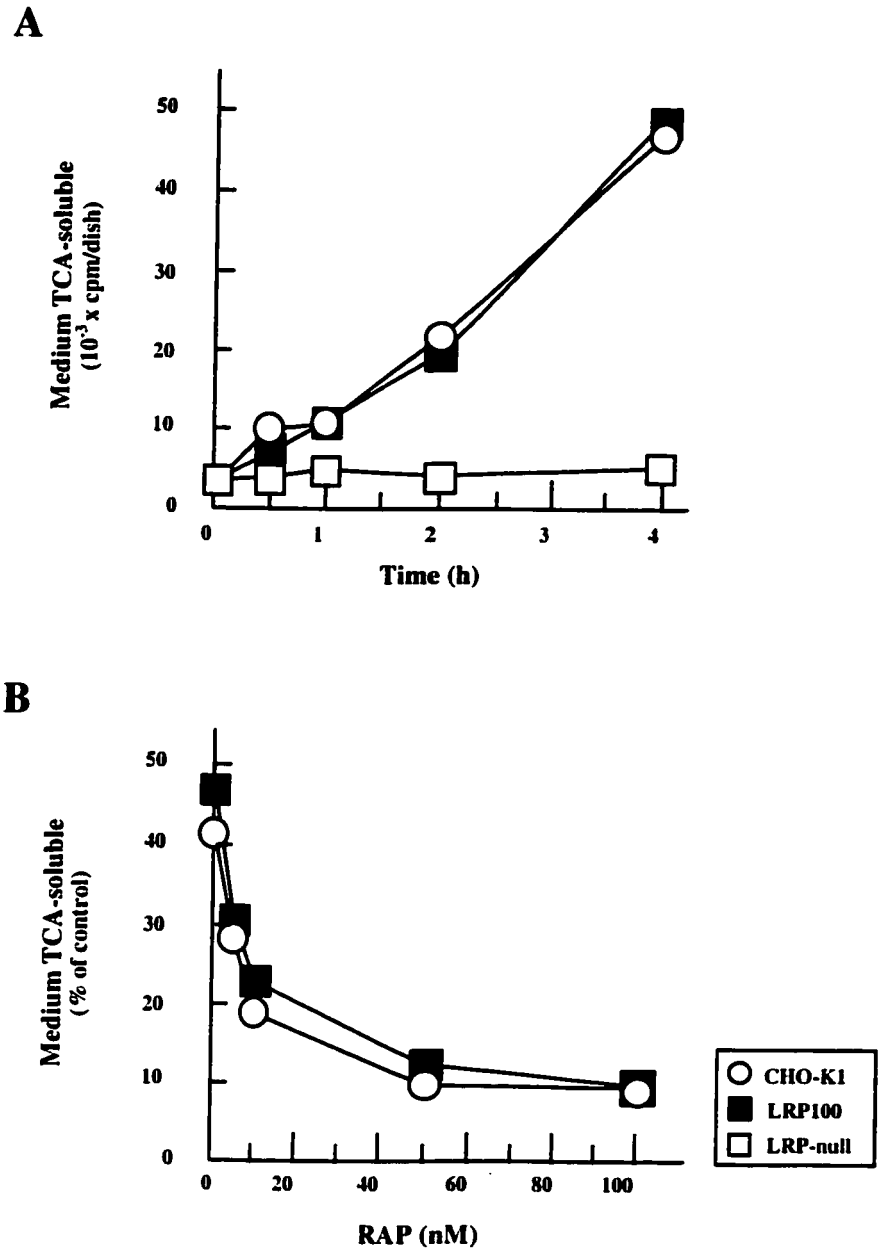

Figure 26. Stable expression of LRP100 in an LRP deficient cell line restores ^{125}I-α_2M binding and degradation.

Cells (230 μg protein/well) were incubated with ^{125}I-α_2M* (5.8 x 10^3 cpm/ng, 20 μg/ml) at 37 °C. *A*, trichloroacetic acid-soluble, non free-iodine radioactivity in the medium was determined at the indicated times; *B*, inhibitory effect of RAP on α_2M* degradation was determined by co-incubation of ^{125}I-α_2M* with increasing concentrations of RAP for 4 hours at 37 °C.

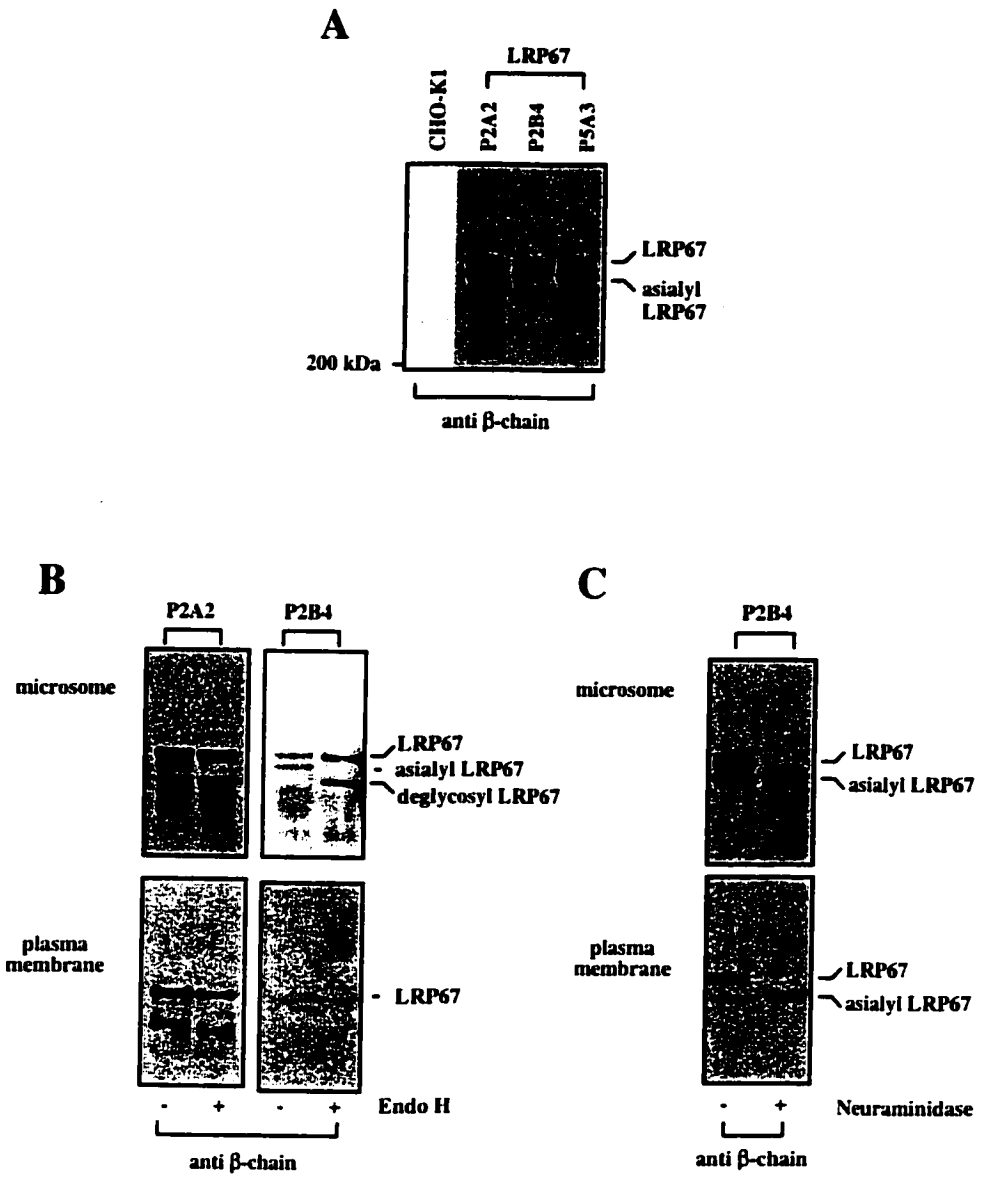

Figure 27. Stably expressed LRP67 is presented at the plasma membrane.

A, immunoblots of LRP67 stably expressed in transfected 13-5-1 cells. 5 μg of cell extract protein was loaded in each lane. *B*, Endo H digestion of LRP67 associated with microsomal (*top*) or plasma membranes (*bottom*). *C*, neuraminidase digestion of LRP67 associated with microsomal (*top*) or plasma membrane (*bottom*). LRP proteins were determined by immunoblotting using anti-α-chain or anti-β-chain antibody.

(figure 27C, bottom panel) that is endo H resistant and neuraminidase sensitive. This suggests that the asialylated form of LRP67 is the immature ER precursor form with sialylated LRP67 being the mature form.

Stably expressed LRP67 restores PEA sensitivity to an LRP-null cell line – All three LRP67 clones tested were found to be capable of internalizing PEA as shown by a decrease in [^{35}S] methionine/cysteine label incorporation into total protein (figure 28). Three cell lines exhibited sensitivity to PEA to varying degrees. Clone P2A2, a high expressor, exhibited the highest PEA sensitivity, comparable to that of CHO-K1 cells. In clones P2B4 and P5A3, both low expressors, toxicity to PEA was only partially restored. The difference in PEA toxicity may be attributable either to differing levels of LRP67 expression or of plasma membrane presentation exhibited by each cell line.

Stable expression of LRP67 restores ^{125}I–α_2-macroglobulin binding to an LRP-null cell line when expressed at a sufficiently high level – The ability of cells stably expressing LRP67 to bind and degrade ^{125}I-α_2M* was analyzed. Of the cell lines tested, P2A2, the cell line exhibiting the higher expression level was able to bind a greater quantity of ^{125}I-α_2M* over four hours (figure 29A) and a greater amount of RAP was required in order to inhibit the binding of ^{125}I-α_2M* (figure 29B).

Membrane fractionation and neuraminidase digestion shows stable LRP25 transformants are correctly targeted to the plasma membrane – Analyses similar to that of LRP67 were performed for three stable transformants expressing LRP25. Clonal cell lines exhibiting

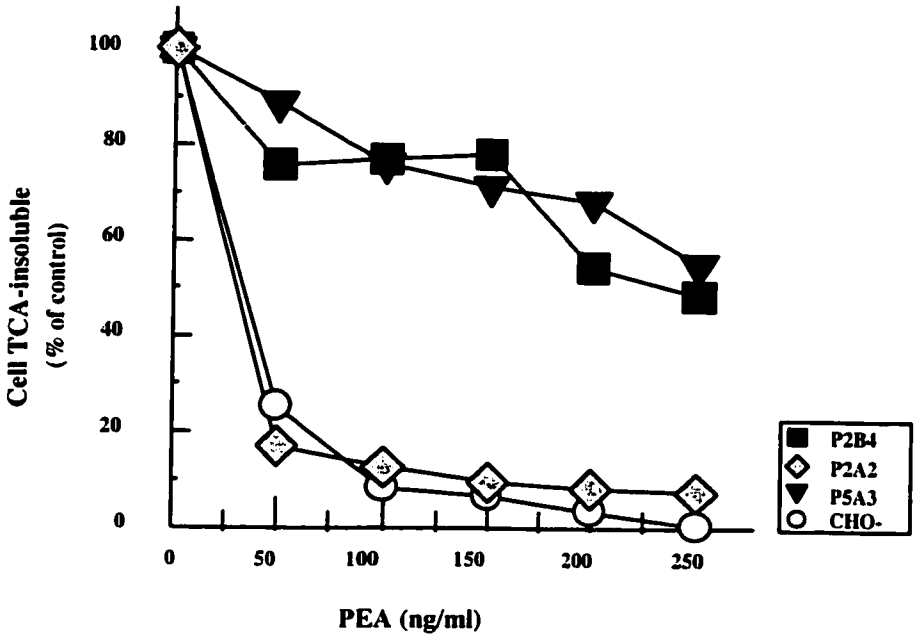

Figure 28. Stable expression of LRP67 in an LRP deficient cell line restores sensitivity to PEA.

Cells were seeded at 10^5 cell/well in 24 well dishes and allowed to adhere for 24 hours. The cells were incubated an additional 18 hours in the presence of the indicated concentrations of PEA prior to metabolic labeling for 1 hour with [^{35}S] methionine/cysteine (100 μCi/ml). Incorporation of [^{35}S] methionine/cysteine into total cellular protein was determined by quantifying the TCA-insoluble fraction by scintillation counting.

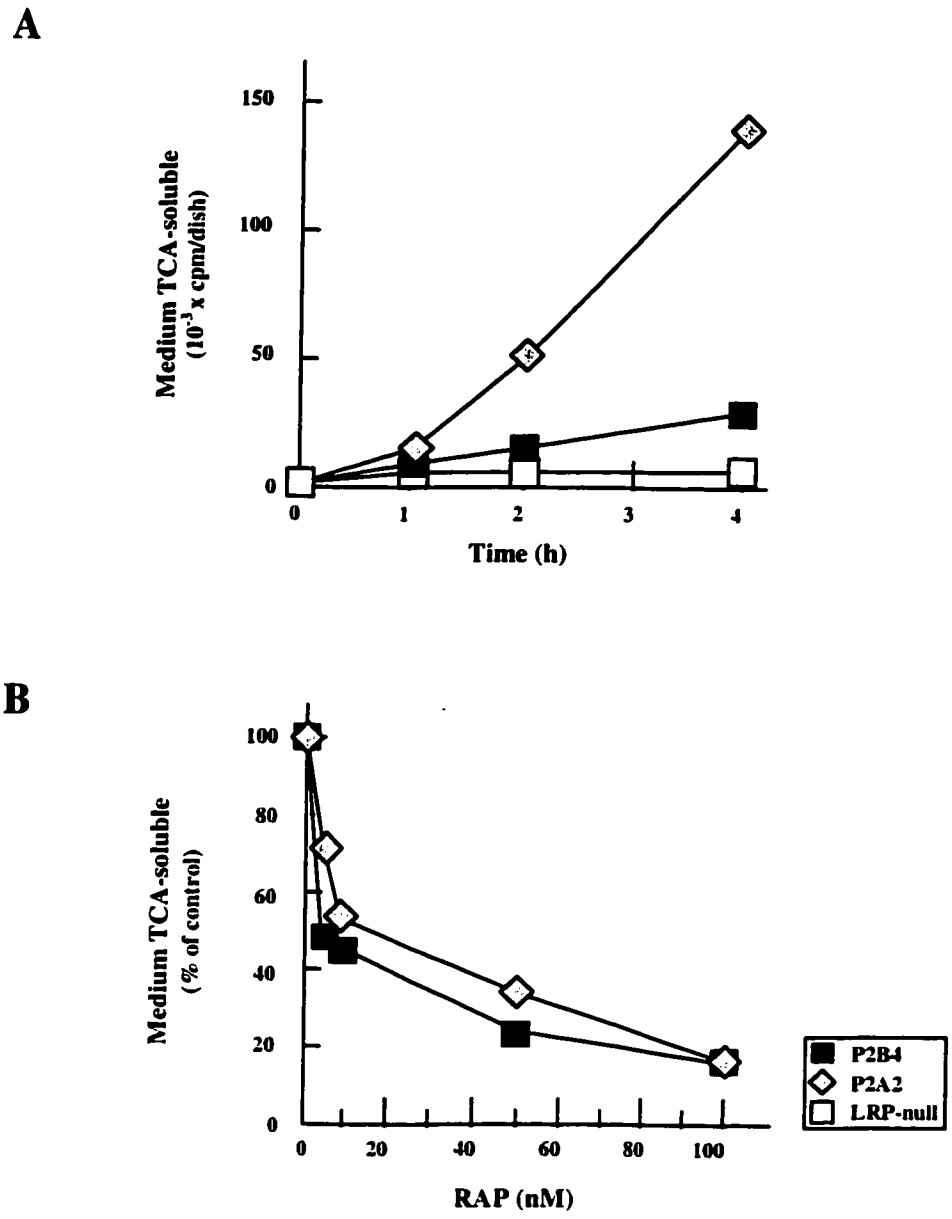

Figure 29. Stable expression of LRP67 in an LRP deficient cell line partially restores ^{125}I-α_2M degradation activity.

Cells (230 μg protein/well) were incubated with ^{125}I-α_2M* (5.8 x 10^3 cpm/ng, 20 μg/ml) at 37 °C. *A*, trichloroacetic acid-soluble, non free-iodine radioactivity in the medium was determined at the indicated times; *B*, inhibitory effect of RAP on α_2M* degradation was determined by co-incubation of ^{125}I-α_2M* with increasing concentrations of RAP for 4 hours at 37 °C.

different expression levels (figure 30A) were chosen for these experiments. For each cell line, the mature form of LRP25 exhibited endo H resistance (figure 30B) and neuraminidase sensitivity (figure 30C). Localization of LRP25 to the plasma membrane as determined by subcellular fractionation similar to that performed for LRP67 and LRP100 supports the finding that LRP25 is presented at the plasma membrane.

Stable expression of LRP25 partially restores PEA sensitivity in certain clones – Surprisingly, two of the LRP25 clones tested, P4B2 and P7B3, were found to modestly restore PEA sensitivity to LRP-null cells, while clone P6B4 remained resistant to the toxin (figure 31). As with LRP67, the restoration of function to the LRP-null cell line was closely correlated to the expression level of the recombinant protein (figure 31A) with clone P7B3 exhibiting the highest expression level and being the most sensitive to PEA.

Stable expression of LR25 partially restores ^{125}I–α_2-macroglobulin binding and degradation to an LRP-null cell line when expressed at a sufficiently high level – The ability of cells stably expressing LRP25 to bind and degrade $^{125}I\alpha_2M^*$ was analyzed. Of the cell lines tested, P7B3, the cell line exhibiting the higher expression level was able to bind a greater quantity of ^{125}I-α_2M^* over four hours (figure 32A) and required a greater RAP concentration in order to inhibit the binding of ^{125}I-α_2M^* (figure 32B).

Recombinant full-length LRP essentially restores ligand binding activity while two LRP truncation mutants LRP67 and LRP25 partially restore LRP activity to an LRP-null cell line – Analysis of the α_2M^* binding and degradation data indicated that the ability of

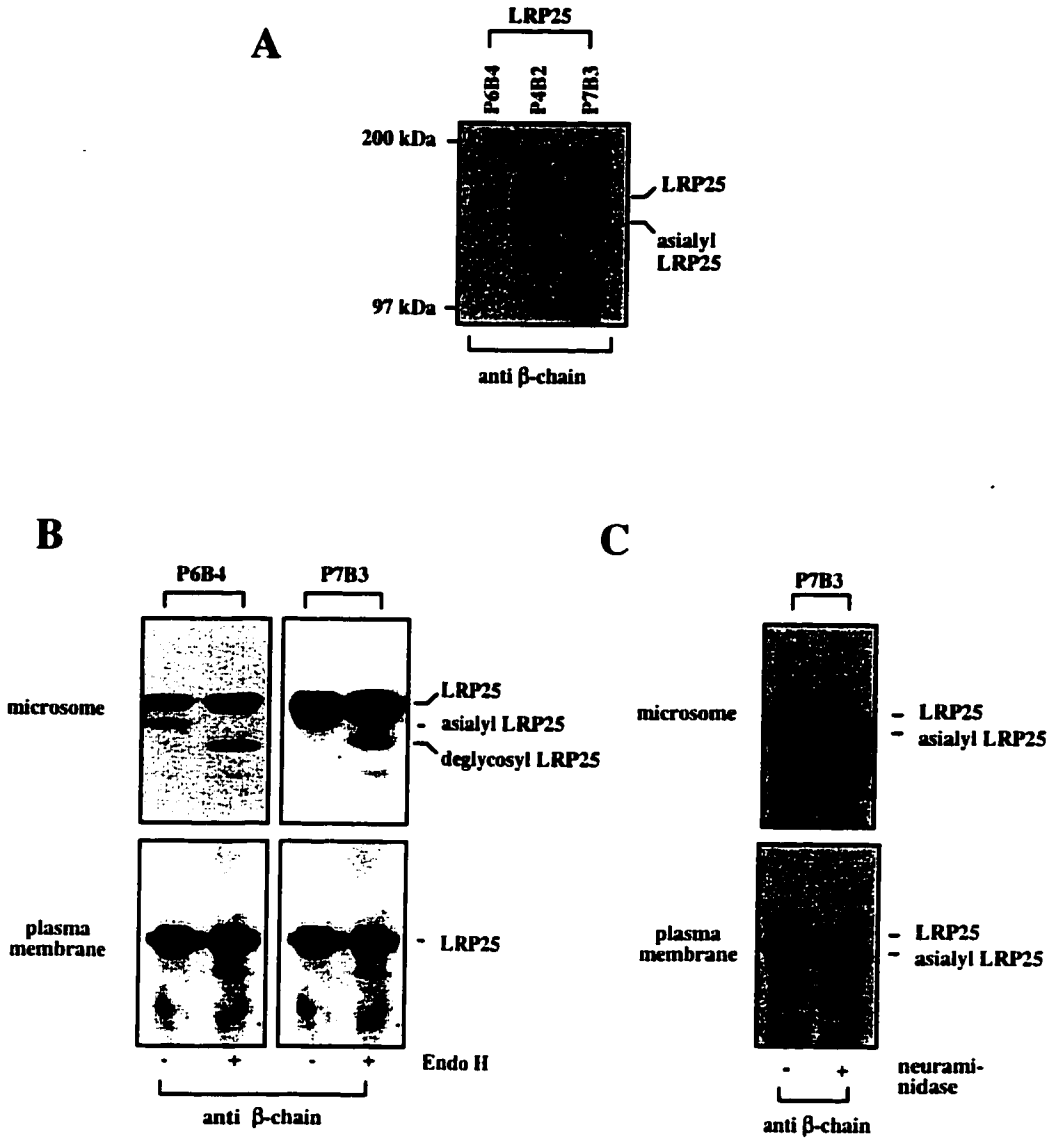

Figure 30. Stably expressed LRP25 is presented at the plasma membrane.

A, immunoblots of LRP25 stably expressed in 13-5-1 cells. 5 μg of cell extract protein was loaded in each lane. *B*, Endo H digestion of LRP25 associated with microsomal (*top*) or plasma membranes (*bottom*). *C*, neuraminidase digestion of LRP25 associated with microsomal (*top*) or plasma membrane (*bottom*). LRP proteins were determined by immunoblotting using anti-α-chain or anti-β-chain antibody.

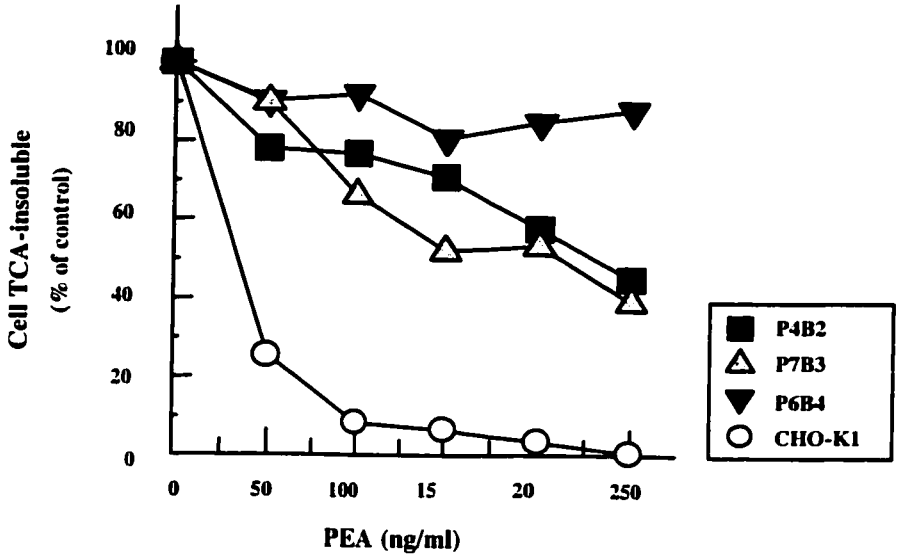

Figure 31. Stable expression of LRP25 in an LRP deficient cell line partially restores sensitivity to PEA in certain clonal cell lines.

Cells were seeded at 10^5 cell/well in 24 well dishes and allowed to adhere for 24 hours. The cells were incubated an additional 18 hours in the presence of the indicated concentrations of PEA. Total protein was metabolically labeled for 1 hour with [^{35}S] methionine/cysteine (100 μCi/ml). Incorporation of label into total protein was determined by quantifying label in TCA-insoluble fraction.

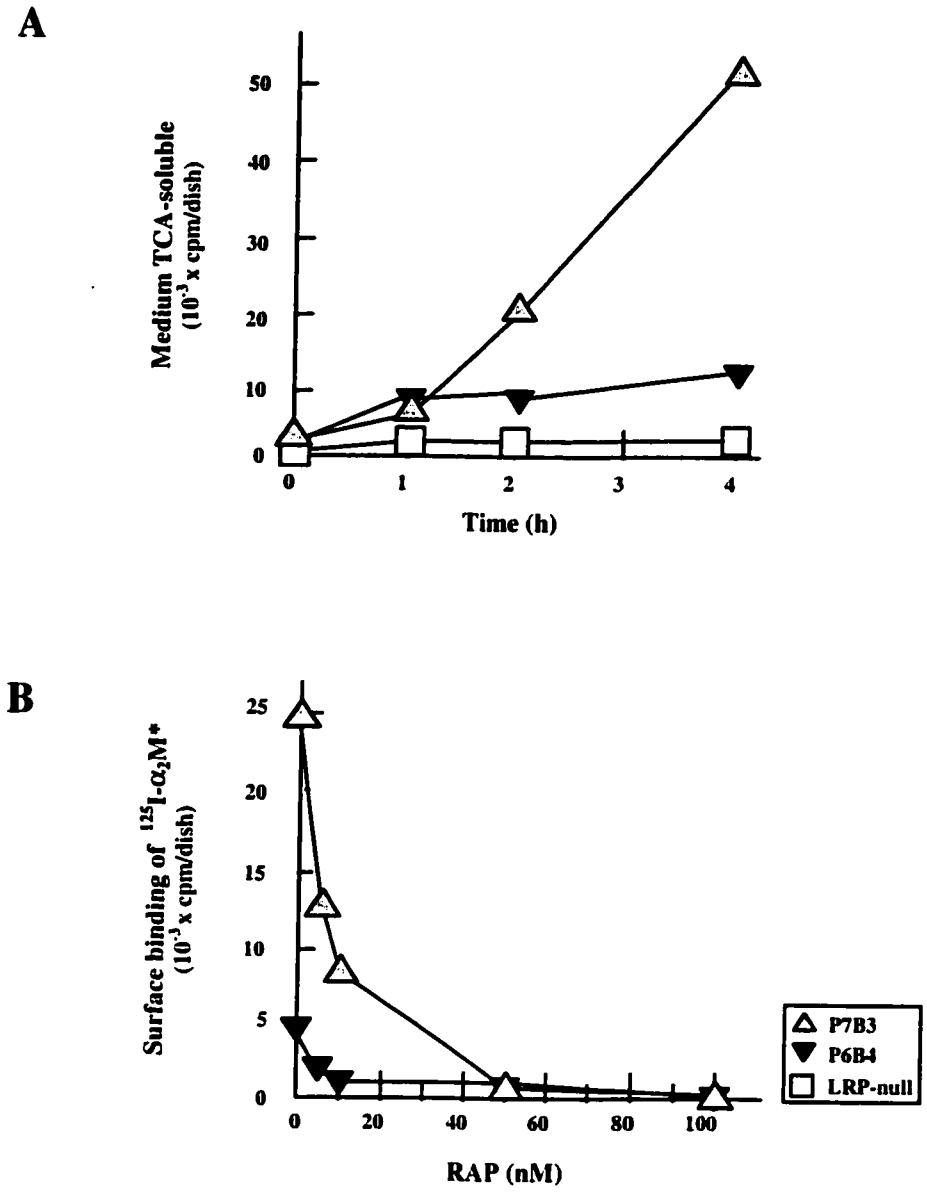

Figure 32. Stable expression of LRP25 in an LRP deficient cell line partially restores ^{125}I-α_2M binding activity in certain clonal cell lines.

Cells (230 μg protein/well) were incubated with ^{125}I-α_2M* (5.8 x 10^3 cpm/ng, 20 μg/ml) at 37 °C. *A*, trichloroacetic acid-soluble, non free-iodine radioactivity in the medium was determined at the indicated times; *B*, inhibitory effect of RAP on α_2M* binding was determined by co-incubation of ^{125}I-α_2M* with increasing concentrations of RAP for 4 hours at 37 °C.

full-length chicken LRP to degrade ^{125}I-α_2M* was approximately equivalent to that of the endogenous receptor (table II). The degradation data was corrected for the differing levels of expression and it was found that LRP67-mediated degradation was roughly comparable to that of LRP100 (approximately 66%), while LRP25 was much less efficient (approximately 10%).

Cell lines	^{125}I-α_2M* binding (ng/mg protein)	^{125}I-α_2M* degradation (ng/mg protein)	Corrected ^{125}I-α_2M* degradation[a] (ng/ng α_2M bound)	PEA toxicity[b]	Corrected PEA toxicity[c]
CHO-K1 (parental line)	2.15	42.6	19.8 (100%)	97 ± 1 (n=3)	45
LRP-null (13-5-1)	0	6.37	N/A[d]	−2 ± 5 (n=3)	N/A
LRP100 P2B3	2.86	49.2	17.2 (87%)	79 ± 3 (n=3)	27 (66%)
LRP67					
P2B4	3.13	39.5	12.6 (64%)	45	14 (31%)
P2A2	23.9	120	5.02 (25%)	92	3.8 (9%)
P5A3	1.28	31.4	24.5 (124%)	32	25 (6%)
LRP25					
P7B3	16.1	106	6.58 (33%)	47	2.9 (6%)
P4B2	35.7	156	4.37 (22%)	42	1.2 (3%)
P6B4	2.58	18	6.98 (35%)	10	3.9 (9%)

Table 2. Degradation of ^{125}I-α_2M and PEA toxicity index in CHO cells transfected with wild type and mutant chicken LRP cDNAs.

The wild-type CHO-K1 cell, mutant LRP-null cell (13-5-1) and stably transfected cells expressing LRP100 (P2B3), LRP67 (P2B4, P2A2, P5A3) or LRP25 (P7B3, P4B2, P6B4) were incubated with ^{125}I-α_2M* (8.9×10^3 cpm/ng) for 2 h at 4 °C to determine surface binding or for 4 h at 37 °C to determine degradation. The same cells were incubated with PEA (200 ng/ml) for 18 h and labeled with [^{35}S]methionine/cysteine for 1 h to determine protein synthesis. N/A, not applicable.

Footnotes to Table

[a]Corrected ^{125}I-α_2M* degradation values were calculated by dividing the amount of ^{125}I-α_2M* (ng/mg protein) degraded at 37 °C by the amount (ng/mg protein) of ^{125}I-α_2M* bound at 4 °C.

[b]Values in parentheses represent the percentage of the normal α_2M degradation activity of LRP.

[c]Corrected PEA toxicity values were calculated by dividing the decrease in protein synthesis (% of no PEA) by the amount of ^{125}I-α_2M* (ng/mg protein) bound at 4 °C. Values in parentheses represent the percentage of the normal LRP-related PEA toxicity.

[d]N/A, not applicable.

4.2 Ligand binding cluster deletion mutants

Generation of ligand binding cluster deletion mutants – The LRP deletion mutants were refined to encode specific deletions to clusters of ligand binding modules. Figure 33 shows the strategy used in the generation of these constructs. Figure 34 shows a schematic diagram of the complement-type/class A cluster deletion variants. LRPΔII and LRPΔIII encode deletions of the putative ligand binding clusters two and three respectively. Both mutants retain the proteolytic processing site and were, therefore, expected to become mature heterodimers. These deletion mutants were constructed based on convenient naturally occurring restriction sites found within the chicken LRP sequence. As a result, these constructs also encode deletions within individual EGF modules and possess crippled or broken β-propeller domains. LRPΔIII-IV encodes a construct possessing intact individual β-propellers, but these are arranged in an asymmetric fashion, the fifth β-propeller from the N-terminal is unpaired, and lacks an adjacent EGF module. The final construct, LRPΔIII-IVβ was generated by PCR in order to encode the same arrangement of complement-type/class A clusters as LRP ΔIII-IV, except that the β-propeller and two EGF modules have been reinserted to now encode a symmetrical arrangement of β-propellers.

Transient transfections of the complement-type/class A module cluster deletion mutant LRPΔII – Transiently transfected LRPΔII was highly expressed and was resolved to the

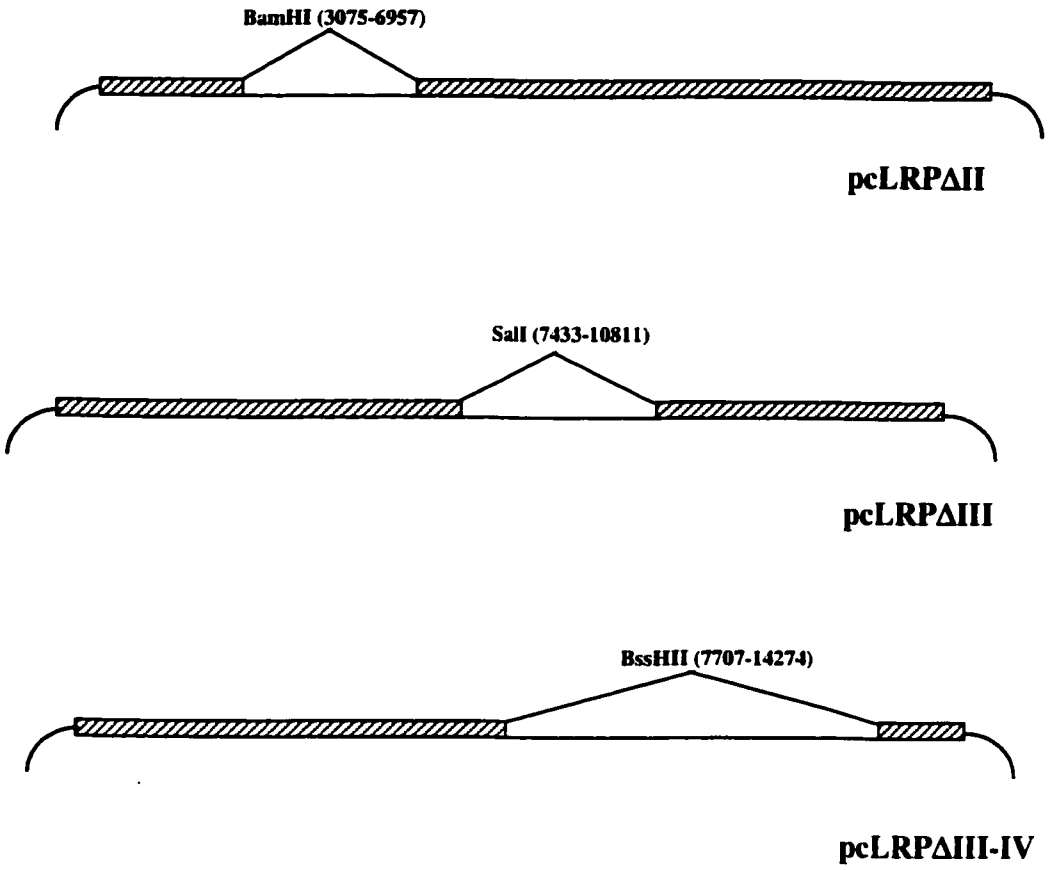

Figure 33. Construction of LRP expression plasmids encoding internal deletions of putative ligand binding clusters.

Strategy used in cloning pcLRPΔII, pcLRPΔIII, and pcLRPΔIII-IV. The convenient restriction sites indicated were used in generating constructs. *Dashed lines* represent regions deleted.

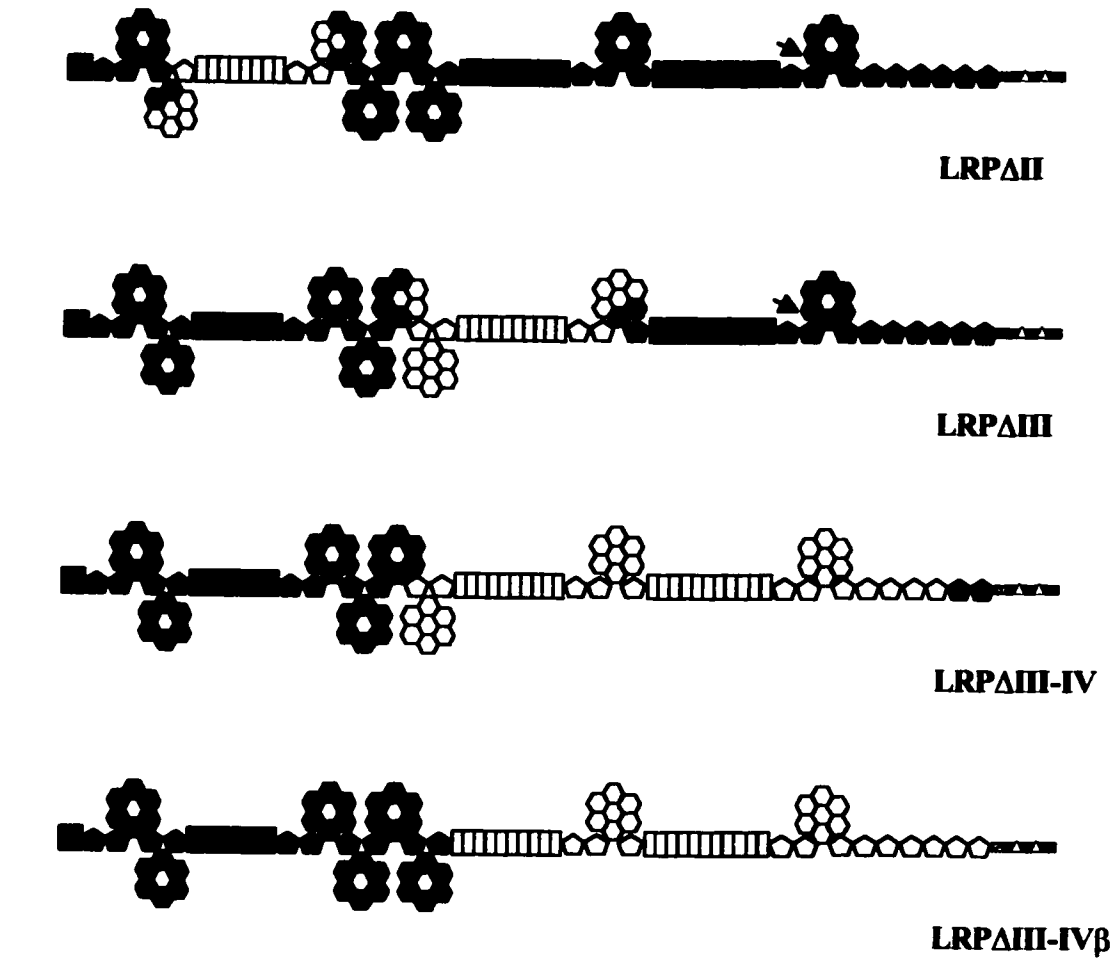

Figure 34. Structure of LRPΔII, LRPΔIII, LRPΔIII-IV, and LRPΔIII-IVβ variants.

Schematic representation of variants encoding ligand binding cluster deletion mutants. Structures have been depicted to emphasize ligand binding complement type complement-type/class A modules and EGF precursor type modules. *Open shapes* indicate regions deleted.

correct molecular mass by SDS-PAGE (figure 35A). Despite the presence of the β-chain, the majority of the receptor was seen in proteolytically unprocessed form. Similar results were observed when stable transformants were generated (figure 35B). Two stable clones were chosen for study, a high expressor, LRPΔII-3 and a low expressor, ΔII-7. Only LRPΔII-3 is detectable by plasma membrane biotinylation (figure 36A). Although it is not known whether the antibody binds to each variant with the same efficiency, a 2.5-fold greater expression level of LRPΔII-3 than LRP100 was required for plasma membrane detection by this method (figure 37). The low expressor, LRPΔII-7 was undetectable by plasma membrane biotinylation (figure 36B) despite being detectable in proteolytically unprocessed form when the same membrane is probed using the anti-β-chain antibody (figure 36C). The β-chain was undetectable using the plasma membrane biotinylation method due to a high level of background (data not shown).

LRPΔII appears to be less stable than LRP100 – Pulse-chase experiments were performed to determine the half-life of LRPΔII stably expressed in LRP-null cells. The quantity of [^{35}S]-labeled LRPΔII-3 appears to decrease by approximately 40-50% eight hours following pulse-chase (figure 38). This is in contrast with LRP100 that exhibited virtually no decrease after this time and only a 20 % decrease after 12 hours of chase (figure 23). This increase in the rate of disappearance of [^{35}S]-labeled LRPΔII from the cells suggests premature degradation of this variant may occur within these cells perhaps because of a folding defect.

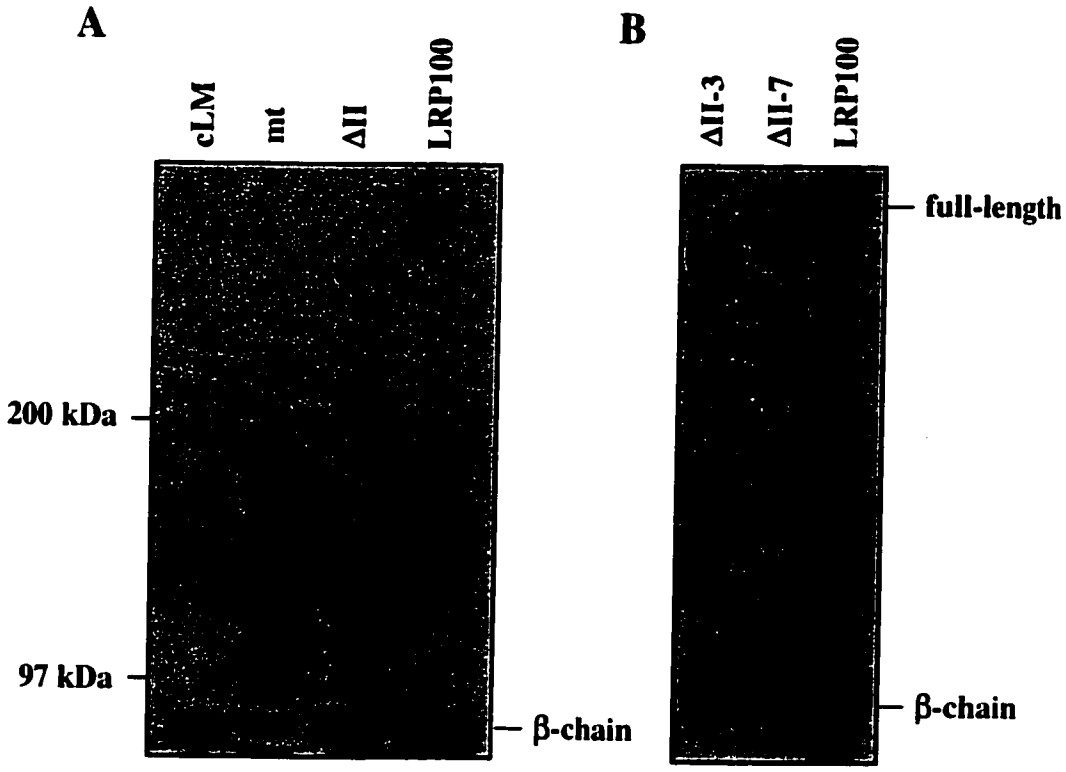

Figure 35. LRPΔII is inefficiently processed in CHO-derived, LRP-null cells.

*A.*Cell extracts were prepared 48 hours following transient transfection of pcLRPΔII into LRP-null cells. Samples were resolved by SDS-PAGE under reducing conditions and the expressed LRP proteins were detected by immunoblotting using the anti-β chain antibody. *B.*Confluent monolayers (60-mm dishes) of LRP-null cells stably transfected with pcLRPΔII were harvested and analyzed as described in A. *cLM*, chicken liver membrane extracts; *mt*, mock transfected.

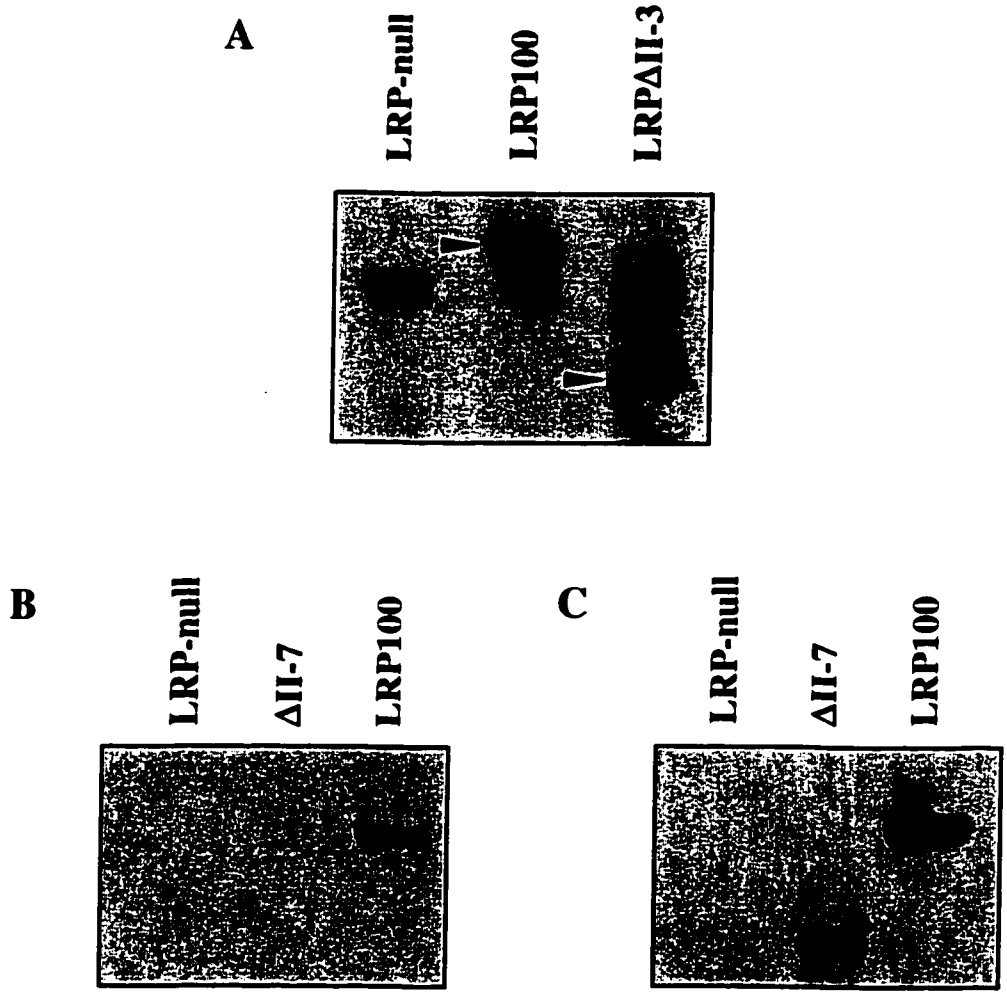

Figure 36. Plasma membrane presentation of LRPΔII is dependent on expression level.

Cell extracts from cells stably expressing LRPΔII were prepared. Samples were resolved by SDS-PAGE under reducing conditions, transferred onto nitrocellulose membrane, and detected by immunoblotting using the anti-β chain antibody. *A*, clone LRPΔII-3, a high expressor is detected by plasma membrane biotinylation. *B*. clone LRPΔII-7, a low expressor is not detected by plasma membrane biotinylation. *C*. both LRPΔII-3 and LRPΔII-7 cell lines express LRPΔII as shown by immunoblot of the same membranes used for *A* and *B*.

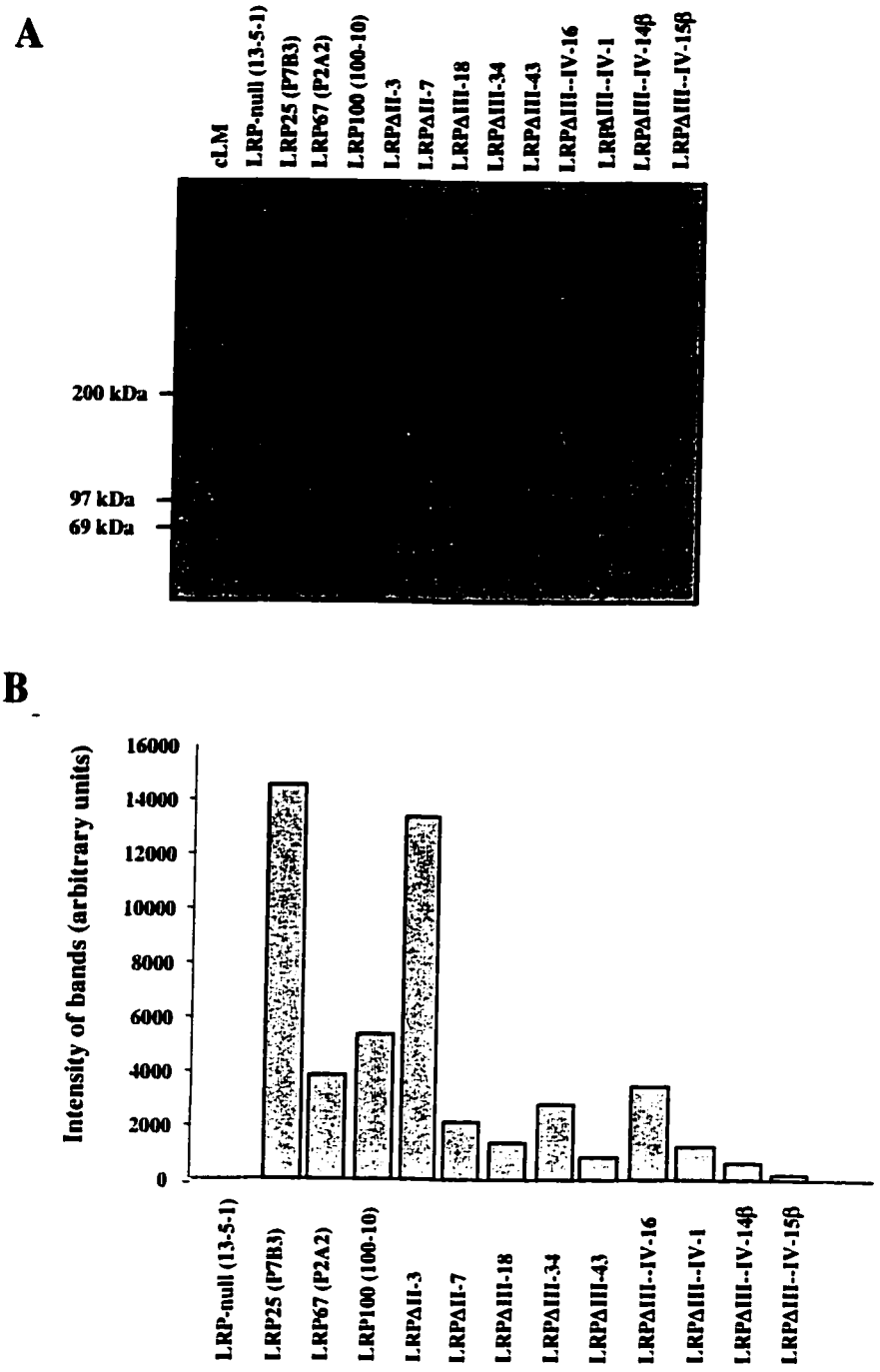

Figure 37. Comparison of expression level for cell lines expressing different LRP variants.

Stable cell lines were grown to confluency in 100-mm dishes. Cell extracts were prepared and an equal amount of total protein (200 μg) was loaded in each lane. The samples were resolved by SDS-PAGE under reducing conditions and LRP proteins were detected by immunoblotting using the anti-β-chain antibody.

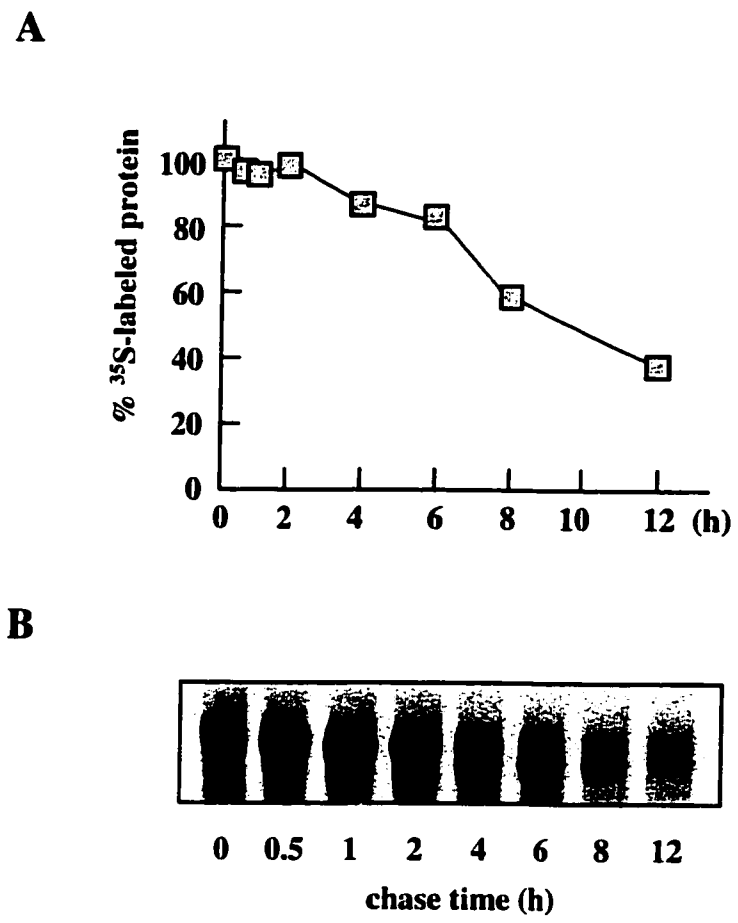

Figure 38. LRPΔII disappears more rapidly than full-length LRP100.

Confluent cell monolayers (30-mm dishes) were metabolically labeled with [^{35}S]methionine/cysteine (200 μCi/ml) for 1 hour and chased for the indicated times. Cells were solubilized and the LRP proteins were immunoprecipitated using the anti-β-chain antibody. Samples were resolved by SDS-PAGE under reducing conditions and LRP proteins detected by fluorography.

Intracellular trafficking itinerary of LRPΔII – To address the question of intracellular trafficking and plasma membrane presentation of LRPΔII, pulse-chase experiments were performed in conjunction with endoglycosidase digestions in order to follow intracellular movement of the receptor over time (figure 39). While LRP100 acquired endo H resistance following 2 hours, the majority of LRPΔII-3 remained sensitive to endoH at 2 hours and only became resistant after the 4 hour time point. PNGase F treatment resulted in detection of a doublet at 2 hours. This doublet corresponds to a mixture of full-length proreceptor as well as a small amount of α-chain. The doublet remained visible up to 6 hours, although by 4 hours the majority of LRPΔII α-chain had acquired endo H resistance. Thus, LRPΔII-3 appeared to be slightly delayed in its ER exit. Together with data suggesting a more rapid disappearance from the cells, the prolonged ER retention suggests a folding problem may occur with this mutant.

Transient transfection of complement-type/class A cluster mutant LRPΔIII – Transient transfections of LRPΔIII were also performed (figure 40). The recombinant protein was expressed and exhibited the correct molecular weight. Both the β-chain, as well as a large quantity of unprocessed proreceptor can be seen when the membrane is probed with an anti-β-chain antibody.

LRPΔIII is not transported to the plasma membrane – Stable transformants were generated, and two cell lines, LRPΔIII-18 and LRPΔIII-34 were used to perform plasma

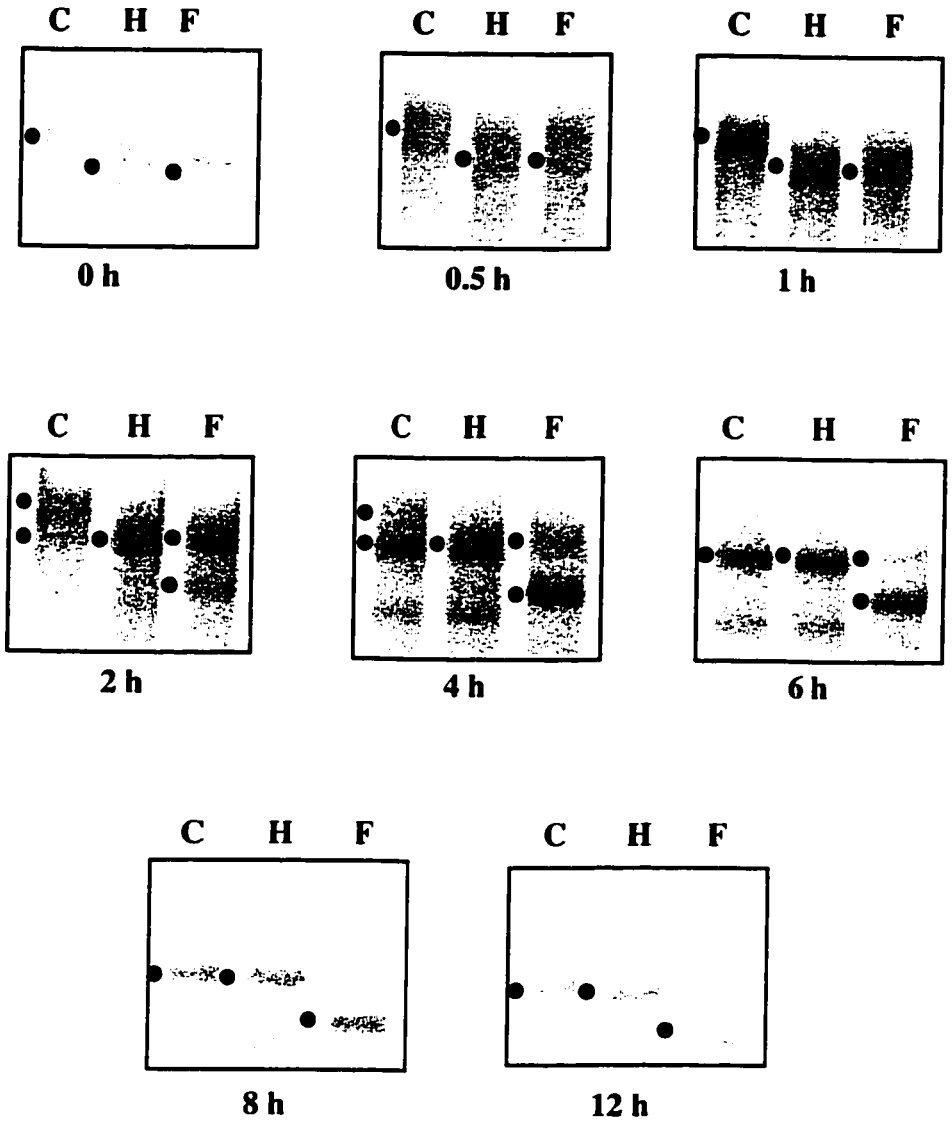

Figure 39. LRPΔII exhibits delayed ER exit.

Cells were plated in 30-mm dishes and allowed to grow to a confluent monolayer. The cells were metabolically labeled with [^{35}S]methionine/cysteine (100 μCi/ml) for 1 hour and harvested at the indicated times. Cells were solubilized and the LRP proteins were immunoprecipitated using the anti-β-chain antibody. Immunoprecipitated LRP proteins were treated with endoglycosidase H (*H*), PNGase F (*F*) or buffer alone. Samples were resolved by SDS-PAGE under reducing conditions and LRP proteins detected by fluorography.

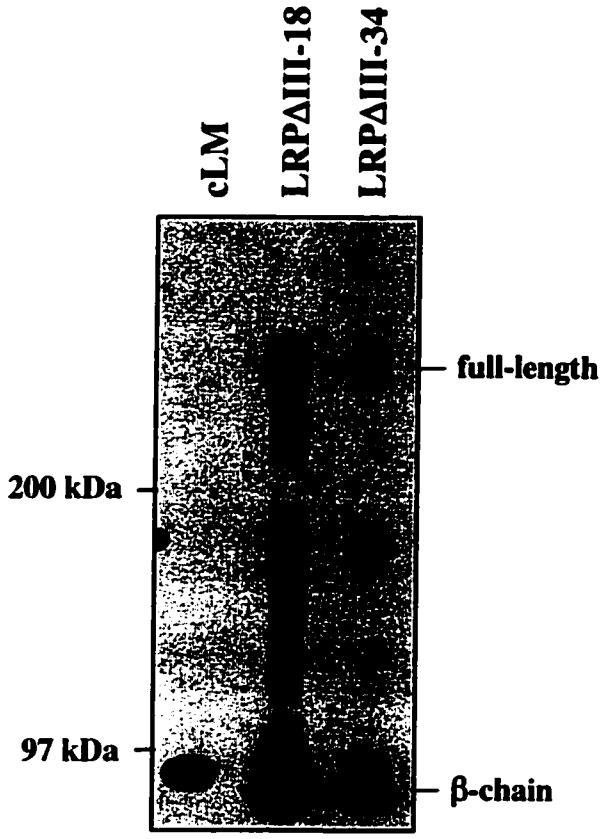

Figure 40. LRPΔIII is inefficiently processed in CHO-derived, LRP-null cells.

Confluent monolayers (60-mm dishes) of LRP-null cells stably transfected with pcLRPΔIII were harvested. Cell extracts were prepared, samples were resolved by SDS-PAGE under reducing conditions, and the expressed LRP proteins were detected by immunoblotting using the anti-β chain antibody.

membrane biotinylation experiments. LRP protein was undetectable by plasma membrane biotinylation for either cell line (figure 41A). Once again, the proreceptor was detectable by probing the same membranes with anti-β-chain antibody (figure 41B). Pulse-chase analysis combined with endoH digestions suggest that LRPΔIII-18 remains sensitive to the enzyme up to 6 hours indicating impaired ER exit (figure 42). Like LRPΔII, this mutant also possesses crippled β-propellers. It is possible that disrupting these highly ordered domains causes folding problems resulting in impaired ER exit and premature cellular degradation.

Transient transfections of complement-type/class A module cluster mutant LRPΔIII-IV – Transient transfections were performed with LRPΔIII-IV, a mutant that encodes a deletion of complement-type/class A clusters three and four. Although the β-propellers in this mutant are kept whole, they are present in an unpaired or asymmetrical arrangement (figure 34). The mutant encodes a deletion of the furin cleavage site and the receptor is expressed as a single polypeptide chain that does not undergo cleavage into α-chain and β-chain. The recombinant protein was expressed and was resolved to the correct molecular weight on SDS-PAGE (figure 43). Stable transformants were generated, several clones were isolated, and a single clone, LRPΔIII-IV-16 was found to possess a level of expression similar to that of LRP100 (figure 37).

LRPΔIII-IV remains endo H sensitive up to 12 hours – The intracellular itinerary of LRPΔIII-IV-16 was verified by performing metabolic labeling in conjunction with endo

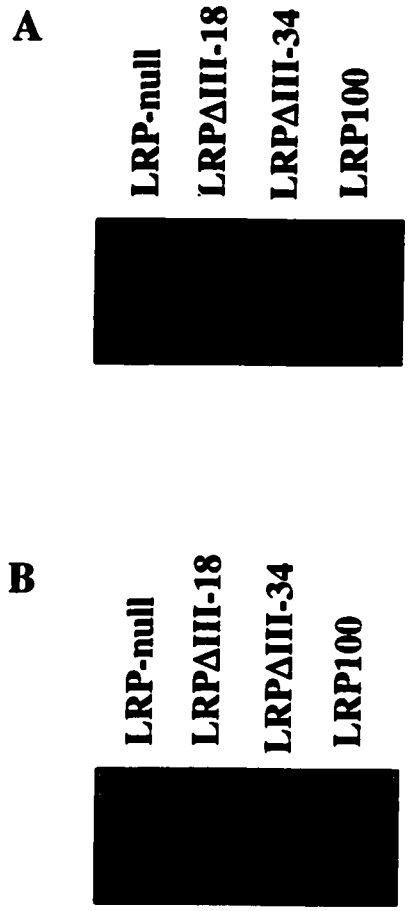

Figure 41. LRPΔIII is not biotinylated at the plasma membrane.

Confluent cell monolayers (60-mm dishes) were treated with biotin for 1 hour at 4 °C. Cell extracts were prepared and the LRP proteins were immunoprecipitated using the anti-β chain antibody. Samples were resolved by SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane. Biotin-labeled proteins were detected using streptavidin conjugated HRP and visualized by enhanced chemiluminescence detection.

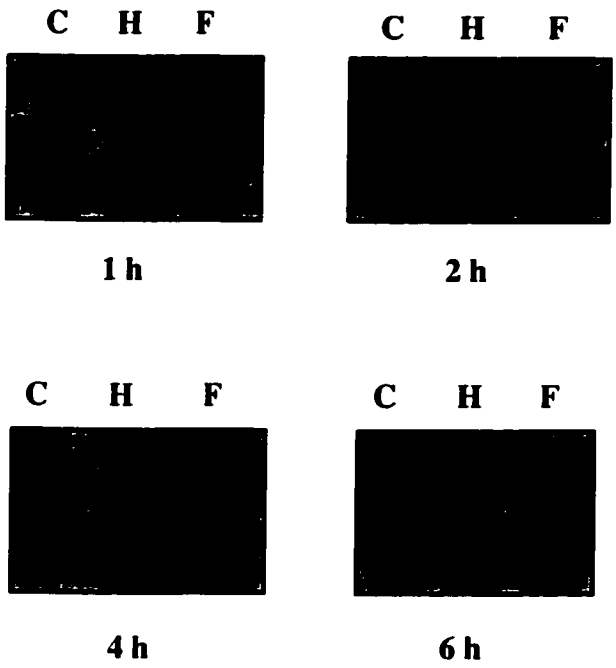

Figure 42. LRPΔIII exhibits impaired ER exit.

Cells were plated in 30-mm dishes and allowed to grow to a confluent monolayer. The cells were metabolically labeled with [^{35}S]methionine/cysteine (100 μCi/ml) for 1 hour and harvested at the indicated times. Cells were solubilized and the LRP proteins were immunoprecipitated using the anti-β-chain antibody. Immunoprecipitated LRP proteins were treated with endoglycosidase H (*H*), PNGase F (*F*) or buffer alone (*C*). Samples were resolved by SDS-PAGE under reducing conditions and LRP proteins detected by fluorography.

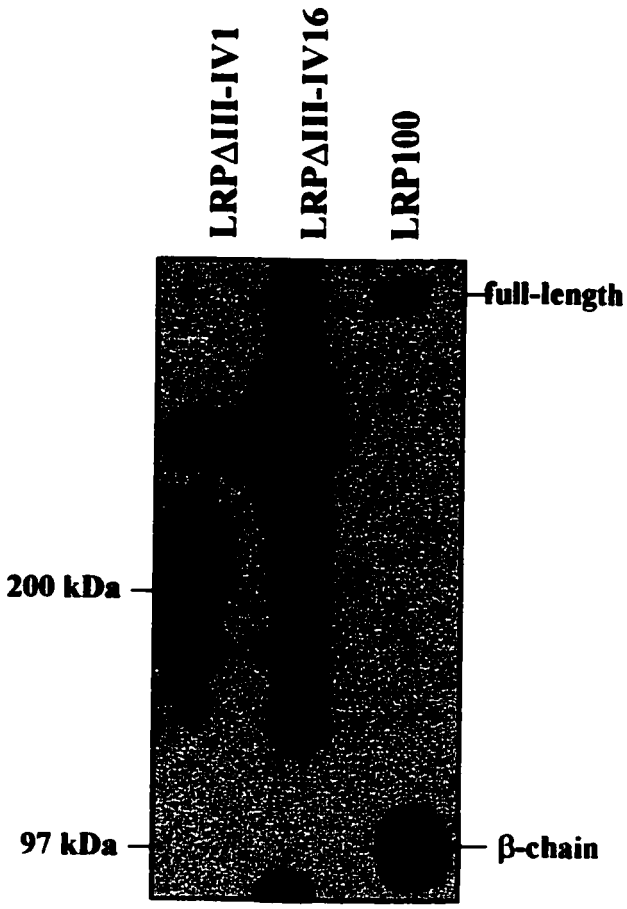

Figure 43. LRPΔIII-IV is highly expressed.

LRPΔIII-IV stably expressed in 13-5-1 cells was screened by immunoblot. Cell extracts were prepared and samples were resolved by SDS-PAGE under reducing conditions. The expressed LRP proteins were detected by immunoblotting using the anti-β chain antibody.

H digestions. This mutant remained endo H sensitive for up to 12 hours (figure 44). In addition, by this time most of the protein appears to have been degraded, suggesting that the majority of the receptor was unable to exit the ER before being degraded by the cell. This result was somewhat unexpected as both LRP25 and LRP67, mutants encoding similar large internal deletions were presented at the plasma membrane and were capable of fully or partially restoring LRP function.

LRP67 and LRP25 are presented at the plasma membrane and exhibit intracellular trafficking similar to LRP100 – The intracellular trafficking and plasma membrane presentation of LRP67 and LRP25 was compared to that of the LRPΔII, LRPΔIII, and LRPΔIII-IV mutants. These LRP variants are functional proteins that were shown to bind α_2M*, RAP and PEA. Neither LRP 67 nor LRP25 contains an intracellular processing site. As a result, the bands representing endo H treated samples migrate with the same electrophoretic mobility as the control. Figure 45 shows endo H digestion of metabolically labeled samples performed over the indicated time course. At 0 and 1 hour all three variants are both endo H and PNGase F sensitive, seen as a shift in electrophoretic mobility compared to control lanes. At 2 hours, all three variants become endo H resistant as had previously been seen for the functional full-length protein.

Addition of a single β-propeller restores intracellular trafficking and plasma membrane presentation to a transport incompetent deletion mutant – Examination of the structures of the deletion mutants tested suggests one possible explanation for the impaired plasma

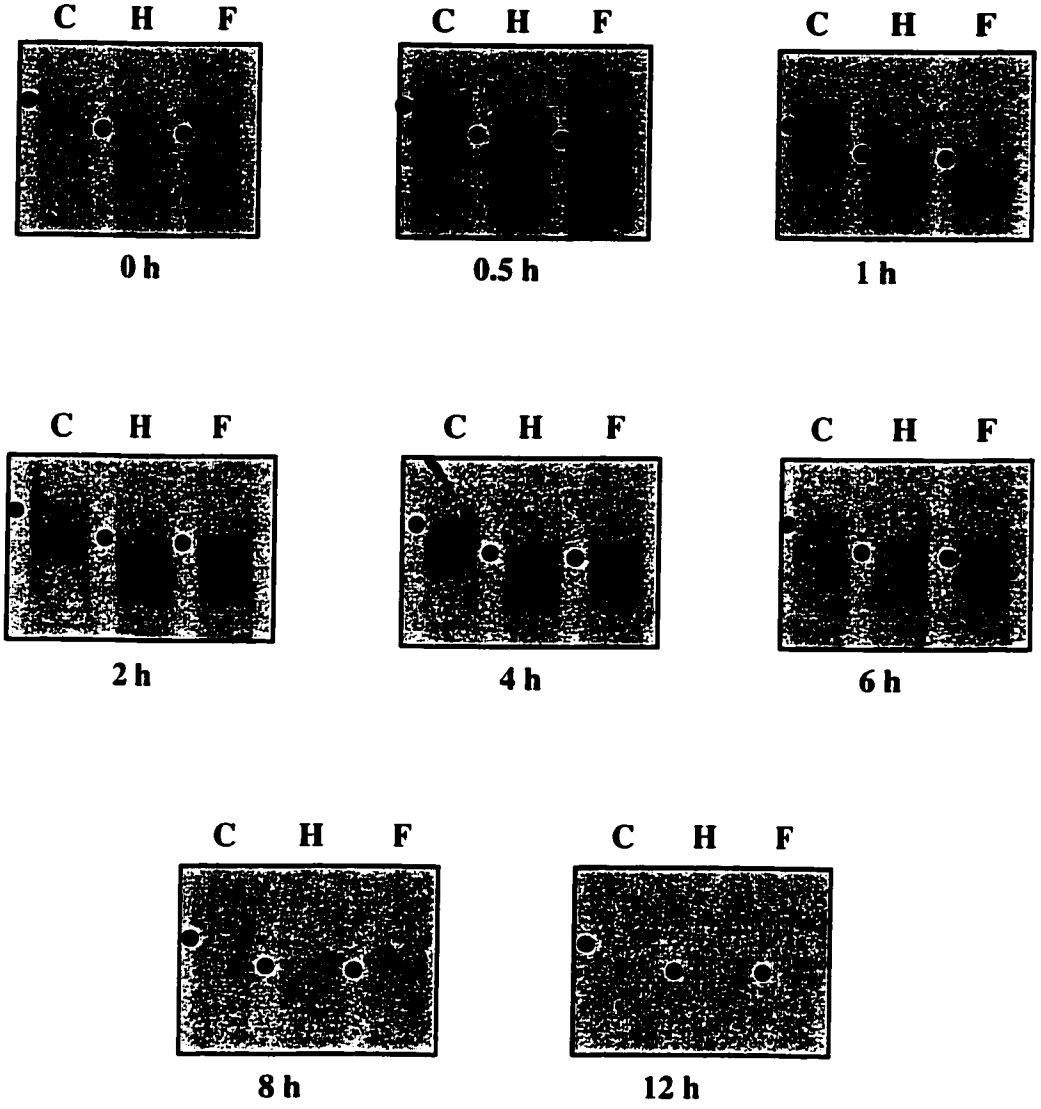

Figure 44. LRPΔIII-IV exhibits impaired ER exit.

Cells were plated in 30-mm dishes and allowed to grow to a confluent monolayer. The cells were metabolically labeled with [^{35}S]methionine/cysteine (100 μCi/ml) for 1 hour and harvested at the indicated times. Cells were solubilized and the LRP proteins were immunoprecipitated using the anti-β-chain antibody. Immunoprecipitated LRP proteins were treated with endoglycosidase H (*H*), PNGase F (*F*) or buffer alone (*C*). Samples were resolved by SDS-PAGE under reducing conditions and LRP proteins detected by fluorography.

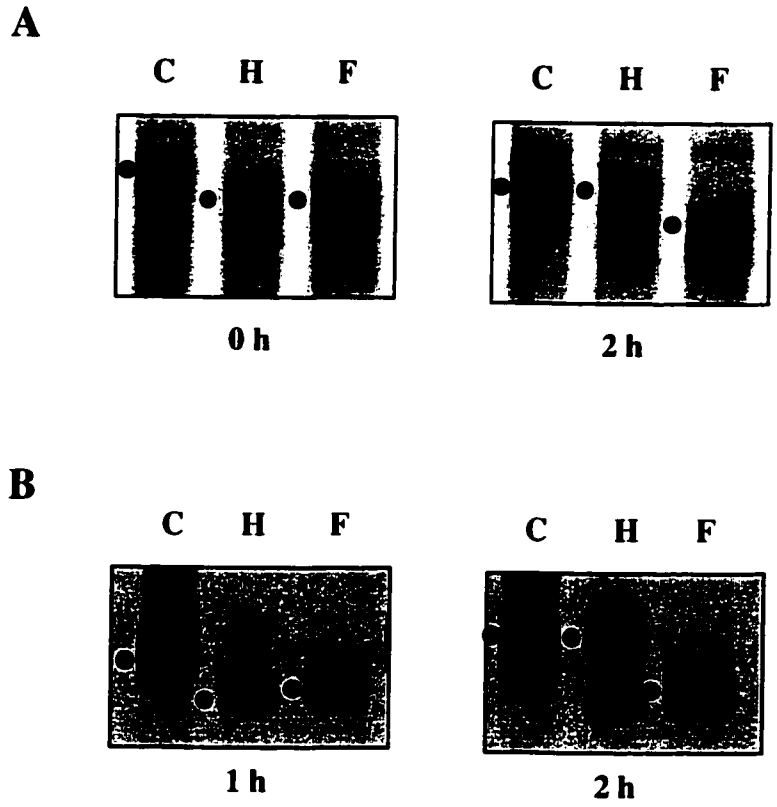

Figure 45. Intracellular trafficking and plasma membrane presentation of stably transfected LRP variants LRP25, and LRP67.

Cells were plated in 30-mm dishes and allowed to grow to a confluent monolayer. The cells were metabolically labeled with [^{35}S]methionine/cysteine (100 μCi/ml) for 1 hour and harvested at the indicated times. Cells were solubilized and the LRP proteins were immunoprecipitated using the anti-β-chain antibody. Immunoprecipitated LRP proteins were treated with endoglycosidase H (*H*), PNGase F (*F*) or buffer alone (*C*). Samples were resolved by SDS-PAGE under reducing conditions and LRP proteins detected by fluorography. *A*, LRP67; *B*, LRP25.

membrane presentation is a disruption of a symmetrical arrangement of β-propeller domains. Figure 11 shows that deletion mutants LRP25 and LRP67 possess a symmetrical arrangement of unbroken β-propeller domains each flanked by at least a single EGF module. The ligand-binding cluster deletion mutants LRPΔII and LRPΔIII encode a broken β-propeller domain caused by the use of convenient restriction sites during cloning (figure 33). The mutant LRPΔIII-IV does not encode broken β-propeller, but encodes an asymmetrical arrangement of these domains. To address this possibility, a construct was generated in which a symmetrical β-propeller arrangement was restored by reintroducing the sixth β-propeller domain flanked by two EGF modules (figure 33). This new mutant, designated LRPΔIII-IVβ, was stably expressed in LRP deficient cells. LRPΔIII-IVβ-14 attained endo H resistance within 2 hours much like the wild type LRP100 (figure 46).

LRPΔIII-IVβ is present and biotinylated at the plasma membrane – Plasma membrane biotinylation experiments performed at 4 °C show that the clone LRPΔIII-IVβ-14 is detected at the plasma membrane (figure 47) despite having a 5-fold lower expression level than LRPΔIII-IV-16, a clone that could not be detected at the plasma membrane (figure 37). These data suggest that restoration of the symmetrical arrangement of β-propeller modules somehow aid in the plasma membrane presentation of LRP and that these domains may somehow be important in maintaining structural integrity necessary for unimpaired intracellular transport and plasma membrane presentation.

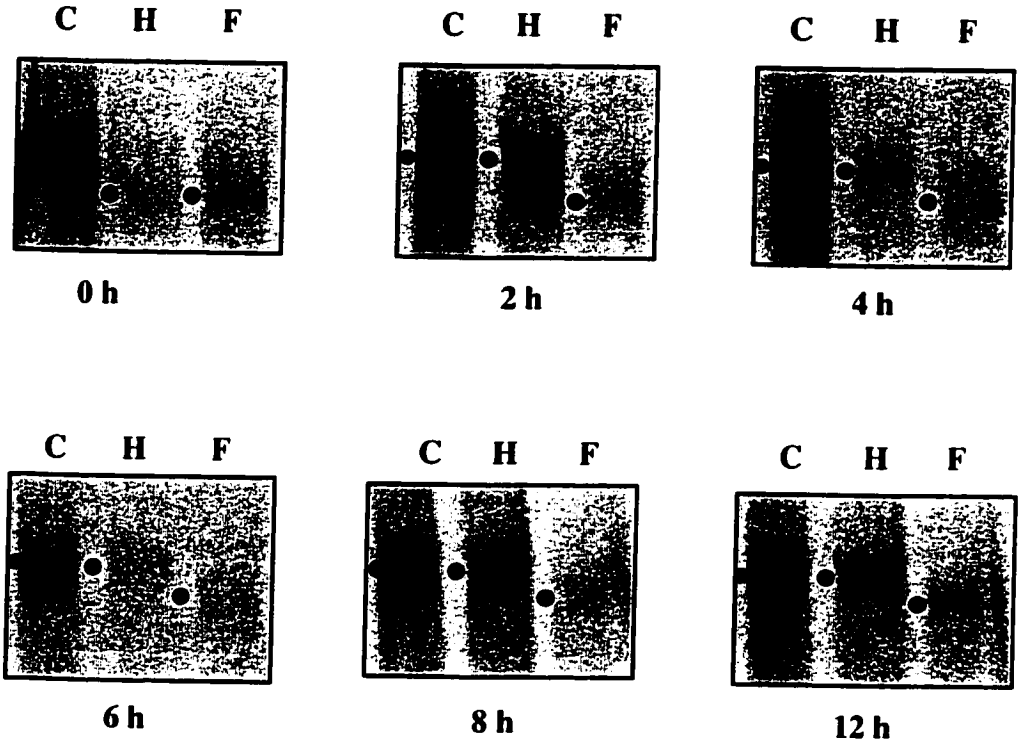

Figure 46. Insertion of a single β-propeller domain with flanking EGF modules restores plasma membrane presentation to transport impaired LRPΔIII-IV mutant.

Cells were plated in 30-mm dishes and allowed to grow to a confluent monolayer. The cells were metabolically labeled with [^{35}S]methionine/cysteine (100 μCi/ml) for 1 hour and harvested at the indicated times. Cells were solubilized and the LRP proteins were immunoprecipitated using the anti-β-chain antibody. Immunoprecipitated LRP proteins were treated with endoglycosidase H (*H*), PNGase F (*F*) or buffer alone (*C*). Samples were resolved by SDS-PAGE under reducing conditions and LRP proteins detected by fluorography.

Figure 47. LRPΔIII-IV is biotinylated at the plasma membrane and presented at the plasma membrane.

Confluent cell monolayers (60-mm dishes) were treated with biotin for 1 hour at 4 °C. Cell extracts were prepared and the LRP proteins were immunoprecipitated using the anti-β chain antibody. Samples were resolved by SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane. Biotin-labeled proteins were detected using streptavidin conjugated HRP and visualized by enhanced chemiluminescence detection.

5. Discussion

5.1 LRP expression and functional studies

Although LRP is composed largely of modules of known individual tertiary structure, our understanding of the molecular interactions that occur between the receptor and its ligands remains inadequate. The unusually large size as well as the multifunctional nature of LRP has made functional analysis a somewhat challenging task.

The primary objective of this study was to generate an expression system, and to use this expression system in order to delineate ligand binding sites within LRP. This work reports on the generation of stable transformants expressing LRP100, the full-length chicken LRP isoform, as well as LRP67 and LRP25, two mutants encoding internal deletions of the receptor. Functional analyses of these recombinant variants has led to the identification of at least part of the sequence elements required for the binding of three LRP ligands namely, RAP, α_2M, and PEA. Furthermore, the identification of trafficking impaired LRP mutants, LRPΔII, LRPΔIII, and LRPΔIII-IV, has suggested a novel role for the β-propeller domains within the spacer regions in promoting correct LRP folding.

5.1.1 Binding of α_2M*

To date, much of the ligand binding activity of LRP has been ascribed to complement-type/class A cluster II (summarized in figure 8). Ligands including RAP and tPA:PAI1 are known to bind within this region. Studies performed using proteolytic fragments or LRP minireceptors have also assigned α_2M binding specifically to cluster II,

although additional evidence suggests that ligand binding activity may not be conferred solely by this region. A study using LRP proteolytic fragments has suggested the involvement of several flanking epidermal growth factor type modules (56) in α_2M* binding. In support of this, it was found that a membrane anchored LRP minireceptor encoding cluster II, but not the flanking epidermal growth factor type modules, failed to bind α_2M* (91).

By restoring ^{125}I-α_2M* binding activity to an LRP-deficient cell line upon receptor overexpression, our current studies have irrevocably confirmed that LRP is indeed the receptor for α_2M (figure 26A). As suggested by functional studies using cell lines expressing LRP25, the structural determinants required for binding of α_2M:proteinase complexes may be, at least in part, encoded within the N-terminal 22% of the receptor. Since LRP25 encodes only the first three of a total of eight complement type/class A modules in cluster II, our data further suggests that these three modules are sufficient to partially restore α_2M* binding activity. This same region is also known to bind RAP (161). Inhibition of α_2M* binding by RAP in cells expressing LRP25 (figure 26B) suggests at least a portion of the binding site on LRP for these two ligand may be shared.

5.1.2 Binding of RAP

It has been postulated that the ability of RAP to inhibit the binding of all known LRP ligands may be due to allosteric effects caused by several RAP molecules binding a single LRP molecule (162). This is supported by experimental data that shows RAP is

able to bind multiple sites within LRP. The existence of independent RAP binding sites on cluster II and IV of LRP has been shown by several groups in studies using anchored LRP minireceptors (91), soluble LRP fragments (143), or proteinase and CNBr digests of LRP (56). Experiments using soluble LRP fragments encoding each of the four complement type/class A modules also showed strong RAP binding to clusters II and IV and somewhat less avid binding to cluster III (161). This latter study suggested the existence of a total of five independent RAP-binding sites within these regions, two each within clusters II and IV, and one within cluster IV.

The data obtained in this study using cell lines that express LRP25 suggest that, at least a portion of one RAP binding site may reside within the first three complement-type/class A modules of cluster II.

5.1.3 Binding of PEA

There is convincing evidence indicating that low concentrations of PEA enter cells specifically via LRP-mediated endocytosis. Studies have shown that the binding of PEA can be inhibited by RAP (163). In addition, the few existing mutant cell lines lacking endogenous LRP expression appear to be resistant to the toxin (88,140). Restoration of PEA toxicity to cells expressing recombinant LRP100 confirms its role in mediating PEA entry into cells (figure 25). In addition, this study was the first to attempt defining the sequence elements required for entry of PEA into cells. Internal deletions encoded for by LRP67 and LRP25 could not entirely abolish the effect of the toxin. It must be noted, however, that even in cells that express an extremely elevated level of

LRP25 the effect of PEA was only partially restored to approximately 10% of the level seen in the parental cell line, CHO-K1 (cell line P6B4, table 2). It is possible that the affinity of PEA for LRP is a function of receptor length as the toxicity appears to correlate directly with the length of the recombinant receptor expressed. This expression system may prove useful in the future for localizing PEA binding sites as well as for understanding the exact mechanism of action of the toxin. Since PEA in believed to exert toxicity by undergoing retrograde transport from the plasma membrane to the cytosol, further modification of our LRP constructs may prove useful in understanding how this occurs.

5.1.4 LRP expression level

Despite the ability of the two minireceptors LRP25 and LRP67 to partially restore LRP function for those ligands tested, this only occurred at the highest level of expression. As the binding studies were complicated by the variation in level of expression and in level of plasma membrane presentation of LRP proteins it became necessary to correct the PEA toxicity by the number of α2M* binding sites as determined by ^{125}I-α_2M* binding at 4 °C. For example, only those cell lines that express the smallest receptor, LRP25, at extremely high level are capable of partially (approximately 10%) restoring receptor function (cell line P6B4, table 2). It is possible that for the two mutants LRP25 and LRP67, trafficking is not as efficient as for LRP100 resulting in a high level of expression being required in order for enough of each minireceptor to be presented at the plasma membrane. For both LRP25 and LRP67, endo H studies show two isoforms of

each variant exist in the stable cell lines tested (figure 45). It is also possible that each of the ligands tested binds less strongly to the two minireceptors and, therefore, a greater amount of each minireceptor is required to be present at the plasma membrane in order to restore function.

5.1.5 Recombinant LRP expression in Cos-7 cells

Despite a high level of transient expression in Cos-7 cells, this cell line appeared incapable of efficiently presenting the three variants LRP25, LRP67, and LRP100 at the plasma membrane. In addition, the full-length receptor was inefficiently processed into α- and β-chains indicating it was not efficiently transported to the late Golgi compartment when expressed in this cell line. In addition to a predominant 600 kDa band visible by immunoblotting, biochemical analyses of the carbohydrate moieties suggested that recombinant LRP was retained within the ER. Previous studies have shown that the human LDL receptor was expressed as a functional protein in Cos cells (53,164). It is also somewhat puzzling that within Cos-7 cells the endogenous LRP appeared to be efficiently processed and presented at the plasma membrane. The latter observation makes it unlikely that the cellular trafficking machinery is saturated by the high expression level of recombinant protein. Co-expression of RAP or furin failed to restore plasma membrane presentation of the mature receptor suggesting that neither of these proteins is limiting (figures 16 and 17). It is possible, however, that the Cos-7 cells are incapable of efficiently recognizing and processing the chicken LRP isoform. Currently,

the reason for the inability of this cell line to efficiently process LRP remains unexplained.

5.1.6 Recombinant LRP expression in CHO-derived cells

When LRP was expressed in CHO-K1 or CHO-derived 13-5-1 cells, it was found to be more efficiently processed into α and β-chains. Although LRP is the only member of the LDL receptor gene family to undergo post-translational proteolytic processing, there are several examples of plasma membrane proteins that undergo such an event. One notable example is the insulin receptor. It has been shown that defective processing of the insulin receptor caused by point mutations results in impaired insulin binding in transfected CHO cells as well as in certain insulin-resistant diabetes mellitus patients (89,90). The function of proteolytic processing in LRP remains unclear. Functional expression of the minireceptors LRP67 and LRP25, that lack the processing site, suggest proteolytic processing may not be critical for receptor function, however. This is supported by studies expressing an LRP minireceptor lacking the processing site (91), or full-length LRP encoding a point mutation at the processing site that prevented proteolytic processing, but did not appear to inhibit plasma membrane presentation or ligand-binding function (92).

5.1.7 Expression of endogenous LRP in adipocytes

The high level of endogenous LRP seen in adipocytes also remains unexplained. LRP is thought to function primarily in the removal of proteases from the circulation though it is unlikely that adipose is the target tissue for protease clearance, since this function has been ascribed mainly to the liver (165). There is also evidence that a small portion of the lipoprotein subclass HDL may bind to LRP via an unknown mechanism perhaps playing a role in the maintenance of cellular lipid homeostasis (166). Adipose tissue, however, is unlikely to be a major site of lipoprotein clearance.

Since the synthesis of cholesterol is limited in human adipocytes, this tissue must obtain this essential lipid via exogenous lipoproteins (167). It is possible that LRP may contribute to the accumulation of cholesterol within adipocytes. Since chylomicrons are thought to be too large to cross the endothelial barrier, the exact mechanism by which this might occur remains unclear at the present time.

Adipocytes secrete factors required for the production of the triacylglycerol synthesis stimulating peptide acylation stimulating protein (168). Acylation stimulating protein is derived from the fragment of complement system protein, C3 (169). C3 was recently shown to bind LRP in ligand blots and it is possible that LRP may locally concentrate factors required for the generation of acylation stimulating protein (20). If generated locally, acylation stimulating protein could, in turn, serve to enhance storage of triacylglycerol in adipocytes. Further studies will be required in order to begin to address these questions.

5.2 LRP intracellular trafficking studies

Several additional deletion mutants were generated in an attempt to further delineate ligand binding regions within LRP. The three minireceptors LRPΔII, LRPΔIII, LRPΔIII-IV were constructed in order to ascribe ligand binding activity to specific complement-type/class A clusters of LRP. All of the mutant receptors were constructed by removing cDNAs encoding segments within the LRP extracellular domain at convenient restriction sites. Following the successful expression of LRP100, LRP67, and LRP25 it was somewhat surprising to observe that several of these new minireceptors exhibited impaired intracellular trafficking and plasma membrane presentation. A close examination of the primary amino acid sequence revealed an interesting distinction between the two groups of minireceptors. Minireceptors LRP25, LRP67, and LRP100 were efficiently expressed and contained intact and paired β-propeller domains (figure 11). In contrast those minireceptors that exhibited impaired intracellular trafficking exhibited incomplete β-propeller domains, as with LRPΔII and LRPΔIII, or unpaired β-propeller domains, as with LRPΔIII-IV (figure 34). This suggested that those minireceptors with paired and unbroken β-propeller arrangements were efficiently processed and transported to the plasma membrane, while disruption of the β-propellers themselves or of their paired arrangement within the protein resulted in retention within the ER compartment.

5.2.1 β-propeller arrangement may be important for efficient LRP trafficking

The requirement for a symmetrical arrangement of β-propellers was most dramatically demonstrated with the minireceptor LRPΔIII-IVβ that differs from LRPΔIII-IV only in the insertion of a single β-propeller domain and EGF module (figure 34). The insertion of these elements not only restored plasma membrane presentation (figure 47), but also restored a normal rate of intracellular trafficking comparable to that of LRP100 (figure 46). It is interesting to note that a functional LRP minireceptor expressed by Willnow et al. (91) and designed to study ligand interactions with LRP cluster II was designed to encoded this region together with four of the adjacent β-propellers. To date, the function of the spacer regions found in all the members of the LDL receptor gene family has remained unknown. These results suggest a possible novel function for the YWTD-containing β-propeller domains in mediating efficient folding of LRP within the ER.

5.2.2 Importance of β-propeller domains in the LDL receptor gene family

The β-propeller domain is a common structural module of proteins. It is surpassed in frequency only by the immunoglobulin, the EGF-like, the fibronectin-type III, the complement control, the C-type lectin domains, and the leucine-rich modules (reviewed in 170). Solved β-propeller domain structures are now known for several functionally diverse proteins including the βγ subunit of G proteins (171), the clathrin heavy chain (172), neuraminidase (173), methanol dehydrogenase (174), and cytochrome cd1 (175).

Although the function and cellular localization of these individual proteins differ, it is thought that the β-propeller domains within all these proteins are involved in mediating protein-protein interactions. For example, the β-propeller domain of the βγ subunit of G proteins interacts with the Gα subunit and downstream signaling components (171). The N-terminal domain of clathrin possesses a groove on its β-propeller, which is thought to bind intracellular scaffolding proteins such as β-arrestin 2 or the AP3 β subunit (176).

Although its exact function has remained elusive, the importance of intact YWTD-containing β-propeller domains in the LDL receptor has been known for some time. It has been observed that even single point mutations within the one β-propeller domain of the LDL receptor often lead to the genetic disorder FH. Phenotypically, this disease is characterized by elevated LDL cholesterol in the circulation mostly caused by individual point mutations within the LDL receptor. These mutations are divided into five classes and are associated with defects in biosynthesis, plasma membrane presentation, ligand binding, receptor:ligand endocytosis or dissociation of ligand resulting in premature receptor degradation (reviewed in 177). Of particular interest are point mutations causing defects in ER-to-Golgi transport (class 2A and 2B mutations) and in receptor recycling (class 5). Within the single YWTD-containing β-propeller domain of the LDL receptor, 39 missense mutations have been found in individuals with FH (class 2A mutations). Three naturally occurring point mutations within the β-propeller domain of the LDL receptor (G525D, G528D, and G544V) are known to cause impaired trafficking from the ER to the Golgi compartments (177,178). It is interesting to note that all three mutations involve substitution of glycine, a residue that is often involved in tight turns and whose disruption may lead to a loss of function. Another naturally occurring

point mutation (W556S) is associated with prolonged ER retention, increased binding of the molecular chaperone BiP, and enhanced degradation of the mutant LDL receptor (86). Again, this is caused by a non-conservative substitution of a bulky tryptophan residue for a serine. It was also reported previously that a single point mutation within a β-propeller domain of LRP caused impaired ER exit. The furin cleavage site within the eighth β-propeller domain of LRP was mutated to encode the sequence RNRS in place of the RNRR sequence found in the wild type chicken LRP. The rate of ER exit was found to be markedly decreased in the mutant (92). Taken together, these results strongly suggest that the β-propeller motifs within LRP may play an important role in the ER quality control system which allows the correct and efficient folding of the protein.

5.2.3 The importance of other domains in folding

Although the present study suggests disruption of the orderly arrangement of β-propeller domains within LRP is associated with prolonged ER retention, it must be noted that structural elements important for efficient intracellular trafficking are not solely confined to these domains. Deletion mutations within the first complement-type/class A module are also associated with impaired ER-to-Golgi transport (class 2B mutation) (177). In addition, the Watanabe heritable hyperlipidemic rabbit model possesses an in-frame deletion that occurs within the sixth complement-type/class A module and is also associated with impaired ER-to-Golgi trafficking of the LDL receptor (179). It would also appear, therefore, that disruption of the complement type/class A modules is detrimental to efficient processing of the receptor.

5.2.4 Post-translational events regulating LRP plasma membrane presentation

Although it is possible that mutations lead directly to misfolding of these regions, the possibility that mutations indirectly cause ER retention by impairing binding with intracellular factors such as ER chaperones cannot be excluded.

It has been shown that RAP is essential for maturation of LRP and that this ER chaperone molecule binds avidly to clusters II, and IV and somewhat less avidly to cluster III (161). It is, therefore, conceivable that in the case of LRPΔII and LRPΔIII, removal of a portion of the molecule involved in RAP interaction might lead to the observed delay in ER exit.

It is also possible that the removal of the majority of LRP glycosylation sites, the majority of which are distributed between cluster II and cluster III, may result in ER retention of several variants. Although N-linked glycosylation is generally believed to be involved in ER quality control, this hypothesis remains to be tested experimentally in the case of LRP.

The mechanism of LRP degradation is currently unknown. It is possible that the apparent increase in degradation seen with LRPΔII is caused by defective receptor recycling some of which is caused by a point mutation within the β-propeller domain in the LDL receptor (class 5 mutation). This causes slightly delayed ER exit, impaired release of LDL and premature degradation of the ligand-receptor complex. A V408M substitution within the fifth complement-type/class A module of the LDL receptor results in slightly delayed plasma membrane presentation and increased degradation very similar to that seen with the LRPΔII variants (180).

Unlike most other proteins that contain a single β-propeller domain, LRP possesses eight, the first six of which are arranged in pairs. These data suggest that both a symmetrical arrangement as well as the correct folding of individual β-propellers may be important for efficient ER-to-Golgi transport. In the case of LRP, the multiple β-propeller domains may be involved in conferring rigidity or structural integrity to this enormous protein. The YWTD-containing β-propeller domains of LRP are large and highly ordered and it is possible that even a single point mutation could be detrimental. Further mutagenesis studies will be required to determine the precise role these domains play in maintaining LRP structure.

6. Conclusion

Much remains to be understood in the molecular mechanisms of receptor regulation of the factors affecting disease processes. At the present time, the role of LRP in the pathogenesis of both cardiovascular disease and Alzheimer's disease is far from being clearly understood. A more thorough understanding of the structure of this enormous receptor protein and its mechanisms of interaction with different ligands will almost certainly be useful in understanding its role in disease as well as that of the entire family of LDL receptor proteins.

Several points reported in this work are noteworthy. This is the first report showing stable and functional expression of a full-length LRP isoform in a mammalian expression system. The availability of an LRP deficient cell line, has made it possible to study both structure-function aspects as well as the intracellular trafficking itinerary of LRP. Using this model, partial ligand binding function was ascribed to the N-terminus of LRP for several ligands including RAP, α_2M, and PEA. The latter two had previously been shown to bind LRP, but the site of their interaction with the receptor was not known. Since the first cluster of class A repeats does not bind ligands, this suggests that the first three modules of the second cluster provide a minimum requirement for the ligands tested in this study.

A putative function has also been ascribed to the spacer regions of LRP containing YWTD or YWTD-like motifs. This previously ignored domain found in all members of the LDL receptor family may somehow be involved in maintaining the structural integrity of these receptors and appears to be required for efficient intracellular

transport and plasma membrane presentation. Although the picture will undoubtedly be more complex than the initial experiments presented in this work suggest, it is conceivable that the spacer regions found within LRP and other members of the LDL receptor gene family might play an important structural role.

Much work remains to be done in order to understand the ligand binding specificity of LRP. The second cluster of class A modules is clearly implicated in the binding of several LRP ligands (56). Comparison of the amino acid sequences of these modules within both LRP and the LDL receptor suggest the repeating acidic motifs found therein may be good candidates for mutagenesis studies. Recent structural work on the LDL receptor has shown the spacer region does indeed fold into a β-propeller domain and that this domain is stabilized by a flanking EGF module (181). In light of the data presented in this study, it would be worthwhile to examine the relationship between the EGF modules and β-propeller domains within LRP and their effect on correct folding of the receptor.

Statement of contribution of collaborators

For all of the experimental work in this thesis I have taken charge of experimental design, conducted the experiments, and interpreted the data.

The original full-length LRP cDNA (LRP100) and deletion mutants LRP67 and LRP25 were constructed prior the beginning of my thesis work by Dr. Zemin Yao.

The LRPΔIII-IVβ construct and stable cell line were generated by Robert Brown.

I also acknowledge the following collaborators in conducting the experiments shown.

Roger McLeod – figures 22, 27, 30

Kerry Ko – figure 20A

References

1. Brown, M. S. and Goldstein, J. L. 1986. A receptor-mediated pathway for cholesterol homeostasis. [Review] [133 refs]. *Science* 232:34-47.

2. Hjalm, G., Murray, E., Crumley, G., Harazim, W., Lundgren, S., Onyango, I., Ek, B., Larsson, M., Juhlin, C., Hellman, P. *et al.* 1996. Cloning and sequencing of human gp330, a Ca(2+)-binding receptor with potential intracellular signaling properties. *European Journal of Biochemistry* 239:132-137.

3. Takahashi, S., Kawarabayasi, Y., Nakai, T., Sakai, J., and Yamamoto, T. 1992. Rabbit very low density lipoprotein receptor: a low density lipoprotein receptor-like protein with distinct ligand specificity. *Proceedings of the National Academy of Sciences of the United States of America* 89:9252-9256.

4. Kim, D. H., Iijima, H., Goto, K., Sakai, J., Ishii, H., Kim, H. J., Suzuki, H., Kondo, H., Saeki, S., and Yamamoto, T. 1996. Human apolipoprotein E receptor 2. A novel lipoprotein receptor of the low density lipoprotein receptor family predominantly expressed in brain. *Journal of Biological Chemistry* 271:8373-8380.

5. Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H., and Stanley, K. K. 1988. Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO Journal* 7:4119-4127.

6. Kim, D.H., Inagaki, Y., Suzuki, T., Ioka, R.X., Yoshioka, S.Z., Magoori, K., Kang, M.J., Cho, Y., Nakano, A.Z., Liu, Q., Fujino, T., Suzuki, H., Sasano, H., Yamamoto, T.T. 1998. A new low density lipoprotein receptor related protein, LRP5, is expressed in hepatocytes and adrenal cortex, and recognizes apolipoprotein E. *Journal of Biochemistry* 124(6):1072-1076.

7. Brown, S.D., Twells, R.C., Hey, P.J., Cox, R.D., Levy, E.R., Soderman, A.R., Metzker, M.L., Caskey, C.T., Todd, J.A., Hess, J.F. 1998. Isolation and characterization of LRP6, a novel member of the low density lipoprotein receptor gene family. *Biochemical & Biophysical Research Communications* 248(3):879-888.

8. Liu, C.X., Li, Y., Obermoeller-McCormick, L.M., Schwartz, A.L., Bu, G. 2001. The putative tumor suppressor LRP1B, a novel member of the low density lipoprotein (LDL) receptor family, exhibits both overlapping and distinct properties with the LDL receptor-related protein. *Journal of Biological Chemistry* 276(31):28889-28896.

9. Hussain, M. M., Strickland, D. K., and Bakillah, A. 1999. The mammalian low-density lipoprotein receptor family. [Review] [206 refs]. *Annual Review of Nutrition* 19:141-172.

10. Gliemann, J. 1998. Receptors of the low density lipoprotein (LDL) receptor family in man. Multiple functions of the large family members via interaction with complex ligands. [Review] [128 refs]. *Biological Chemistry* 379:951-964.

11. Springer, T. A. 1998. An extracellular beta-propeller module predicted in lipoprotein and scavenger receptors, tyrosine kinases, epidermal growth factor precursor, and extracellular matrix components. *Journal of Molecular Biology* 283:837-862.

12. Goldstein, J. L. and Brown, M. S. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *Journal of Biological Chemistry* 249:5153-5162.

13. Mahley, R. W., Huang, Y., and Rall, S. C., Jr. 1999. Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia). Questions, quandaries, and paradoxes. [Review] [127 refs]. *Journal of Lipid Research* 40:1933-1949.

14. Nykjaer, A., Bengtsson-Olivecrona, G., Lookene, A., Moestrup, S. K., Petersen, C. M., Weber, W., Beisiegel, U., and Gliemann, J. 1993. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds lipoprotein lipase and beta-migrating very low density lipoprotein associated with the lipase. *Journal of Biological Chemistry* 268:15048-15055.

15. Kounnas, M. Z., Chappell, D. A., Wong, H., Argraves, W. S., and Strickland, D. K. 1995. The cellular internalization and degradation of hepatic lipase is mediated by low density lipoprotein receptor-related protein and requires cell surface proteoglycans. *Journal of Biological Chemistry* 270:9307-9312.

16. Herz, J., Clouthier, D. E., and Hammer, R. E. 1992. LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation [published erratum appears in Cell 1993 May 7;73(3):428]. *Cell* 71:411-421.

17. Bu, G., Maksymovitch, E. A., and Schwartz, A. L. 1993. Receptor-mediated endocytosis of tissue-type plasminogen activator by low density lipoprotein receptor-related protein on human hepatoma HepG2 cells. *Journal of Biological Chemistry* 268:13002-13009.

18. Strickland, D. K., Ashcom, J. D., Williams, S., Burgess, W. H., Migliorini, M., and Argraves, W. S. 1990. Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *Journal of Biological Chemistry* 265:17401-17404.

19. Kristensen, T., Moestrup, S. K., Gliemann, J., Bendtsen, L., Sand, O., and Sottrup-Jensen, L. 1990. Evidence that the newly cloned low-density-lipoprotein receptor related protein (LRP) is the alpha 2-macroglobulin receptor. *FEBS Letters* 276:151-155.

20. Meilinger, M., Gschwentner, C., Burger, I., Haumer, M., Wahrmann, M., Szollar, L., Nimpf, J., and Huettinger, M. 1999. Metabolism of activated complement component C3 is mediated by the low density lipoprotein receptor-related protein/alpha(2)-macroglobulin receptor. *Journal of Biological Chemistry* 274:38091-38096.

21. Hofer, F., Gruenberger, M., Kowalski, H., Machat, H., Huettinger, M., Kuechler, E., and Blass, D. 1994. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. *Proceedings of the National Academy of Sciences of the United States of America* 91:1839-1842.

22. Hodits, R. A., Nimpf, J., Pfistermueller, D. M., Hiesberger, T., Schneider, W. J., Vaughan, T. J., Johnson, K. S., Haumer, M., Kuechler, E., and Winter, G. 1995. An antibody fragment from a phage display library competes for ligand binding to the low density lipoprotein receptor family and inhibits rhinovirus infection. *Journal of Biological Chemistry* 270:24078-24085.

23. Kounnas, M. Z., Morris, R. E., Thompson, M. R., FitzGerald, D. J., Strickland, D. K., and Saelinger, C. B. 1992. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *Journal of Biological Chemistry* 267:12420-12423.

24. Herz, J., Goldstein, J. L., Strickland, D. K., Ho, Y. K., and Brown, M. S. 1991. 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *Journal of Biological Chemistry* 266:21232-21238.

25. Moestrup, S. K. and Gliemann, J. 1991. Analysis of ligand recognition by the purified alpha 2-macroglobulin receptor (low density lipoprotein receptor-related protein). Evidence that high affinity of alpha 2-macroglobulin-proteinase complex is achieved by binding to adjacent receptors. *Journal of Biological Chemistry* 266:14011-14017.

26. Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R. E., Richardson, J. A., and Herz, J. 1999. Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell* 97:689-701.

27. Willnow, T. E., Hilpert, J., Armstrong, S. A., Rohlmann, A., Hammer, R. E., Burns, D. K., and Herz, J. 1996. Defective forebrain development in mice lacking gp330/megalin. *Proceedings of the National Academy of Sciences of the United States of America* 93:8460-8464.

28. Nimpf, J., Stifani, S., Bilous, P. T., and Schneider, W. J. 1994. The somatic cell-specific low density lipoprotein receptor-related protein of the chicken. Close kinship to mammalian low density lipoprotein receptor gene family members. *Journal of Biological Chemistry* 269:212-219.

29. Yochem, J. and Greenwald, I. 1993. A gene for a low density lipoprotein receptor-related protein in the nematode Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America* 90:4572-4576.

30. Kounnas, M. Z., Church, F.C., Argraves, W. S., Strickland, D. K. 1996. Cellular internalization and degradation of antithrombin III-thrombin, heparin cofactor II-thrombin, and alpha(1)-antitrypsin-trypsin complexes is mediated by the low density lipoprotein receptor-related protein. *Journal of Biological Chemistry* 271:6523-6529.

31. Knauer, M. F., Kridel, S. J., Hawley, S. B., Knauer, D. J. 1997. The efficient catabolism of thrombin-protease nexin 1 complexes is a synergistic mechanism that requires both the LDL receptor-related protein and cell surface heparins. *Journal of Biological Chemistry* 272:29039-29045.

32. Kounnas, M. Z., Moir, R. D., Rebeck, G. W., Bush, A. I., Argraves, W. S., Tanzi, R. E., Hyman, B. T., and Strickland, D. K. 1995. LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell* 82:331-340.

33. Warshawsky, I., Broze, G. J. Jr, Schwartz, A. L. 1994. The low density lipoprotein receptor-related protein mediates the cellular degradation of tissue factor pathway inhibitor. *Proceedings of the National Academy of Sciences of the United States of America* 91:6664-6668.

34. Bu, G., Williams, S., Strickland, D. K., and Schwartz, A. L. 1992. Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor is an hepatic receptor for tissue-type plasminogen activator. *Proceedings of the National Academy of Sciences of the United States of America* 89:7427-7431.

35. Kounnas, M. Z., Henkin, J., Argraves, W. S., Strickland, D. K. 1993. Low density lipoprotein receptor-related protein/α_2-macroglobulin receptor mediates cellular uptake of pro-urokinase. *Journal of Biological Chemistry* 268:21862-21867.

36. Van Leuven, F., Cassiman, J. J., Van der Beghe, H. 1986. Human pregnancy zone protein and alpha 2-macroglobulin. High affinity binding of complexes to the same receptor on fibroblasts and characterization by monoclonal antibodies. Journal of Biological Chemistry 261:16622-16625.

37. Barmina, O. Y., Walling, H. W., Fiacco, G. J., Freije, J. M., Lopez-Otin, C., Jeffrey, J. J., and Partridge, N. C. 1999. Collagenase-3 binds to a specific receptor and requires the low density lipoprotein receptor-related protein for internalization. *Journal of Biological Chemistry* 274:30087-30093.

38. Mikhailenko, I., Kounnas, M. Z., and Strickland, D. K. 1995. Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor mediates the cellular internalization and degradation of thrombospondin. A process facilitated by cell-surface proteoglycans. *Journal of Biological Chemistry* 270:9543-9549.

39. Saenko, E. L., Yakhyaev, A. V., Mikhailenko, I., Strickland, D. K., and Sarafanov, A. G. 1999. Role of the low density lipoprotein-related protein receptor in mediation of factor VIII catabolism. *Journal of Biological Chemistry* 274:37685-37692.

40. Beisiegel, U., Weber, W., Ihrke, G., Herz, J., and Stanley, K. K. 1989. The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature* 341:162-164.

41. Kowal, R. C., Herz, J., Goldstein, J. L., Esser, V., and Brown, M. S. 1989. Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins. *Proceedings of the National Academy of Sciences of the United States of America* 86:5810-5814.

42. Reblin, T., Niemeier, A., Meyer, N., Willnow, T. E., Kronenberg, F., Dieplinger, H., Greten, H., and Beisiegel, U. 1997. Cellular uptake of lipoprotein[a] by mouse embryonic fibroblasts via the LDL receptor and the LDL receptor-related protein. *Journal of Lipid Research* 38:2103-2110.

43. Willnow, T. E., Goldstein, J. L., Orth, K., Brown, M. S., and Herz, J. 1992. Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance. *Journal of Biological Chemistry* 267:26172-26180.

44. Hiesberger, T., Huttler, S., Rohlmann, A., Schneider, W., Sandhoff, K., and Herz, J. 1998. Cellular uptake of saposin (SAP) precursor and lysosomal delivery by the low density lipoprotein receptor-related protein (LRP). *EMBO Journal* 17:4617-4625.

45. Shakibaei, M., Frevert, U. 1996. Dual interaction of the malaria circumsporozoite protein with the low density lipoprotein receptor-related protein (LRP) and heparan sulfate proteoglycans. *Journal of Experimental Medicine* 184:1699-1711.

46. Moestrup, S. K., Kaltoft, K., Sottrup-Jensen, L., and Gliemann, J. 1990. The human alpha 2-macroglobulin receptor contains high affinity calcium binding sites important for receptor conformation and ligand recognition. *Journal of Biological Chemistry* 265:12623-12628.

47. Muramatsu, H., Zou, K., Sakaguchi, N., Ikematsu, S., Sakuma, S., Maramatsu, T. 2001. LDL receptor-related protein as a component of the midkine receptor. *Biochemical & Biophysical Research Communications* 270(3):936-941.

48. Riddell, D. R., Siripurapu, V., Vinogradov, D. V., Gliemann, J., and Owen, J. S. 1998. Blood platelets do not contain the low-density receptor-related protein (LRP). *Biochemical Society Transactions* 26:S244.

49. Moestrup, S. K., Gliemann, J., and Pallesen, G. 1992. Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell & Tissue Research* 269:375-382.

50. Van Leuven, F., Stas, L., Hilliker, C., Lorent, K., Umans, L., Serneels, L., Overbergh, L., Torrekens, S., Moechars, D., and De Strooper, B. 1994. Structure of the gene (LRP1) coding for the human alpha 2-macroglobulin receptor lipoprotein receptor-related protein. *Genomics* 24:78-89.

51. Herz, J., Kowal, R. C., Goldstein, J. L., and Brown, M. S. 1990. Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *EMBO Journal* 9:1769-1776.

52. Krieger, M. and Herz, J. 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). [Review] [253 refs]. *Annual Review of Biochemistry* 63:601-637.

53. Esser, V., Limbird, L. E., Brown, M. S., Goldstein, J. L., and Russell, D. W. 1988. Mutational analysis of the ligand binding domain of the low density lipoprotein receptor. *Journal of Biological Chemistry* 263:13282-13290.

54. Dolmer, K., Huang, W., and Gettins, P. G. 2000. NMR solution structure of complement-like repeat CR3 from the low density lipoprotein receptor-related protein. Evidence for specific binding to the receptor binding domain of human alpha(2)-macroglobulin. *Journal of Biological Chemistry* 275:3264-3269.

55. Davis, C. G., Goldstein, J. L., Sudhof, T. C., Anderson, R. G., Russell, D. W., and Brown, M. S. 1987. Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. *Nature* 326:760-765.

56. Moestrup, S. K., Holtet, T. L., Etzerodt, M., Thogersen, H. C., Nykjaer, A., Andreasen, P. A., Rasmussen, H. H., Sottrup-Jensen, L., and Gliemann, J. 1993. Alpha 2-macroglobulin-proteinase complexes, plasminogen activator inhibitor type-1-plasminogen activator complexes, and receptor-associated protein bind to a region of the alpha 2-macroglobulin receptor containing a cluster of eight complement-type repeats. *Journal of Biological Chemistry* 268:13691-13696.

57. Steiner, D. F., Smeekens, S. P., Ohagi, S., and Chan, S. J. 1992. The new enzymology of precursor processing endoproteases. [Review] [102 refs]. *Journal of Biological Chemistry* 267:23435-23438.

58. Robertson, B. J., Moehring, J. M., and Moehring, T. J. 1993. Defective processing of the insulin receptor in an endoprotease-deficient Chinese hamster cell strain is corrected by expression of mouse furin. *Journal of Biological Chemistry* 268:24274-24277.

59. Bravo, D. A., Gleason, J. B., Sanchez, R. I., Roth, R. A., and Fuller, R. S. 1994. Accurate and efficient cleavage of the human insulin proreceptor by the human proprotein-processing protease furin. Characterization and kinetic parameters using the purified, secreted soluble protease expressed by a recombinant baculovirus. *Journal of Biological Chemistry* 269:25830-25837.

60. White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Dull, T. J., Ullrich, A., and Kahn, C. R. 1988. Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. *Cell* 54:641-649.

61. Kaburagi, Y., Yamamoto-Honda, R., Tobe, K., Ueki, K., Yachi, M., Akanuma, Y., Stephens, R. M., Kaplan, D., Yazaki, Y., and Kadowaki, T. 1995. The role of the NPXY motif in the insulin receptor in tyrosine phosphorylation of insulin receptor substrate-1 and Shc. *Endocrinology* 136:3437-3443.

62. Backer, J. M., Schroeder, G. G., Cahill, D. A., Ullrich, A., Siddle, K., and White, M. F. 1991. Cytoplasmic juxtamembrane region of the insulin receptor: a critical role in ATP binding, endogenous substrate phosphorylation, and insulin-stimulated bioeffects in CHO cells. *Biochemistry* 30:6366-6372.

63. Chen, W. J., Goldstein, J. L., and Brown, M. S. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *Journal of Biological Chemistry* 265:3116-3123.

64. Vignoud, L., Albiges-Rizo, C., Frachet, P., and Block, M. R. 1997. NPXY motifs control the recruitment of the alpha5beta1 integrin in focal adhesions independently of the association of talin with the beta1 chain. *Journal of Cell Science* 110:1421-1430.

65. Filardo, E. J., Brooks, P. C., Deming, S. L., Damsky, C., and Cheresh, D. A. 1995. Requirement of the NPXY motif in the integrin beta 3 subunit cytoplasmic tail for melanoma cell migration in vitro and in vivo. *Journal of Cell Biology* 130:441-450.

66. Li, Y., Paz, M. M., van Kerkhof, P., Strous, G. J., and Bu, G. 2000. The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. *Journal of Biological Chemistry* 275:17187-17194.

67. Owen, D. J., Luzio, J. P. 2000. Structural insights into clathrin-mediated endocytosis. [Review] [62 refs] *Current Opinion in Cell Biology.* 12:467-474.

68. Huang, W., Dolmer, K., and Gettins, P. G. 1999. NMR solution structure of complement-like repeat CR8 from the low density lipoprotein receptor-related protein. *Journal of Biological Chemistry* 274:14130-14136.

69. Daly, N. L., Scanlon, M. J., Djordjevic, J. T., Kroon, P. A., and Smith, R. 1995. Three-dimensional structure of a cysteine-rich repeat from the low-density lipoprotein receptor. *Proceedings of the National Academy of Sciences of the United States of America* 92:6334-6338.

70. Daly, N. L., Djordjevic, J. T., Kroon, P. A., and Smith, R. 1995. Three-dimensional structure of the second cysteine-rich repeat from the human low-density lipoprotein receptor. *Biochemistry* 34:14474-14481.

71. Fass, D., Blacklow, S., Kim, P. S., and Berger, J. M. 1997. Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module [see comments]. *Nature* 388:691-693.

72. Neer, E. J. and Smith, T. F. 2000. A groovy new structure [comment]. *Proceedings of the National Academy of Sciences of the United States of America* 97:960-962.

73. Fox, J. W., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Mann, K., Timpl, R., Krieg, T., and Engel, J. 1991. Recombinant nidogen consists of three globular domains and mediates binding of laminin to collagen type IV. *EMBO Journal* 10:3137-3146.

74. http://www.rcsb.org/pdb/cgi/explore.cgi?pid=4289984597685&page=0&pdbId=1LPX

75. Helenius, A. 1994. How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. [Review] [134 refs]. *Molecular Biology of the Cell* 5:253-265.

76. Hammond, C., Braakman, I., and Helenius, A. 1994. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proceedings of the National Academy of Sciences of the United States of America* 91:913-917.

77. Sousa, M. and Parodi, A. J. 1995. The molecular basis for the recognition of misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. *EMBO Journal* 14:4196-4203.

78. Hannink, M. and Donoghue, D. J. 1986. Cell surface expression of membrane-anchored v-sis gene products: glycosylation is not required for cell surface transport. *Journal of Cell Biology* 103:2311-2322.

79. Noiva, R. and Lennarz, W. J. 1992. Protein disulfide isomerase. A multifunctional protein resident in the lumen of the endoplasmic reticulum. [Review] [61 refs]. *Journal of Biological Chemistry* 267:3553-3556.

80. Willnow, T. E., Rohlmann, A., Horton, J., Otani, H., Braun, J. R., Hammer, R. E., and Herz, J. 1996. RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor-related endocytic receptors. *EMBO Journal* 15:2632-2639.

81. Willnow, T. E., Armstrong, S. A., Hammer, R. E., and Herz, J. 1995. Functional expression of low density lipoprotein receptor-related protein is controlled by receptor-associated protein in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 92:4537-4541.

82. Medh, J. D., Fry, G. L., Bowen, S. L., Pladet, M. W., Strickland, D. K., and Chappell, D. A. 1995. The 39-kDa receptor-associated protein modulates lipoprotein catabolism by binding to LDL receptors. *Journal of Biological Chemistry* 270:536-540.

83. Kounnas, M. Z., Argraves, W. S., and Strickland, D. K. 1992. The 39-kDa receptor-associated protein interacts with two members of the low density lipoprotein receptor family, alpha 2-macroglobulin receptor and glycoprotein 330. *Journal of Biological Chemistry* 267:21162-21166.

84. Battey, F. D., Gafvels, M. E., FitzGerald, D. J., Argraves, W. S., Chappell, D. A., Strauss, J. F., III, and Strickland, D. K. 1994. The 39-kDa receptor-associated protein regulates ligand binding by the very low density lipoprotein receptor. *Journal of Biological Chemistry* 269:23268-23273.

85. Bu, G., Geuze, H. J., Strous, G. J., and Schwartz, A. L. 1995. 39 kDa receptor-associated protein is an ER resident protein and molecular chaperone for LDL receptor-related protein. *EMBO Journal* 14:2269-2280.

86. Jorgensen, M. M., Jensen, O. N., Holst, H. U., Hansen, J. J., Corydon, T. J., Bross, P., Bolund, L., and Gregersen, N. 2000. Grp78 is involved in retention of mutant low density lipoprotein receptor protein in the endoplasmic reticulum. *Journal of Biological Chemistry, 275. (43):33861. -33868.* .

87. Obermoeller, L. M., Chen, Z., Schwartz, A. L., and Bu, G. 1998. Ca2+ and receptor-associated protein are independently required for proper folding and disulfide bond formation of the low density lipoprotein receptor-related protein. *Journal of Biological Chemistry* 273:22374-22381.

88. Willnow, T. E., Moehring, J. M., Inocencio, N. M., Moehring, T. J., and Herz, J. 1996. The low-density-lipoprotein receptor-related protein (LRP) is processed by furin in vivo and in vitro. *Biochemical Journal* 313:71-76.

89. Kakehi, T., Hisatomi, A., Kuzuuya, H., Yoshimasa, Y., Okamoto, M., Yamada, K., Nishimura, H., Kosaki, A., Nawata, H., Ibayashi, H., Imura, H. 1988. Defective processing of insulin-receptor precursor in cultured lymphocytes from a patient with extreme insulin resistance. *Journal of Clinical Investigation* 81:2020-2022.

90. Yoshimasa, Y., Seino, S., Whittaker, J., Kakehi, T., Kosaki, A., Kuzuya, H., Imura, H., Bell, G. I., Steiner, D. F. 1988. Insulin-resistant diabetes due to a point mutation that prevents insulin proreceptor processing. *Science* 240:784-787.

91. Willnow, T. E., Orth, K., and Herz, J. 1994. Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *Journal of Biological Chemistry* 269:15827-15832.

92. Ko, K. W., McLeod, R. S., Avramoglu, R. K., Nimpf, J., FitzGerald, D. J., Vukmirica, J., and Yao, Z. 1998. Mutation at the processing site of chicken low density lipoprotein receptor-related protein impairs efficient endoplasmic reticulum

exit, but proteolytic cleavage is not essential for its endocytic functions. *Journal of Biological Chemistry* 273:27779-27785.

93. Corvera, S., Graver, D. F., Smith, R. M. 1989. Insulin increases the cell surface concentration of α_2-macroglobulin receptors in 3T3-L1 adipocytes. Altered transit of the receptor among intracellular endocytic compartments. *Journal of Biological Chemistry* 264:10133-10138.

94. Descamps, O., Bilheimer, D., and Herz, J. 1993. Insulin stimulates receptor-mediated uptake of apoE-enriched lipoproteins and activated alpha 2-macroglobulin in adipocytes. *Journal of Biological Chemistry* 268:974-981.

95. Bu, G., Sun, Y., Schwartz, A. L., and Holtzman, D. M. 1998. Nerve growth factor induces rapid increases in functional cell surface low density lipoprotein receptor-related protein. *Journal of Biological Chemistry* 273:13359-13365.

96. LaMarre, J., Wolf, B. B., Kittler, E. L., Quesenberry, P. J., and Gonias, S. L. 1993. Regulation of macrophage alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein by lipopolysaccharide and interferon-gamma. *Journal of Clinical Investigation* 91:1219-1224.

97. Gaeta, B. A., Borthwick, I., Stanley, K. K. 1994. The 5'-flanking region of the alpha 2MR/LRP gene contains an enhancer-like cluster of Sp1 binding sites. *Biochimica et Biophysica Acta* 1219(2): 307-313.

98. Jensen, H.K., Holst, H., Jensen, L.G., Jorgensen, M.M., Andreasen, P.H., Jensen, T.G., Andresen, B.S., Heath, F., Hansen, P.S., Neve, S., Kristiansen, K., Faergeman, O., Kolvraa, S., Bolund, L.,Gregersen, N. 1997 A common W556S mutation in the LDL receptor gene of Danish patients with familial hypercholesterolemia encodes a transport-defective protein. *Atherosclerosis* 131(1):67-72.

99. Russell, D. W. Esser, V. Hobbs, H. H. 1989. Molecular basis of familial hypercholesterolemia. [Review] [47 refs] *Arteriosclerosis.* 9(1 Suppl):I8-13.

100. Berhanu, P., Anderson, C., Hickman, M., Ciaraldi, T. P. 1997. Insulin signal transduction by a mutant human insulin receptor lacking the NPEY sequence. Evidence for an alternate mitogenic signaling pathway that is independent of shc phosphorylation. *Journal of Biological Chemistry* 272:22884-22890.

101. Hamer, I., Haft, C. R., Paccaud, J. P., Maeder, C., Taylor, S., and Carpentier, J. L. 1997. Dual role of a dileucine motif in insulin receptor endocytosis. *Journal of Biological Chemistry* 272:21685-21691.

102. Kil, S. J., Hobert, M., and Carlin, C. 1999. A leucine-based determinant in the epidermal growth factor receptor juxtamembrane domain is required for the efficient transport of ligand-receptor complexes to lysosomes. *Journal of Biological Chemistry* 274:3141-3150.

103. Herz, J., Kowal, R. C., Ho, Y. K., Brown, M. S., and Goldstein, J. L. 1990. Low density lipoprotein receptor-related protein mediates endocytosis of monoclonal antibodies in cultured cells and rabbit liver. *Journal of Biological Chemistry* 265:21355-21362.

104. Trommsdorff, M., Borg, J. P., Margolis, B., and Herz, J. 1998. Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *Journal of Biological Chemistry* 273:33556-33560.

105. Oleinikov, A. V., Zhao, J., Makker, S. P. 2000. Cytosolic adaptor protein Dab2 is an intracellular ligand of endocytic receptor gp600/megalin. *Biochemical Journal.* 347 Pt 3:613-621.

106. Goretzki L., Mueller B.M.1998. Low-density-lipoprotein-receptor-related protein (LRP) interacts with a GTP-binding protein. *Biochemical Journal*, 336 (Pt 2):381-386.

107. Quinn, K. A., Grimsley, P. G., Dai, Y. P., Tapner, M., Chesterman, C. N., and Owensby, D. A. 1997. Soluble low density lipoprotein receptor-related protein (LRP) circulates in human plasma. *Journal of Biological Chemistry* 272:23946-23951.

108. Rutledge, E. A., Green, F. A., Enns, C. A. 1994. Generation of the soluble transferrin receptor requires cycling through an endosomal compartment. *Journal of Biological Chemistry.* 269(50):31864-31868.

109. Gechtman, Z., Alonso, J. L., Raab, G., Ingber, D. E., Klagsbrun, M. 1999. The shedding of membrane-anchored heparin-binding epidermal-like growth factor is regulated by the raf/mitogen-activated protein kinase cascade and by cell adhesion and spreading. *Journal of Biological Chemistry* 274:28828-28835.

110. Solano, D.C., Sironi, M., Bonfini, C., Solerte, S.B., Govoni S., Racchi M. 2000 Insulin regulates soluble amyloid precursor protein release via phosphatidyl inositol 3 kinase-dependent pathway. *FASEB Journal.* 14(7):1015-1022, 2000.

111. Mullberg J. Althoff K. Jostock T. Rose-John S. 2000. The importance of shedding of membrane proteins for cytokine biology. [Review] [81 refs] *European Cytokine Network. 11:27-38.*

112. Ehlers, M. R. and Riordan, J. F. 1991. Membrane proteins with soluble counterparts: role of proteolysis in the release of transmembrane proteins. [Review] [152 refs]. *Biochemistry* 30:10065-10074.

113. Cross, J. C., Werb, Z., and Fisher, S. J. 1994. Implantation and the placenta: key pieces of the development puzzle. [Review] [163 refs]. *Science* 266:1508-1518.

114. Weaver, A. M., Hussaini, I. M., Mazar, A., Henkin, J., and Gonias, S. L. 1997. Embryonic fibroblasts that are genetically deficient in low density lipoprotein receptor-related protein demonstrate increased activity of the urokinase receptor

system and accelerated migration on vitronectin. *Journal of Biological Chemistry* 272:14372-14379.

115. Ishigami, M., Swertfeger, D. K., Granholm, N. A., and Hui, D. Y. 1998. Apolipoprotein E inhibits platelet-derived growth factor-induced vascular smooth muscle cell migration and proliferation by suppressing signal transduction and preventing cell entry to G1 phase. *Journal of Biological Chemistry* 273:20156-20161.

116. Sottrup-Jensen, L. 1989. Alpha-macroglobulins: structure, shape, and mechanism of proteinase complex formation. [Review] [92 refs]. *Journal of Biological Chemistry* 264:11539-11542.

117. Sottrup-Jensen, L., Petersen, T. E., Magnusson, S. 1980. A thiol-ester in a2-macroglobulin cleaved during proteinase complex formation. *FEBS Letters* 121:275-279.

118. Salvesen, G., Enghild, J. J.1993. α-macroglobulins: detection and characterization. *Methods in Enzymology* 223:121-141

119. Ossowski L., Aguirre-Ghiso, J.A. Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. [Review] [58 refs] *Current Opinion in Cell Biology. 12(5):613-620, 2000.*

120. Andreasen, P.A.. Egelund, R., Petersen, H.H. The plasminogen activation system in tumor growth, invasion, and metastasis. [Review] [197 refs] *Cellular & Molecular Life Sciences. 57(1):25-40, 2000*

121. Gotto AM Jr. Pownall HJ. Havel RJ. 1986. Introduction to the plasma lipoproteins. [Review] [169 refs] *Methods in Enzymology. 128:31-41.*

122. Kita, T., Goldstein, J. L., Brown, M. S., Watanabe, Y., Hornick, C. A., and Havel, R. J. 1982. Hepatic uptake of chylomicron remnants in WHHL rabbits: a mechanism genetically distinct from the low density lipoprotein receptor. *Proceedings of the National Academy of Sciences of the United States of America* 79:3623-3627.

123. Aliev, G., Burnstock, G. Watanabe rabbits with heritable hypercholesterolaemia: a model of atherosclerosis.1998 [Review] [199 refs] *Histology & Histopathology.* 13(3):797-817.

124. Rubinsztein, D. C., Cohen, J. C., Berger, G. M., van der Westhuyzen, D. R., Coetzee, G. A., and Gevers, W. 1990. Chylomicron remnant clearance from the plasma is normal in familial hypercholesterolemic homozygotes with defined receptor defects. *Journal of Clinical Investigation* 86:1306-1312.

125. Hussain, M. M., Maxfield, F. R., Mas-Oliva, J., Tabas, I., Ji, Z. S., Innerarity, T. L., and Mahley, R. W. 1991. Clearance of chylomicron remnants by the low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *Journal of Biological Chemistry* 266:13936-13940.

126. Yu, C-W.K., Chen, W., Cooper, A.D. 2001. LDL receptor–related protein mediates cell-surface clustering and hepatic sequestration of chylomicron remnants in LDLR-deficient mice. *Journal of Clinical Investigation* 107:1387-1394.

127. Ji, Z. S., Fazio, S., Lee, Y. L., and Mahley, R. W. 1994. Secretion-capture role for apolipoprotein E in remnant lipoprotein metabolism involving cell surface heparan sulfate proteoglycans. *Journal of Biological Chemistry* 269:2764-2772.

128. Mahley, R. W., Ji, Z. S., Brecht, W. J., Miranda, R. D., and He, D. 1994. Role of heparan sulfate proteoglycans and the LDL receptor-related protein in remnant lipoprotein metabolism. *Annals. of the New York. Academy of Sciences* 737:39-52.

129. Ji, Z. S., Sanan, D. A., and Mahley, R. W. 1995. Intravenous heparinase inhibits remnant lipoprotein clearance from the plasma and uptake by the liver: in vivo role of heparan sulfate proteoglycans. *Journal of Lipid Research* 36:583-592.

130. Willnow, T. E., Sheng, Z., Ishibashi, S., and Herz, J. 1994. Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor antagonist. *Science* 264:1471-1474.

131. Rohlmann, A., Gotthardt, M., Hammer, R. E., and Herz, J. 1998. Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *Journal of Clinical Investigation* 101:689-695.

132. Beisiegel, U., Weber, W., and Bengtsson-Olivecrona, G. 1991. Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein. *Proceedings of the National Academy of Sciences of the United States of America* 88:8342-8346.

133. Chappell, D. A., Fry, G. L., Waknitz, M. A., Iverius, P. H., Williams, S. E., and Strickland, D. K. 1992. The low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor binds and mediates catabolism of bovine milk lipoprotein lipase. *Journal of Biological Chemistry* 267:25764-25767.

134. Nykjaer, A., Nielsen, M., Lookene, A., Meyer, N., Roigaard, H., Etzerodt, M., Beisiegel, U., Olivecrona, G., and Gliemann, J. 1994. A carboxyl-terminal fragment of lipoprotein lipase binds to the low density lipoprotein receptor-related protein and inhibits lipase-mediated uptake of lipoprotein in cells. *Journal of Biological Chemistry* 269:31747-31755.

135. de Faria, E., Fong, L.G., Komaromy, M., Cooper, A.D. 1996, Relative roles of the LDL receptor, the LDL receptor-like protein, and hepatic lipase in chylomicron remnant removal by the liver. Journal of Lipid Research, 37:197-209.

136. Ji, Z. S., Lauer, S. J., Fazio, S., Bensadoun, A., Taylor, J. M., and Mahley, R. W. 1994. Enhanced binding and uptake of remnant lipoproteins by hepatic lipase-

secreting hepatoma cells in culture. *Journal of Biological Chemistry* 269:13429-13436.

137. Jackson, M.E., Simpson, J.C., Girod, A., Pepperkok R., Roberts L.M., Lord J.M., 1999. The KDEL retrieval system is exploited by Pseudomonas exotoxin A, but not by Shiga-like toxin-1, during retrograde transport from the Golgi complex to the endoplasmic reticulum. *Journal of Cell Science.* 112 (Pt 4):467-475.

138. Taupiac, M-P., Alami, M., Beaumelle, B. 1996. Translocation of full-length pseudomonas exotoxin from endosomes is driven by ATP hydrolysis but requires prior exposure to acidic pH. *Journal of Biological Chemistry* 271:26170-26173.

139. FitzGerald, D. and Pastan, I. 1993. Pseudomonas exotoxin and recombinant immunotoxins derived from it. [Review] [43 refs]. *Annals. of the New York. Academy of Sciences* 685:740-745.

140. FitzGerald, D. J., Fryling, C. M., Zdanovsky, A., Saelinger, C. B., Kounnas, M., Winkles, J. A., Strickland, D., and Leppla, S. 1995. Pseudomonas exotoxin-mediated selection yields cells with altered expression of low-density lipoprotein receptor-related protein [published erratum appears in J Cell Biol 1995 Aug;130(4):1015]. *Journal of Cell Biology* 129:1533-1541.

141. Ben-Yehudah, A., Yarkoni, S., Nechushtan, A., Belostotsky, R., Lorberboum-Galski, H. 1999.Linker-based GnRH-PE chimeric proteins inhibit cancer growth in nude mice. *Medical Oncology.* 16:38-45.

142. Ulery, P. G., Beers, J., Mikhailenko, I., Tanzi, R. E., Rebeck, G. W., Hyman, B. T., Strickland, D. K. 2000. Modulation of beta-amyloid precursor protein processing by the low density lipoprotein receptor-related protein (LRP). Evidence that LRP contributes to the pathogenesis of Alzheimer's disease. *Journal of Biological Chemistry.* 275:7410-7415.

143. Horn, I. R., van Den Berg, B. M., van der Meijden, P. Z., Pannekoek, H., and van Zonneveld, A. J. 1997. Molecular analysis of ligand binding to the second cluster of complement-type repeats of the low density lipoprotein receptor-related protein. Evidence for an allosteric component in receptor-associated protein-mediated inhibition of ligand binding. *Journal of Biological Chemistry,* 272:13608-13613.

144. Obermoeller L,M,. Warshawsky I. Wardell M,R,. Bu G. 1997 Differential functions of triplicated repeats suggest two independent roles for the receptor-associated protein as a molecular chaperone. *Journal of Biological Chemistry.* 272(16):10761-10768.

145. Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L., Pericak-Vance, M.A. 1993 Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science,* 261:921-923.

146. Strittmatter, W.J., Saunders, A.M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G.S., Roses, A.D. 1993 Apolipoprotein E: High avidity binding to b-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America, 90:1977-1980.*

147. Ladu, M.J., Reardon, C., Van Eldik, L., Fagan, A.M., Bu, G., Holtzman, D., Getz, G.S. 2000 Lipoproteins in the central nervous system. [Review] [58 refs] *Annals of the New York Academy of Sciences.* 903:167-75.

148. Weisgraber, K.H., Mahley, R.W. 1996 Human apolipoprotein E: the Alzheimer's disease connesction. *FASEB Journal*, 10:1485-1494.

149. Blacker, D., Wilcox, M.A., Laird, N.M., Rodes, L., Horvath, S.M., Go, R.P.C., Perry, R., Watson, B., Bassett, S.S., McInnis, M.G., Albert, M.S., Hyman, B.T., Tanzi, R.E.1998 Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nature Genetics*, 19:357-360.

150. Kamboh, M.I., Ferrell, R.E., DeKosky, S.T.1998, Genetic association studies between Alzheimer's disease and two polymorphisms in the low density lipoprotein receptor-related protein gene. Neuroscience Letters, 244(2):65-68.

151. Cohn, J.S., Marcoux, C., Davignon, J., 1999. Detection, quantification, and characterization of potentially atherogenic triglyceride-rich remnant lipoproteins. [Review] [185 refs] *Arteriosclerosis, Thrombosis & Vascular Biology.* 19(10):2474-2486.

152. Mahley, R.W., Innerarity, T.L., Weisgraber, K.H., Rall, S.C., Hui, D.Y., Lalazar, A., Boyles, J.K., Taylor, J.M., Levy-Wilson, B. 1986. Cellular and molecular biology of lipoprotein metabolism: characterization of lipoprotein receptor-ligand interactions. *Cold Spring harbor Symposia on Quantitative Biology*, Vol. LI:821-828.

153. Kissebah, A.H., Alfarsi, S., Adams, P.W.1981. Integrated regulation of very low density lipoprotein triglyceride and apolipoprotein-B kinetics in man: normolipidemic subjects, familial hypertriglyceridemia and familial combined hyperlipidemia, *Metabolism* 30: 856.

154. McLeod, R. S., Zhao, Y., Selby, S. L., Westerlund, J., and Yao, Z. 1994. Carboxyl-terminal truncation impairs lipid recruitment by apolipoprotein B100 but does not affect secretion of the truncated apolipoprotein B-containing lipoproteins. *Journal of Biological Chemistry* 269:2852-2862.

155. Wang, S., McLeod, R. S., Gordon, D. A., and Yao, Z. 1996. The microsomal triglyceride transfer protein facilitates assembly and secretion of apolipoprotein B-containing lipoproteins and decreases cotranslational degradation of apolipoprotein B in transfected COS-7 cells. *Journal of Biological Chemistry* 271:14124-14133.

156. Ashcom, J. D., Tiller, S. E., Dickerson, K., Cravens, J. L., Argraves, W. S., and Strickland, D. K. 1990. The human alpha 2-macroglobulin receptor: identification of a 420-kD cell surface glycoprotein specific for the activated conformation of alpha 2-macroglobulin. *Journal of Cell Biology* 110:1041-1048.

157. Barrett, A.J.1981 α_2-Macroglobulin. *Methods in Enzymology*, 80:737-754.

158. Salvesen, G., Enghild, J.J.1993 α-Macroglobulins: detection and characterization. *Methods in Enzymology*, 223:121-141.

159. Goldstein, J. L., Basu, S. K., and Brown, M. S. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods in Enzymology* 98:241-260.

160. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry* 87:206-210.

161. Obermoeller, L.M., Warshawsky, I., Wardell, M.R., Bu, G. 1997 Differential functions of triplicated repeats suggest two independent roles for the receptor-associated protein as a molecular chaperone. *Journal of Biological Chemistry.* 272(16):10761-10768.

162. Bu, G. 1998 Receptor-associated protein: a specialized chaperone and antagonist for members of the LDL receptor gene family. *Current Opinion in Lipidology.* 9(2):149-155.

163. Mucci, D., Forristal, J., Strickland, D., Morris, R., FitzGerald, D., Saelinger, C.B.1995 Level of receptor-associated protein moderates cellular susceptibility to Pseudomonas exotoxin A. *Infection and Immunity*, 63(8):2912-2918.

164. Russell, D.W., Brown, M.S., Goldstein, J.L., 1989 Different combinations of cysteine-rich repeats mediate binding of low density lipoprotein receptor to two different proteins. *Journal of Biological Chemistry.* 264(36):21682-8, 1989.

165. Mahley, R.W., Ji, Z.S. 1999, Remnant lipoprotein metabolism: key pathways involving cell-surfaceheparan sulfate proteoglycans and apolipoprotein E. [Review] [126 refs] *Journal of Lipid Research*, 40(1): 1-16.

166. Ji, Z.S., Dichek, H.L., Miranda, R.D., Mahley, R.W. 1997. Heparan sulfate proteoglycans participate in hepatic lipase and apolipoprotein E-mediated binding and uptake of plasma lipoproteins, including high density lipoproteins. *Journal of Biological Chemistry*, 272(50): 31285-31292

167. Radeau, T., Robb, M., Lau, P., Borthwick, J., McPherson, R. 1998. Relationship of adipose tissue cholesteryl ester transfer protein (CETP) mRNA to plasma concentrations of CETP in man. *Atherosclerosis*, 139(2):369-376.

168. Sniderman, A.D., Maslowska, M., Cianflone, K. 2000. Of mice and men (and women) and the acylation-stimulating protein pathway. [Review] [19 refs] Current Opinion in Lipidology, 11(3):291-296.

169. Baldo A., Sniderman A.D., St-Luce S., Avramoglu R.K., Maslowska, M., Hoang, B., Monge, J.C., Bell, A., Mulay, S., Cianflone, K. 1993 The adipsin-acylation stimulating protein system and regulation of intracellular triglyceride synthesis. *Journal of Clinical Investigation.* 92(3):1543-7.

170. Bork, P., Downing, A.K., Kieffer, B., Campbell, I.D. 1996 Structure and distribution of modules inextracellular proteins. [Review] [95 refs] *Quarterly Reviews of Biophysics.* 29(2):119-167.

171. Wall, M.A., Coleman, D.E., Lee, E., Iniguez-Lluhi, J.A., Posner, B.A., Gilman, A.G., Sprang, S.R. 1995 The structure of the G protein heterotrimer Gi alpha 1 beta 1 gamma 2. *Cell* 83(6):1047-1058.

172. ter Haar, E., Musacchio, A., Harrison, S.C., Kirchhausen, T. 1998 Atomic structure of clathrin: a beta propeller terminal domain joins an alpha zigzag linker. *Cell* 95(4):563-573.

173. Gaskell, A., Crennell, S., Taylor, G.1995 The three domains of a bacterial sialidase: a beta-propeller, an immunoglobulin module and a galactose-binding jelly-roll. *Structure* 3(11):1197-1205.

174. Ghosh, M., Anthony, C., Harlos, K., Goodwin, M.G., Blake, C. 1995 The refined structure of thequinoprotein methanol dehydrogenase from Methylobacterium extorquens at 1.94 A. *Structure* 3(2):177-187.

175. Fulop, V., Moir, J.W., Ferguson, S.J., Hajdu, J.1993 Crystallization and preliminary crystallographic study of cytochrome cd1 nitrite reductase from Thiosphaera pantotropha. *Journal of Molecular Biology* 232(4):1211-1212.

176. ter Haar, E., Harrison, S.C., Kirchhausen, T. 2000 Peptide-in-groove interactions link target proteins to the beta-propeller of clathrin. *Proceedings of the National Academy of Sciences of the United States of America. 97(3):1096-1100.*

177. Hobbs, H.H., Russell, D.W., Brown, M.S., Goldstein, J.L. 1990 The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. [Review] [122 refs] *Annual Review of Genetics* 24:133-170.

178. Esser, V., Russell, D.W. 1988 Transport-deficient mutations in the low density lipoprotein receptor.Alterations in the cysteine-rich and cysteine-poor regions of the protein block intracellular transport. *Journal of Biological Chemistry.* 263(26):13276-13281.

179. Schneider, W.J., Brown, M.S., Goldstein, J.L.1983 Kinetic defects in the processing of the low density lipoprotein receptor in fibroblasts from WHHL rabbits and a

family with familial hypercholesterolemia. *Molecular Biology and Medicine.* 1:353-367.

180. Leitersdorf, E., Van der Westhuyzen, D.R., Coetzee, G.A., Hobbs, H.H. 1989 Two common low density lipoprotein receptor gene mutations cause familial hypercholesterolemia in Afrikaners. *Journal of Clinical Investigation.* 84:954-961.

181. Jeon, H., Meng, W., Takagi, J., Eck, M.J., Springer, T.A., Blacklow, S.C. 2001 Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair. *Nature Structural Biology.* 8(6):499-504.

Curriculum vitae

Identification

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Education

CEGEP

D.E.C. Pure and applied sciences, CEGEP Vanier 1982-84

Undergraduate

B.Sc. Chemistry, Concordia University 1985-1989
Specialization in analytical chemistry

Undergraduate Research Project 1989
Molar absorptivity constants of horse heart porphyrin cytochrome C

Graduate

M. Sc. Biochemistry, Concordia University 1992-1994

Courses:
Analytical Biochemistry
Protein Engineering and Design
Cell Membrane Biochemistry
Advanced Enzyme Kinetics

Thesis title: Mechanisms of uptake and secretion of apolipoprotein B containing triacylglycerol-rich lipoproteins in a liver cell model

Ph.D. Biochemistry, University of Ottawa 1995-

Courses:
Biochemistry of Lipoproteins
Advanced Topics in Nutrition and Regulation of Metabolism

Work experience

1989-1995 Laboratory Technician, Division of Cardiology. Royal Victoria Hospital, McGill University, Montreal, Quebec

From 1989-1992 worked on the purification and characterization of acylation stimulating protein.

Experience in this area includes column chromatography (size exclusion, ion exchange, and affinity chromatography) and TLC, FPLC, HPLC, SDS-PAGE, IEF, 2-D electrophoresis, western blots, ELISA, development of and purification of polyclonal antibodies, enzyme activity assays, ion-spray mass spectroscopy and amino acid sequencing and composition analysis as well as tissue culture of fibroblasts.

From 1992-1995 worked on the uptake mechanism of triacylglycerol-rich lipoproteins and neutral lipid regulation of apolipoprotein B secretion in a liver cell model.

Additional work experience in this area includes tissue culture of hepatocytes, isolation of human lipoproteins and subcellular fractions using density gradient centrifugation, chemical and enzymatic determination of neutral lipids from cell extracts and in plasma, enzyme activity assays of lipases and ELISA of apolipoprotein B.

During this period I was also responsible for the design of both my experiments and those of the two summer students supervised.

1989 Laboratory technician, Department of Chemistry and Biochemistry, Concordia University, Montreal, Quebec

Responsible for the purification of native horse heart cytochrome C using ion exchange chromatography.

Teaching

Laboratory sessions demonstrated

September 1992	Intermediate Inorganic Chemistry Concordia University
January 1993	Introductory General Chemistry Concordia University
September 1993	Introductory Physical Chemistry laboratory and tutorial sessions Concordia University

Students supervised

1993	Studied mechanism of uptake of triacylglycerol-rich lipoproteins in the presence of substances which disrupt the formation of proteoglycans at the cell surface.
1994	Studied the regulation of apolipoprotein B secretion from hepatocytes in the presence of a potential inhibitor of diacylglycerol transferase.

Abstracts and publications

Abstracts

1. Cianflone, K., Sawyez, C., **Kohen Avramoglu, R.**, Huff, M.W.: A role for proteoglycans in the HTg-VLDL induced accumulation of triglyceride and cholesterol ester in HepG2 cells. FASEB J. 7(3): 384A, 1993.

2. Cianflone, K., Sawyez, C., **Kohen Avramoglu, R.**, Huff, M.W.: A role for proteoglycans in the HTg-VLDL induced accumulation of triglyceride and cholesterol ester in HepG2 cells. Clin. Invest. Med. Sept/Oct 1993

3. **Kohen Avramoglu, R.**, Cianflone, K., Sniderman, A.D.: Intracellular cholesterol ester accumulation in HepG2 cells and apoB secretion (CFBS, June, 1994)

4. Cianflone, K., **Kohen Avramoglu, R.**, Sniderman, A.D.: Intracellular cholesterol ester accumulation in HepG2 cells and apoB secretion Circ. 90(4): (Part 2): I-185, 1994

5. **Kohen Avramoglu, R.**, Wang, Y., Nimpf, J., Ko, K., Yao, Z.: Expression of somatic LDL receptor related protein (LRP) of chicken (ASCB, December, 1996)

6. **Kohen Avramoglu, R.**, Nimpf, J., Ko. K.W.S., Wang Y., FitzGerald, D., Yao, Z.,: Functional expression of the chicken LDL receptor-related protein in a mutant cell line restores sensitivity to *Pseudomonas* exotoxin A (CFBS, June 1997)

7. **Kohen Avramoglu, R.**, Nimpf. J., Ko, K.W.S., McLeod, R., Wang, Y., FitzGerald, D., Yao, Z.,: Functional expression of recombinant chicken LDL receptor-related protein (LRP) mutants in an LRP deficient CHO cell line (CLC, 1997)

8. **Kohen Avramoglu, R.**, Nimpf. J., Ko, K.W.S., McLeod, R., Wang, Y., FitzGerald, D., Yao, Z.,: Functional expression of recombinant chicken LDL receptor-related protein mutants in a deficient cell line (ASCB, 1997)

Peer reviewed publications

1. Baldo, A., Sniderman, A.D., St-Luce, S., **Kohen Avramoglu, R.**, Maslowska, M., Hoang, B., Monge, JC., Bell, A., Mulay, S., Cianflone, K.: The adipsin-acylation stimulating protein system and regulation of intracellular triglyceride synthesis. J. Clin. Invest. 92 (3): 1543-1557, 1993.

2. **Kohen Avramoglu, R.**, Sniderman, A.D., Cianflone, K.: The role of neutral lipid accessible pool in the regulation of secretion of B100 lipoprotein particles by HepG2 cells. J. Lipid Res. 36: 2513-2528, 1995

3. Cianflone, K., **Kohen Avramoglu, R.**, Sawyez, C., Huff, M.W.: Inhibition of lipoprotein lipase induced cholesterol ester accumulation in human hepatoma HepG2 cells. Atherosclerosis 120: 101-114, 1196

4. Cianflone, K., Zhang, Z., Vu, H., **Kohen Avramoglu, R.**, Kalant., D., Sniderman, A.D.: The effect of individual amino acids on ApoB100 and Lp(a) secretion by HepG2 cells. J. Biol. Chem. 271 (46): 29136-29145, 1996.

5. **Avramoglu RK.**, Nimpf J. McLeod RS. Ko KW. Wang Y. FitzGerald D. Yao Z. Functional expression of the chicken low density lipoprotein receptor-related protein in a mutant chinese hamster ovary cell line restores toxicity of Pseudomonas exotoxin A and degradation of alpha2-macroglobulin, Journal of Biological Chemistry, 273(11):6057-65, 1998 Mar 13.

6. Tran K. Boren J. Macri J. Wang Y. McLeod R. **Avramoglu RK.**, Adeli K. Yao Z. Functional analysis of disulfide linkages clustered within the amino terminus of human apolipoprotein B. Journal of Biological Chemistry. 273(13):7244-51, 1998 Mar 27.

7. Ko KW. McLeod RS. **Avramoglu RK.**, Nimpf J. FitzGerald DJ. Vukmirica J. Yao Z. Mutation at the processing site of chicken low density lipoprotein receptor-related protein impairs efficient endoplasmic reticulum exit, but proteolytic cleavage is not essential for its endocytic functions, Journal of Biological Chemistry 273(43):27779-85, 1998 Oct 23.

8. Kerry W. S. Ko, **Rita Kohen Avramoglu**, Roger S. McLeod, Jelena Vukmirica, and Zemin YaoThe Insulin-Stimulated Cell Surface Presentation of Low Density Lipoprotein Receptor-Related Protein in 3T3-L1 Adipocytes Is Sensitive to Phosphatidylinositide 3-Kinase Inhibition, Biochemistry 40, 752-759, 2001.