Low-Density Lipoprotein Receptor-Related Protein
Novel Aspects Revealed

Patricia Spijkers

Cover illustration: Sabine Spijkers
Photograph taken at lab day 2004 at Austerlitz

Printed by Febodruk B.V., Enschede

ISBN: 90-393-4001-3

Low-Density Lipoprotein Receptor-Related Protein
Novel Aspects Revealed

Low-density Lipoprotein Receptor-Related Protein – nieuwe aspecten onthuld
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit van Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen, in gevolge het besluit van het College voor Promoties in het openbaar te verdedigen op dinsdag 20 september 2005 des middags te 14.30 uur

door

Patricia Petronella Elisabeth Maria Spijkers

Geboren op 6 september 1978 te ’s-Hertogenbosch

Promotor: Prof. Dr. Ph.G. de Groot

Co-promotor: Dr. P.J. Lenting

Faculteit Geneeskunde, Universiteit van Utrecht

The study described in this thesis was supported by a grant of the Dutch Organization of Scientific Research (NWO) (no. 902-26-236).

Financial support by NWO and the Stichting voor Afweerstoornissen Nederland for the publication of this thesis is gratefully acknowledged.

Additional financial support by the University of Utrecht, Dr. Ir. van der Laar Stichting, Pfizer B.V., Internationale Stichting voor Alzheimer Onderzoek, and Tebu-Bio is also gratefully acknowledged.

Contents

Abbreviations

α_2M	α_2-macroglobulin
Aβ	amyloid β-peptide
AD	Alzheimer's Disease
APC	antigen presenting cell
ApoE	Apolipoprotein E
ApoER2	ApoE receptor 2
APP	Aβ precursor protein
BBB	blood-brain barrier
BEC	brain endothelial cells
C4BP	C4b-binding protein
CCP	complement control protein
CD	cluster of differentiation
DIAβ40	Aβ40 peptide with Dutch/Iowa mutation
EGF	epidermal growth factor
FITC	fluorescein isothiocyanate
GST	glutathione-S-transferase
GVHD	graft-versus-host disease
HSPG	heparan sulfate proteoglycan
HUVEC	human umbilical vein endothelial cells
ICAM	intercellular adhesion molecule
IL	interleukin
LDL	low-density lipoprotein
LRP	LDL receptor-related protein
MLR	mixed lymphocyte reaction
NGF	nerve growth factor
NIF	neutrophil inhibitory factor
NK	natural killer
NMDA	N-methyl-D-aspartate
PBMC	peripheral blood mononuclear cell
PDGF	platelet-derived growth factor
PFA	paraformaldehyde
PHA	phytohaemagglutinin
PKA	protein kinase A
PMA	phorbol-12-myristate-13 acetate
PMN	polymorphonuclear cell
PNP	p-nitrophenyl phosphate
RAGE	receptor for advanced glycation end products
RAP	receptor-associated protein
rC4BP	recombinant C4BP
RU	resonance units
SPR	surface plasmon resonance
TCA	trichloracetic acid
TFPI	tissue-factor pathway inhibitor
TNF-α	tumor necrosis factor-α
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor
VLDL	very low-density lipoprotein
VWF	Von Willebrand Factor

Een reis van duizend mijl begint
met de eerste stap
(*Lao-Tse*)

chapter 1

General Introduction

Multiligand Receptors

Cell surface receptors play a crucial role in maintaining cellular homeostasis by linking the extracellular environment and intracellular compartments. In addition, they can act as an anchor in the adhesion process to other cells or the extracellular matrix. Whereas receptors have long been thought to recognize one specific ligand, a broad range of receptors has now been identified that are able to bind multiple ligands. Moreover, these ligands may even belong to structurally and functionally unrelated families. As a result of this complexity, the receptor must therefore be accompanied by a tightly organized regulatory system to ensure that the appropriate cellular responses are initiated. For example, multiligand receptors may be specialized in distinct functions, like the removal of obsolete proteins, or may be differentially expressed by particular cell types. Also, the multiligand receptor could be part of a heterologous protein complex that controls a specific physiological process[1;2].

Low-Density Lipoprotein (LDL)-Receptor Family

The LDL-receptor family is a classical group of multiligand cell-surface receptors that share structural and functional homology (*Fig 1*). The first member that has been characterized was the LDL receptor, which plays a key role in cholesterol homeostasis[3]. At present, 12 members are described in humans: LDL receptor, Apolipoprotein E receptor 2 (ApoER2), very low density lipoprotein (VLDL) receptor, LDL receptor-related protein (LRP), LRP1B, megalin, LRP3, LRP4/protein 7 with multiple epidermal growth factor (EGF)-like repeats (MEGF7), LRP5/7, LRP6, LDL receptor relative (LR) 11/ sorting protein-related receptor containing LDL receptor class A repeats (SorLA) and ST7/LRP12 (reviewed in Li et al[4] and Herz[5;6]). In general, members contain the following structural domains (*Fig 1*). Their extracellular domain contains ligand-binding regions that consist of clusters of cysteine-rich complement-type repeats. These repeats are separated by EGF precursor repeats and YWTD modules. The latter two domains are thought to be involved in pH-dependent ligand dissociation from the receptors in the endosomes after internalization[7;8]. The receptors are anchored in the cell membrane via a single-chain transmembrane domain, which is followed by a relatively short intracellular tail that harbors motifs for endocytosis and signal transduction.

The statement that all members of the LDL receptor family are multiligand receptors, does not imply that they recognize identical ligands and provoke the same cellular responses. In contrast, ligand binding and cellular reaction may be highly regulated, *e.g.* by differential cellular expression patterns or variable splicing. An overview of tissue-specific expression, ligands and function of the members is summarized in table 1.

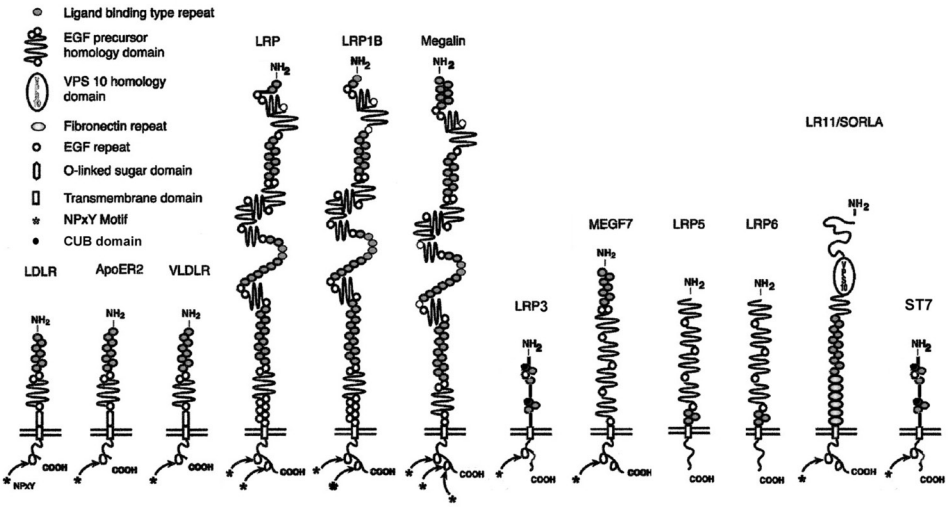


Figure 1. Schematic representation of the human LDL receptor family. Currently, 12 members are known with structurally homologous domains (depicted in the upper left box). Figure adapted and modified from Herz et al[6].

Structure of LRP

In the late 80's, three groups independently described a new protein that displays homology to the LDL receptor. Herz et al[9] searched for cDNA clones containing the highly conserved cysteine-rich complement-type repeats that are present in LDL receptor. This resulted in the discovery of a ~15 kb cDNA stretch, encoding for a 4525 amino acid protein, designated LRP. Simultaneously, Gliemann et al[10;11] and Ashcom et al[12] purified a high affinity receptor for activated α_2-macroglobulin (α_2M) from human hepatocytes, which was therefore called α_2M receptor. Protein sequence analysis revealed that the α_2M receptor and LRP were the same receptor[13].

Table 1. Characteristics of the human LDL receptor family.

Receptor	Examples of expression	Examples of ligands	Examples of functions	References
LDL receptor	liver	ApoE, ApoB	lipid metabolism in liver	3;14
ApoER2/LRP8	brain, platelets	reelin, ApoE, β2-GPI*	brain development, platelet activation	15
VLDLR	heart, muscle, adipose tissue	lipoproteins, reelin	lipid metabolism in extrahepatic tissues, brain development	16
LRP	see table 2	see table 3	see text	11;9
LRP1B/LRP-DIT*	brain, thyroid, salivary glands	uPA* / PAI-1*, APP*	cell migration	17;18
Megalin	kidney	vitamin D binding protein, ApoE, uPA / PAI-1	protein absorption from the urine, vitamin D catabolism	19
LRP3	skeletal muscle, ovary	?	?	20
LRP4/MEGF7	brain	?	VLDL receptor-like signaling?	21
LRP5/7	brain, aorta, bone, liver	Wnt	Wnt signaling, osteogenesis	22;23
LRP6	brain, embryonic tissues	Wnt	Wnt signaling	22;24
LR11/SorLA	brain, liver	ApoE, head activator peptide	protein catabolism, cell proliferation	25
ST7/LRP12	heart, skeletal muscle	?	transformation?	26;27

* APP, amyloid precursor protein; β2-GPI, β2-glycoprotein-I; DIT, deleted in tumors; PAI-1, plasminogen-activator inhibitor-1; uPA, urokinase-type plasminogen activator.

Whereas LDL receptor has a molecular weight of 130 kDa, LRP is a considerably larger protein. It is processed as a 600-kDa single-chain protein that is cleaved by furin in the *trans* Golgi apparatus[28;29]. The two chains, a 515-kDa heavy or α-subunit, and an 85-kDa light or β-subunit, are expressed at the cell surface as a non-covalently linked heterodimer. Both α- and β-chains are heavily N-linked glycosylated[30].

The 515-kDa extracellular domain harbors four clusters of 2, 8, 10 and 11 complement-type repeats, designated as cluster I to IV. Cluster II and IV have been shown to dominate ligand binding[31;32]. The LRP 85-kDa cytoplasmic tail contains two copies of NP*X*Y motifs, one Y*XX*L motif and two di-leucine motifs. These motifs are generally known as internalization signals, which are also present in the LDL receptor[33]. In addition, they can serve as docking sites for cytosolic adaptor proteins involved in cellular signaling[34].

Besides its localization at the cell surface, a low amount of LRP is also present in plasma[35;36]. This soluble form consists of the full α-chain and the extracellular part of the β-chain, as a result of metalloprotease activity at the cell surface. Furthermore, the cytoplasmic tail also contains a cleavage site, which is sensitive to γ-secretase activity. Cleavage may allow transportation of the disconnected tail to other intracellular compartments and subsequent participation in intracellular processes[37;38].

Tissue Distribution of LRP and Regulation of Expression

LRP protein is expressed by a broad number of cells[39], which are summarized in table 2.

One of the aspects of LRP biology that has been poorly explored so far, relates to the molecular regulation of its expression. In view of its multifunctionality, it seems reasonable to assume that LRP requires strict regulation of its cellular expression. Unlike the LDL-receptor gene, the promotor region of the LRP gene does not contain a sterol-regulatory region[40]. In placenta, cyclic AMP (cAMP) prevents LRP expression during differentiation of cytotrophoblasts into syncytiotrophoblasts[41]. In reverse, maturation of monocytes to macrophages results in an increase of LRP protein expression[42]. In retinal pigment epithelial cells, LRP mRNA and protein expression is increased in response to transforming growth factor (TGF)-β1, TGF-β2 or vascular endothelial growth factor[43]. An increase of cell surface expression may also be a result of mobilization of LRP from an endosomal storage pool to the cell surface, as is the case for insulin-treated adipocytes[44]. Also nerve growth factor (NGF) stimulates rapid translocation of LRP to

the cell surface in neurons[45]. This differentially regulated expression may be a key in specifying LRP's function.

Table 2. Tissue and cell distribution of LRP protein[39].

Gastro-Intestinal Tract	*Blood and other Bone Marrow-Derived Cells*
hepatic parenchymal cells	monocytes
intestinal epithelial cells	macrophages
kupffer cells	polymorphonuclear cells
	langerhans cells in skin
Reproductive System	dendritic cells
syncytiotrophoblasts	erythroblasts
granulosa cells in ovary	
leydig cells in testis	*Central Nervous System*
sertoli cells in testis	astrocytes
stromal cells in ovary	neurons
decidua cells in placenta	retinal muller cells
hofbauer cells in placenta	retinal pigment epithelial cells
mammary epithelial cells	trabecular meshwork cells in eye
	cerebral microvessel endothelial cells
Glandular System	purkinje cells
medullary cells in adrenal gland	
epithelial cells of parathyroid gland	*Other*
	adipocytes
Urinary Tract	bone marrow reticulum cells
dendritic interstitial cells of kidney	chondrocytes
glomerular mesangial cells in kidney	pericytes in blood vessels
	myofibroblasts
Respiratory Tract	smooth muscle Cells
lung macrophages	fibroblasts
laryngeal epithelium	mesothelial cells
bronchial and alveolar epithelium	keratinocytes

LRP Ligands

Following activated α_2M, ApoE-enriched β-VLDL was the second protein that was identified as a ligand for LRP[46;47]. Rapidly, more and more proteins were found to bind to LRP and this number is still increasing. These ligands are structurally and functionally unrelated, although part of them can be categorized based on functional characteristics (*Table 3*)[48;49]. The ligands mainly bind to the clusters II and IV and the isolated recombinant clusters are sufficient to recognize ligands[50]. Further molecular dissection of these clusters showed that a certain ligand binds to only a few repeats and that ligands show different or overlapping binding sites[32;51]. Binding competition between ligands could occur via direct competition or sterical hindrance of adjacent bound ligands.

Table 3. LRP ligands[48;49].

Lipoproteins and Lipases	*Proteases and Inhibitor Complexes*
ApoE	activated α_2-macroglobulin
lipoprotein lipase	pregnancy zone protein
hepatic lipase	α_1-antitrypsin
chylomicron remnants	elastase.α_1-antitrypsin
sphingolipid activator protein	protein C inhibitor
Haemostatic Components	*Complement Factors*
factor VIII/ factor VIIIa	C3
factor IXa	C4BP*
TFPI* (/factor VIIa/TF*(/factor Xa))	
tPA*	*Growth Factors*
uPA*	platelet-derived growth factor
	midkine
Combination of Protease…	connective tissue growth factor
thrombin	
factor IXa	*Toxic Compounds*
factor XIa	pseudomonas exotoxin A
tPA	rhinovirus
uPA	HIV* tat-protein
and Inhibitors	circumsporozoite protein
protease nexin-1	ricin A
protease nexin-2	saporin
heparin cofactor II	trichosanthin
PAI-1*	gentamycin
antithrombin III	polymycin B
C1-inhibitor	
activated α_2-marcroglobulin	*Chaperones and Intracellular Proteins*
α_1-antitrypsin	HSP96*
	RAP*
Matrix Proteins	Calreticulin
thrombospondin-1	
thrombospondin-2	*Other*
matrix metalloprotease-2	lactoferrin
matrix metalloprotease-9	APP* / Aβ* peptides
matrix metalloprotease-13	melanotransferrin
fibronectin	

* Aβ, amyloid β peptide; APP, amyloid precursor protein; C4BP, C4b-binding protein; HIV, human immunodeficiency virus; HSP, heat shock protein; PAI-1, plasminogen-activator inhibitor-1; RAP, receptor-associated protein; TF, tissue factor; TFPI, tissue factor pathway inhibitor; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

Whereas some ligands bind to LRP via hydrophobic interactions, ligand-receptor interaction occurs predominantly via electrostatic interactions. LRP displays negative charges on its surface, whereas ligands are mainly positively charged[1]. All ligand-LRP interactions are calcium-dependent[1]. As in LDL receptor, each EGF precursor and complement-type repeat contain a single calcium ion[52;53]. Incorporation of calcium ions is important in the maintenance of the conformation of LRP and, thereby, for ligand binding[54;55].

A ligand of special interest is receptor-associated protein (RAP). During purification of LRP by ligand-affinity chromatography, a ~ 39 kDa protein was co-

purified[12]. This protein was called receptor-associated protein and binds to LRP with high affinity. It functions as an intracellular chaperone by facilitating protein folding in the endoplasmic reticulum and preventing premature ligand binding during the transport to the cell membrane. RAP is the only protein known to bind to cluster III as well[56]. Later, RAP was also found to show high affinity for the other LDL receptor family members, except LDL receptor itself, which has much lower affinity for RAP. Because of RAP's high affinity for the receptors, it is commonly used as a receptor antagonist.

Other ligands relevant to the scope of this thesis will be discussed later in this chapter.

The Physiological Role of LRP

One intriguing aspect of LRP concerns its role in embryonic development. Whereas deficiency of LDL receptor homologues in general results in vital offspring[57-59], deficiency of LRP is associated with early embryonic death at day 10 of gestation[60]. Apparently, LRP's most homologous relatives, LRP1B and megalin, are unable to compensate for the loss of LRP. This may be caused by a highly restricted cellular expression pattern of LRP1B and megalin, or by an LRP-specific function. This critical role of LRP during embryogenesis may point to the involvement of this receptor in various processes. To facilitate studying LRP function *in vivo*, however, conditional LRP knock-out mice have been constructed using the Cre/Lox system[61].

LRP as an endocytic receptor

One of the first functions that could be assigned to LRP was its role in lipoprotein metabolism. Kowal et al[46] found that LRP mediates the uptake and lysosomal delivery of ApoE-enriched β-VLDL in hepatocytes. LRP's endocytic capacity was not limited to this ligand, but is applied to many other proteins as well. Therefore, LRP is considered mainly as a multiligand clearance receptor. LRP continuously shuttles between the cell surface and endosomes, independent of the presence of ligands[62-64]. Li et al, however, suggest that endocytosis is facilitated by protein kinase A (PKA)-mediated phosphorylation of the intracellular tail of LRP upon ligand binding[65]. Based on characteristics of other endocytic receptors, like the LDL receptor, the two NP*X*Y motifs in the cytoplasmic tail of LRP were thought to be the internalization signals[33]. However, more recent studies on LRP showed that the dominant endocytosis signals are a Y*XX*L and a di-leucine motif that are located in the intracellular tail[63]. Receptor-mediated endocytosis starts

at clathrin-coated pits in the cell membrane. The growing vesicle pinches off from the cell membrane and is transported to endosomes. Because of an acidic environment in endosomes, ligands dissociate from LRP and are transported to lysosomes for degradation. In contrast, LRP efficiently recycles back to the plasma membrane for reuse[4;66;67]. During each cycle, a substantial part of the total LRP pool is targeted to the proteasome for degradation[68].

A considerable number of ligands use other membrane-anchored proteins than LRP as primary docking sites for cellular binding. A major group comprises heparan sulfate proteoglycans (HSPGs), which are abundantly expressed at the cell surface. They consist of a core protein, termed proteoglycan, and heterogeneous heparan sulfate side-chains. Two proteoglycan families are known that can form HSPGs, called syndecans and glycipans. They are inserted in the plasma membrane via a transmembrane domain or a glycosyl phosphatidyl inositol moiety, respectively. Via the heparan sulfate side chains, HSPGs bind to a wide variety of positively charged proteins with relatively high affinity (K_d = 1-100 nM). Because of their abundance, HSPGs function as important ligand capturing proteins, thereby increasing local protein concentration and directing it to its specific receptor (reviewed in ref. 69). One of such a specific receptor is LRP. In the binding and catabolism of many heparin-binding ligands LRP efficiently cooperates with HSPGs. Examples of heparin-binding LRP ligands are: ApoE[70;71], factor VIII[72], factor IXa[73] and thrombospondin-1[74].

LRP and phagocytosis

Macrophages are specialized cell types that have the ability to internalize large particles by a process known as phagocytosis. Binding of a particle triggers a signaling cascade leading to its engulfment and cytoskeletal reorganization. A variety of receptors have been demonstrated to mediate phagocytosis. LRP has been implicated in phagocytosis of apoptotic cells by macrophages[75;76]. Apoptotic cells can be recognized by calreticulin. Since calreticulin also interacts with LRP, it can serve as a physical connection between LRP and apoptotic cells during phagocytosis[77]. Studies using chimeras containing the intracellular tail of LRP demonstrated that this domain of LRP plays a dominant role in the phagocytic process, since mutations in the di-leucine motifs or in the NP*XY*X*X*L motif abolish phagocytosis of sheep red blood cells[75]. This implies that an adaptor protein is required that interacts with the cytoplasmic tail of LRP. The group of Ravichandran indeed found that the intracellular adaptor protein GULP binds to LRP in the phagocytic process[76].

LRP and transcytosis

Transcytosis is an intracellular pathway that mediates transport of proteins from the apical cell membrane to the basolateral cell membrane or vice versa. One specialized transcytosis pathway is transendothelial transport across the blood-brain barrier (BBB). This pathway allows tightly regulated transfer of proteins from blood to the brain tissue via brain endothelial cells to prevent entry of toxic compounds into the brain. Therefore, it has been suggested that transcytosis across the BBB predominantly involves specific receptors. Recently, Demeule et al found that LRP is most probably the candidate receptor for mediating transcytosis of melanotransferrin from the blood across the BBB[78]. Later, also RAP is found to cross the BBB via LRP[79]. However, in contrast to melanotransferrin, RAP is predominantly transported by LRP from the basolateral to the apical membrane, whereas megalin is responsible for transport the other way around. All studies on LRP-mediated transcytosis were unable to provide evidence on receptor-mediated intracellular trafficking of the ligands. While little lysosomal degradation occurs, it is not known whether the ligands remain associated with LRP during transcytosis, or whether ligands are transferred to another receptor that transports them to the other site of the cell surface.

LRP and nuclear targeting

Growth factors employ various mechanisms to exert their effect on cellular proliferation, including their translocation to the nucleus after receptor-mediated internalization. A number of growth factors require their nuclear localization to carry out their mitogenic activity[80-83]. Midkine is a growth factor that is involved in various biological functions, such as neuronal survival, carcinogenesis and tissue remodeling[84]. LRP is the receptor for midkine that mediates its internalization[85]. However, LRP delivers midkine not only to the endosomal/lysosomal degradation pathway, but also to the nucleus. In this latter pathway, nucleolin activity is required to take over the trafficking of midkine to ensure nuclear localization of midkine, which is required for full activity of midkine[86]. Midkine is the only growth factor described to be dependent on LRP for its nuclear localization. However, since platelet-derived growth factor (PDGF) is also transported to the nucleus[87] and is a ligand for LRP as well[88], it is tempting to speculate that LRP is also able to mediate nuclear targeting of PDGF.

LRP as a signaling receptor

In recent years, it has become apparent that LRP not only functions in intracellular transport pathways, but also contributes to cellular signaling processes. Whereas endocytosis requires LRP's localization in clathrin-coated pits, LRP was also found to be present in caveolae[89], a cell membrane domain well known to

be involved in cellular signaling. In contrast to the LDL receptor, the cytoplasmic NP*X*Y motif of LRP plays a minor role in endocytosis. However, this motif has an important function in docking of a wide variety of intracellular adaptor proteins, including Disabled (Dab)-1, Dab-2, Fe65, Shc, c-Jun kinase-interacting protein (JIP), PSD-95, CAPON, SEMCAP-1 and ced-6/GULP via their phosphotyrosine binding or PSD-95/Desclarge/ZO-1 domains[76;90;91]. For interaction with these adaptor proteins, LRP's tail needs to be tyrosine phosphorylated at the NP*X*Y motifs by members of the Src kinase family upon activation by diverse stimuli[92]. Most of these adaptor proteins function in cell adhesion, vesicle trafficking, neurotransmission, migration and proliferation[90]. Besides phosphorylation of tyrosine residues, serine and threonine residues in LRP's tail may also be subjected to phosphorylation[65;93]. The responsible kinase is PKA, which in its turn is activated by cAMP[65]. A guanosine triphosphate-binding protein is able to associate with the cytoplasmic tail of LRP after binding of lactoferrin or urokinase-type plasminogen activator (uPA)[94], causing the observed elevation of cAMP levels. Phosphorylation by PKA results in an increase in the endocytosis rate[65]. Opposing results were obtained by Ranganathan et al[93]. In this study, serine/threonine phosphorylation by protein kinase C causes a decrease in endocytosis velocity. In conclusion, serine/threonine phosphorylation may be involved in regulation of endocytosis rate[65;93], whereas tyrosine phosphorylation may play a role in other signal transduction pathways.

LRP as a component of heterologous cell surface complexes

In the previous paragraphs, LRP has been discussed in relation to its function as a single, non-complexed receptor. However, several studies reported that LRP may also participate in heterologous receptor complexes. Within these complexes, LRP and its co-receptor may affect their functions interdependently.

One example of a heterologous complex is the LRP / PDGF-receptor complex. PDGF is able to bind to both the PDGF receptor and LRP, resulting in PDGF receptor-induced phosphorylation of the intracellular NP*X*Y motif of LRP[88]. This reaction results in association of the adaptor protein Shc to the intracellular tail of LRP. Phosphorylation does not occur when phosphoinositide 3-kinase, a protein downstream of PDGF receptor, was blocked by wortmannin, indicating that the PDGF receptor directs phosphorylation of LRP[89]. The authors also demonstrated that LRP phosphorylation occurs in the caveolae of the cell membrane. PDGF enhances smooth muscle cell migration and proliferation. LRP knock-down experiments showed that in the absence of LRP, PDGF-mediated smooth muscle cell migration is increased[95]. Thus, it is conceivable that LRP prevents overstimulation by PDGF, thereby decreasing migration and proliferation of smooth muscle cells[96]. In this way, LRP can prevent the formation of atherosclerotic plaques[97].

The PDGF receptor is not the only receptor that may cooperate with LRP. Qiu et al[98] described that activated α_2M influences intracellular calcium signaling in neurons. Pretreatment with activated α_2M significantly decreases calcium signals in response to N-methyl-D-aspartate (NMDA) through an LRP-mediated pathway. The authors hypothesize that α_2M ligation to LRP causes downregulation of the NMDA receptor, resulting in an attenuation of extracellular calcium influx through the NMDA receptor. In addition, Bacskai et al found that binding of activated α_2M to LRP in neuronal cells immediately increases calcium influx from extracellular pools through NMDA receptors[99]. This requires dimerization of LRP, and it is also suggested that both receptors are interlinked via the scaffold protein PSD-95[90;100]. Apparently, LRP has a dual function in modulating NMDA-induced calcium influx. It is responsible for the acute elevated calcium levels and for long-term downregulation of the NMDA receptor, thereby causing desensitization.

β2-Integrins

Leukocyte recruitment from the blood is a key event in the regulation of immune responses. Activation of leukocytes by chemokines results in rolling of the cells over the vessel wall, mediated by selectins. Finally, leukocytes arrest, spread and emigrate through to endothelial layer to the subendothelial tissues in a β2-integrin-dependent manner[101;102] (*Fig 2*).

β2-Integrins are cell surface receptors that are specifically expressed by leukocytes[103]. They are believed to be involved in processes, such as inflammation, wound healing, and cell growth and survival. β2-integrins are heterodimeric proteins, consisting of a β2-subunit (CD18) and one of the following α-subunits (CD11): α_L (or CD11a or LFA-1), α_M (or CD11b or MAC-1), α_X (or CD11c or p150,95) and α_D (CD11d) (*Fig 3 and Table 4*)[104;105].

For firm adhesion, a tight interaction between β2-integrins and molecules at the cell surface of endothelial cells is required. To do so, β2-integrins are able to interact with a wide subset of endothelial cell (-associated) and soluble proteins. The main counter receptors on the endothelial-cell surface are intercellular adhesion molecules (ICAM)–1 and –2[106-108]. Other ligands for β2-integrins include fibrinogen[109-111], factor X[112], heparin[113], neutrophil inhibitory factor (NIF)[114] and iC3b[115]. Ligand recognition occurs mainly via the I (*inserted*) domain located in the α-subunit, while β2-subunits are indispensable for proper integrin function. They are involved in correct subcellular localization, activation of signaling pathways, and direct binding and regulation of the affinity for ligands[116;117]. Whereas $\alpha_L\beta2$ is constitutively expressed at the cell surface, $\alpha_M\beta2$ cell surface expression

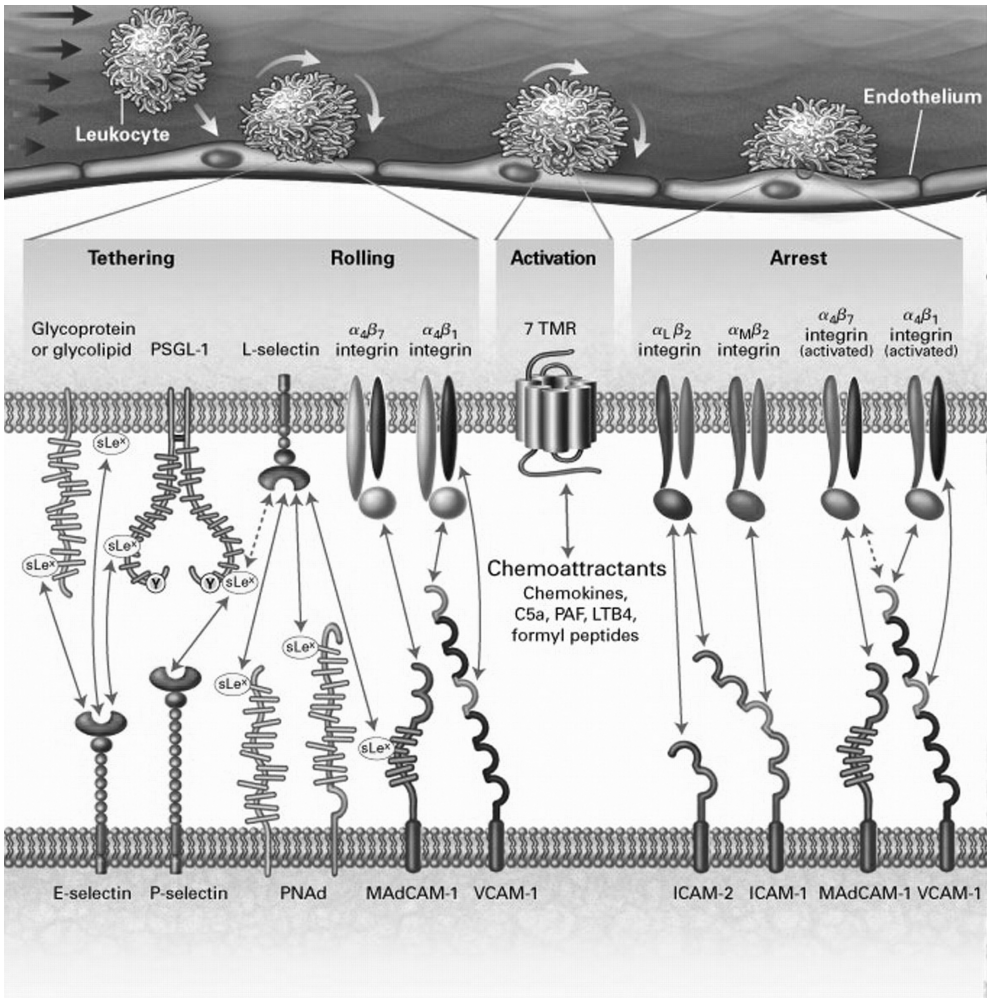


Figure 2. Mechanism of leukocyte adhesion after activation. Leukocytes tether to endothelial cells and start rolling. Tethering is mediated by leukocyte cell surface receptors L-selectin, P-selectin, P-selectin glycoprotein ligand 1 (PSGL-1) and α_4-integrins. The selectins counter receptors at the endothelial cell surface are sialyl-Lewis^x (sLe^x) bearing molecules. Rolling leukocytes become activated by chemokines secreted by endothelial cells via receptors with 7 transmembrane domains (7 TMR). The consequential rapid β2-integrin activation results in firm adhesion and subsequent transmigration to the subendothelial tissues. Figure adapted from Von Andrian et al, 2000[102].

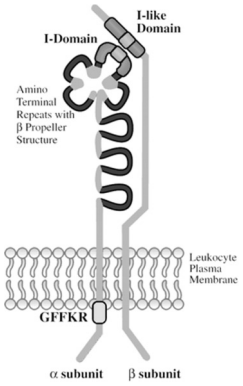


Figure 3. Structure of the β2-integrin heterodimer. Figure adapted from Harris et al, 2000[103].

is increased upon stimulation via a mechanism that involves translocation of $\alpha_M\beta2$ from intracellular storage vesicles to the cell membrane[118].

Normally, leukocytes circulate in blood in a non-adhesive state. However, they must be rapidly transformed into active cells, which are able to bind to their counter receptors via β2-integrins. The precise mechanisms on how integrins are rapidly activated are still not completely understood[119]. In general, it is thought that activation occurs via an inside-out mechanism in which endothelial chemokines are involved. This results in a conformational change in the extracellular domain of the α-subunit[120]. In addition, activation is also associated with lateral movements of β2-integrins to particular regions of the cell membrane (=clustering), thereby increasing their avidity[121]. This process requires reorganization of the cytoskeleton[122], since β2-integrins are tightly associated with it. Thus, both inside-out signaling and clustering are required for optimal ligand binding[123].

Table 4. Main characteristics of β2-integrins. Overview of distribution and ligands of the β2-integrin family members[104;105].

Integrin	Other names	Distribution	Ligands
$\alpha_L\beta2$	CD11a/CD18, LFA-1	B- and T-lymphocytes, monocytes, neutrophils	ICAM-1, ICAM-2, ICAM-3, ICAM-4, ICAM-5, E-selectin, collagen I, JAM-A*
$\alpha_M\beta2$	CD11b/CD18, Mac-1, Mo-1, CR3	monocytes, neutrophils, subset of cytotoxic T-lymphocytes	ICAM-1, ICAM-2, ICAM-4, iC3b, GPIbα*, fibrinogen, VWF*, factor X, heparin, CNTF*, NIF, LPS*, CD23, uPAR*, CD14, CD16, Cyr61, GP63, JAM-C
$\alpha_X\beta2$	CD11c/CD18, p150,95, CR4	monocytes, neutrophils, eosinophils, subset of cytotoxic T-lymphocytes	iC3b, fibrinogen, LPS, CD23, collagen I, uPAR, JAM-C
$\alpha_D\beta2$	CD11d/CD18	monocytes	ICAM-3, VCAM*

* CNTF, ciliary neurotrophic factor; GPIbα, glycoprotein Ibα; JAM, junction adhesion molecule; LPS, lipopolysacharide; uPAR, urokinase-type plasminogen activator receptor; VCAM, vascular cell adhesion molecule; VWF, Von Willebrand Factor.

Amyloid β (Aβ) Peptides in Brain

Amyloid Precursor Protein (APP) is a type I transmembrane protein present in *e.g.* neurons. Due to alternative splicing three isoforms are produced that comprise 770, 751, or 695 amino acids. The largest two isoforms contain a Kunitz-type domain at the C-terminus. At the cell surface, APP can be proteolytically cleaved by α- or β-secretases (*Fig 4*), resulting in release of a fragment (called protease nexin-2) from the cell surface into the cerebrospinal fluid. The carboxy-terminal fragment that remains associated with the membrane, can be further processed to generate Aβ peptides, which are constitutively secreted into the extracellular space[124]. Deposition of Aβ peptides in the brain is central to the pathogenesis of Alzheimer's Disease (AD). Therefore, it is important that Aβ peptides are eliminated from brain tissue to prevent their neurotoxic accumulation[124;125].

LRP is responsible for binding and internalization of both transmembrane and soluble APP[126;127]. When the transmembrane form that contains a Kunitz-type domain, is internalized by LRP, intracellular processing of APP can result in increased secretion of Aβ peptides[126]. The intracellular adaptor protein FE65 is thought to bridge the intracellular tails of LRP and APP[34]. In contrast to this promotional effect of LRP on the progression of AD, LRP can also serve as a protector. By using a murine model, Shibata et al[128] showed that LRP plays an important role in the clearance of Aβ peptide from the brain into plasma. Although LRP is also present in neurons, LRP does not seem to be involved in clearance of

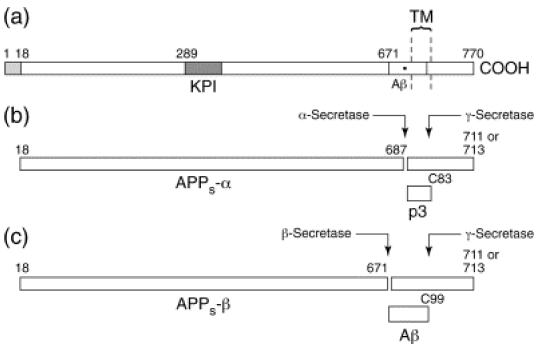


Figure 4. Intracellular processing of APP. *A*. The largest isoform is 770 amino acids (aa), which contains an alternatively spliced exon of 56 aa at position 286. Alternatively, this exon can also comprise 19 aa, resulting in the 751 aa isoform. The Aβ peptide includes 28 aa of the extracellular domain and 12-14 aa of the transmembrane domain (TM). *B*. α-secretase activity results in cleavage at position 687. The remaining transmembrane and intracellular part can be further processed at position 711 of 713 by γ-secretases, resulting in the formation of p3 peptides. *C.* Alternative cleavage at position 671 by β-secretases results in retention of a 99 aa fragment, which can undergo subsequent cleavage by γ-secretases. This action releases the Aβ peptides. Figure adapted from Selkoe[124].

Aβ peptides in the prevention of Aβ peptide deposition in these cells[129]. Aβ peptides can also be internalized as part of a complex with α_2M or ApoE[130] – two LRP ligands that are also associated with AD[131]. The relation between LRP and the development of AD is further exemplified by the observation that reduced LRP expression has been noted in the brains of humans and mice suffering from AD[128;132].

C4b-binding Protein (C4BP)

C4BP is a plasma glycoprotein (150 mg/L) of approximately 570 kDa that is expressed in the liver[133-135]. C4BP has a heterogeneous structure: it contains 6 or 7 identical α-chains and 80% also contains a unique β-chain[136;137] (*Fig 5*). Each α-chain has a molecular weight of 70 kDa, whereas the β-chain is 45 kDa. The chains are interconnected via disulfide bridges, resulting in a spider-like structure[138]. The α- and β-chains are composed of 8 or 3 so-called complement control protein (CCP)- or Sushi domains, respectively.

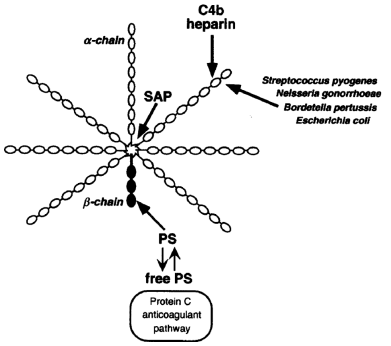


Figure 5. Structure of C4BPαβ. C4BP contains 6 or 7 α-chains and 0 or 1 β-chains (as depicted in this figure). Also shown are ligands of both α- and β-chain. Figure adapted from Blom et al[137].

C4BP has a dual function, since it is associated with both complement and coagulation pathway. In the complement pathway, it controls C4b-mediated reactions in several ways. First, it acts as a cofactor for factor I in the degradation of C4b and C3b[139;140]. Second, C4BP prevents the assembly of C3-convertase by binding C4b, and accelerates the natural decay of the complex[141]. The α-chains bind several proteins, like C4b[138;142], heparin[139;143], serum amyloid P[144] and components of pathogenic bacteria[145]. The β-chain harbors a high affinity binding site for protein S, which acts as a cofactor for anticoagulant activated protein C in the hemostatic process[146;147].

C4BP is an acute phase protein. Upon inflammation its concentration increases 7-fold. Mainly the C4BPα-isoform is raised, which has the consequence that due to stable C4BPαβ levels, the free protein S levels do not decrease during inflammation. To this end, inflammation and coagulation are regulated independently[148].

Recently, LRP was found to be a receptor for C4BP, thereby mediating its cellular uptake *in vitro* and *in vivo*[149]. In addition, the interaction could be inhibited by heparin, suggesting overlapping binding sites for heparin and LRP.

Outline of this Thesis

From historical perspective, LRP has been considered as an endocytic receptor that recognizes a multitude of ligands. More recent studies have demonstrated that LRP exerts other (often cell-type restricted) functions as well. The aim of the present thesis was to further explore the various aspects of LRP biology.

1 LRP has been found to be expressed in granulocytes and monocytes/macrophages. Although the contribution of LRP to macrophage function has been studied to some extent, little is known concerning the role of LRP in monocytes and granulocytes. In chapter 2, we describe studies in which we searched for potential cell-surface-located components in granulocytes and monocytes that interact with LRP. We were able to identify β2-integrins as partners for LRP, and showed that complex formation between both receptors is a prerequisite for optimal leukocyte adhesion to endothelial cells.
2 Despite the abundant literature on the expression patterns of LRP, the presence of LRP in the various leukocyte subsets has remained a poorly investigated issue. In view of its functional importance in cells of the myeloid lineage, it was of interest to elucidate to what extent LRP is expressed in lymphocytic cells. The studies are described in chapter 3, and demonstrated the presence of LRP in CD4+ and CD8+ T-lymphocytes, B-lymphocytes and Natural Killer cells. One remarkable observation in our studies was that LRP is maintained intracellularly in T-lymphocytes, whereas in B-lymphocytes and Natural Killer cells LRP is constitutively present at the cell surface. In addition, we found that LRP is instantly redistributed to the cell surface upon incubation of the T-lymphocytes with allogenic antigen-presenting cells. The functional consequence of this redistribution awaits further studies.
3 There is both biochemical and genetic evidence that LRP is linked to the pathogenesis of Alzheimer's Disease. However, the functional mechanism underlying this link is still unclear. Studies in this regard are complicated by the fact that not only LRP, but also some of its ligands play a role in the development of Alzheimer's Disease. Moreover, LRP is present in various cell types (*ie* neurons and brain capillary endothelium) that contribute to amyloid plaque formation and clearance of amyloid β-peptides. In chapter 4,

studies that focus on the role of LRP present in brain endothelium cells in the clearance of amyloid β-peptides from brain to the circulation are presented. These studies show that high concentrations of amyloid β-peptides direct a downregulation of LRP in brain endothelium cells, resulting in decreased transport of such peptides through the blood-brain barrier and their accumulation in neurotoxic plaques.

4 Recently, we have identified C4b-binding protein (C4BP) as a ligand for LRP *in vitro* and *in vivo*. Since binding of C4BP to LRP is inhibited in the presence of heparin, we considered the possibility that patches of positively charged amino acids within the C4BP α-chain contribute to this interaction. In chapter 5, we describe our studies in this regard. It is shown that mutations within the heparin-binding site of C4BP α-chains result in reduced binding to LRP. In addition, we have found that the cellular uptake of C4BP by LRP is preceded by binding of C4BP to cell-surface-exposed heparan sulfate proteoglycans.

References

1. Krieger, M. and J. Herz. 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu.Rev.Biochem.* 63:601-637.
2. Krieger, M. and D.M. Stern. 2001. Series introduction: multiligand receptors and human disease. *J.Clin.Invest.* 108:645-647.
3. Brown, M.S. and J.L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science* 232:34-47.
4. Li, Y., J. Cam, and G. Bu. 2001. Low-density lipoprotein receptor family: endocytosis and signal transduction. *Mol.Neurobiol.* 23:53-67.
5. Herz, J. 2001. The LDL receptor gene family: (un)expected signal transducers in the brain. *Neuron* 29:571-581.
6. Herz, J. and H.H. Bock. 2002. Lipoprotein receptors in the nervous system. *Annu.Rev.Biochem.* 71:405-434.
7. Mikhailenko, I., W. Considine, K.M. Argraves, D. Loukinov, B.T. Hyman, and D.K. Strickland. 1999. Functional domains of the very low density lipoprotein receptor: molecular analysis of ligand binding and acid-dependent ligand dissociation mechanisms. *J.Cell Sci.* 112:3269-3281.
8. Davis, C.G., J.L. Goldstein, T.C. Sudhof, R.G. Anderson, D.W. Russell, and M.S. Brown. 1987. Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. *Nature* 326:760-765.
9. Herz, J., U. Hamann, S. Rogne, O. Myklebost, H. Gausepohl, and K.K. Stanley. 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 J.* 7:4119-4127.
10. Gliemann, J., O. Davidsen, and S.K. Moestrup. 1989. Characterization, size estimation and solubilization of alpha-macroglobulin complex receptors in liver membranes. *Biochim.Biophys.Acta* 980:326-332.
11. Jensen, P.H., S.K. Moestrup, and J. Gliemann. 1989. Purification of the human placental alpha 2-macroglobulin receptor. *FEBS Lett.* 255:275-280.

12. Ashcom, J.D., S.E. Tiller, K. Dickerson, J.L. Cravens, W.S. Argraves, and D.K. Strickland. 1990. The human alpha 2-macroglobulin receptor: identification of a 420-kD cell surface glycoprotein specific for the activated conformation of alpha 2-macroglobulin. *J.Cell Biol.* 110:1041-1048.
13. Kristensen, T., S.K. Moestrup, J. Gliemann, L. Bendtsen, O. Sand, and L. Sottrup-Jensen. 1990. Evidence that the newly cloned low-density-lipoprotein receptor related protein (LRP) is the alpha 2-macroglobulin receptor. *FEBS Lett.* 276:151-155.
14. Yamamoto, T., C.G. Davis, M.S. Brown, W.J. Schneider, M.L. Casey, J.L. Goldstein, and D.W. Russell. 1984. The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell* 39:27-38.
15. Kim, D.H., H. Iijima, K. Goto, J. Sakai, H. Ishii, H.J. Kim, H. Suzuki, H. Kondo, S. Saeki, and T. Yamamoto. 1996. Human apolipoprotein E receptor 2. A novel lipoprotein receptor of the low density lipoprotein receptor family predominantly expressed in brain. *J.Biol.Chem.* 271:8373-8380.
16. Sakai, J., A. Hoshino, S. Takahashi, Y. Miura, H. Ishii, H. Suzuki, Y. Kawarabayasi, and T. Yamamoto. 1994. Structure, chromosome location, and expression of the human very low density lipoprotein receptor gene. *J.Biol.Chem.* 269:2173-2182.
17. Liu, C.X., Y. Li, L.M. Obermoeller-McCormick, A.L. Schwartz, and G. Bu. 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. *J.Biol.Chem.* 276:28889-28896.
18. Li, Y., J.M. Knisely, W. Lu, L.M. McCormick, J. Wang, J. Henkin, A.L. Schwartz, and G. Bu. 2002. Low density lipoprotein (LDL) receptor-related protein 1B impairs urokinase receptor regeneration on the cell surface and inhibits cell migration. *J.Biol.Chem.* 277:42366-42371.
19. Saito, A., S. Pietromonaco, A.K. Loo, and M.G. Farquhar. 1994. Complete cloning and sequencing of rat gp330/"megalin", a distinctive member of the low density lipoprotein receptor gene family. *Proc.Natl.Acad.Sci.U.S.A.* 91:9725-9729.
20. Ishii, H., D.H. Kim, T. Fujita, Y. Endo, S. Saeki, and T.T. Yamamoto. 1998. cDNA cloning of a new low-density lipoprotein receptor-related protein and mapping of its gene (LRP3) to chromosome bands 19q12-q13. 2. *Genomics* 51:132-135.
21. Nakayama, M., D. Nakajima, T. Nagase, N. Nomura, N. Seki, and O. Ohara. 1998. Identification of high-molecular-weight proteins with multiple EGF-like motifs by motif-trap screening. *Genomics* 51:27-34.
22. Bejsovec, A. 2000. Wnt signaling: an embarrassment of receptors. *Curr.Biol.* 10:R919-R922.
23. Dong, Y., W. Lathrop, D. Weaver, Q. Qiu, J. Cini, D. Bertolini, and D. Chen. 1998. Molecular cloning and characterization of LR3, a novel LDL receptor family protein with mitogenic activity. *Biochem.Biophys.Res.Commun.* 251:784-790.
24. Brown, S.D., R.C. Twells, P.J. Hey, R.D. Cox , E.R. Levy, A.R. Soderman, M.L. Metzker, C.T. Caskey, J.A. Todd, and J.F. Hess. 1998. Isolation and characterization of LRP6, a novel member of the low density lipoprotein receptor gene family. *Biochem.Biophys.Res.Commun.* 248:879-888.
25. Jacobsen, L., P. Madsen, S.K. Moestrup, A.H. Lund, N. Tommerup, A. Nykjaer, L. Sottrup-Jensen, J. Gliemann, and C.M. Petersen. 1996. Molecular characterization of a novel human hybrid-type receptor that binds the alpha2-macroglobulin receptor-associated protein. *J.Biol.Chem.* 271:31379-31383.
26. Qing, J., D. Wei, V.M. Maher, and J.J. McCormick. 1999. Cloning and characterization of a novel gene encoding a putative transmembrane protein with altered expression in some human transformed and tumor-derived cell lines. *Oncogene* 18:335-342.
27. Battle, M.A., V.M. Maher, and J.J. McCormick. 2003. ST7 is a novel low-density lipoprotein receptor-related protein (LRP) with a cytoplasmic tail that interacts with proteins related to signal transduction pathways. *Biochemistry* 42:7270-7282.

28. Herz, J., R.C. Kowal, J.L. Goldstein, and M.S. Brown. 1990. Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *EMBO J.* 9:1769-1776.
29. Willnow, T.E., J.M. Moehring, N.M. Inocencio, T.J. Moehring, and J. Herz. 1996. The low-density-lipoprotein receptor-related protein (LRP) is processed by furin in vivo and in vitro. *Biochem.J.* 313:71-76.
30. Jensen, P.H., J. Gliemann, and T. Orntoft. 1992. Characterization of carbohydrates in the alpha 2-macroglobulin receptor. *FEBS Lett.* 305:129-132.
31. Willnow, T.E., K. Orth, and J. Herz. 1994. Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *J.Biol.Chem.* 269:15827-15832.
32. Horn, I.R., B.M. van den Berg, P.Z. van der Meijden, H. Pannekoek, and A.J. van Zonneveld. 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. *J.Biol.Chem.* 272:13608-13613.
33. Chen, W.J., J.L. Goldstein, and M.S. Brown. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J.Biol.Chem.* 265:3116-3123.
34. Trommsdorff, M., J.P. Borg, B. Margolis, and J. Herz. 1998. Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J.Biol.Chem.* 273:33556-33560.
35. Quinn, K.A., V.J. Pye, Y.P. Dai, C.N. Chesterman, and D.A. Owensby. 1999. Characterization of the soluble form of the low density lipoprotein receptor-related protein (LRP). *Exp.Cell Res.* 251:433-441.
36. Grimsley, P.G., K.A. Quinn, and D.A. Owensby. 1998. Soluble low-density lipoprotein receptor-related protein. *Trends Cardiovasc.Med.* 8:363-368.
37. May, P., Y.K. Reddy, and J. Herz. 2002. Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. *J.Biol.Chem.* 277:18736-18743.
38. Kinoshita, A., T. Shah, M.M. Tangredi, D.K. Strickland, and B.T. Hyman. 2003. The intracellular domain of the low density lipoprotein receptor-related protein modulates transactivation mediated by amyloid precursor protein and Fe65. *J.Biol.Chem.* 278:41182-41188.
39. Moestrup, S.K., J. Gliemann, and G. Pallesen. 1992. Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell.Tissue Res.* 269:375-382.
40. Kutt, H., J. Herz, and K.K. Stanley. 1989. Structure of the low-density lipoprotein receptor-related protein (LRP) promoter. *Biochim.Biophys.Acta* 1009:229-236.
41. Gafvels, M.E., G. Coukos, R. Sayegh, C. Coutifaris, D.K. Strickland, and J.F. Strauss III. 1992. Regulated expression of the trophoblast alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein. Differentiation and cAMP modulate protein and mRNA levels. *J.Biol.Chem.* 267:21230-21234.
42. Watanabe, Y., T. Inaba, H. Shimano, T. Gotoda, K. Yamamoto, H. Mokuno, H. Sato, Y. Yazaki, and N. Yamada. 1994. Induction of LDL receptor-related protein during the differentiation of monocyte-macrophages. Possible involvement in the atherosclerotic process. *Arterioscler.Thromb.* 14:1000-1006.
43. Hollborn, M., G. Birkenmeier, A. Saalbach, I. Iandiev, A. Reichenbach, P. Wiedemann, and L. Kohen. 2004. Expression of LRP1 in retinal pigment epithelial cells and its regulation by growth factors. *Invest.Ophthalmol.Vis.Sci.* 45:2033-2038.
44. Descamps, O., D. Bilheimer, and J. Herz. 1993. Insulin stimulates receptor-mediated uptake of apoE-enriched lipoproteins and activated alpha 2-macroglobulin in adipocytes. *J.Biol.Chem.* 268:974-981.

45. Bu, G., Y. Sun, A.L. Schwartz, and D.M. Holtzman. 1998. Nerve growth factor induces rapid increases in functional cell surface low density lipoprotein receptor-related protein. *J.Biol.Chem.* 273:13359-13365.
46. Kowal, R.C., J. Herz, J.L. Goldstein, V. Esser, and M.S. Brown. 1989. Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins. *Proc.Natl.Acad.Sci.U.S.A.* 86:5810-5814.
47. Kowal, R.C., J. Herz, K.H. Weisgeiber, R.W. Mahley, M.S. Brown, and J.L. Goldstein. 1990. Opposing effects of apolipoproteins E and C on lipoprotein binding to low density lipoprotein receptor-related protein. *J.Biol.Chem*. 268:10771-10779.
48. Strickland, D.K., S.L. Gonias, and W.S. Argraves. 2002. Diverse roles for the LDL receptor family. *Trends Endocrinol.Metab.* 13:66-74.
49. Herz, J. and D.K. Strickland. 2001. LRP: a multifunctional scavenger and signaling receptor. *J.Clin.Invest.* 108:779-784.
50. Neels, J.G., B.M. van den Berg, A. Lookene, G. Olivecrona, H. Pannekoek, and A.J. van Zonneveld. 1999. The second and fourth cluster of class A cysteine-rich repeats of the low density lipoprotein receptor-related protein share ligand-binding properties. *J.Biol.Chem.* 274:31305-31311.
51. Meijer, A.B., J. Rohlena, C. van der Zwaan, R.C. Boertjes, P.J. Lenting, and K. Mertens. 2005. Complement-type repeats involved in the interaction of low density lipoprotein receptor-related protein with factor VIII light chain, activated factor IX, and α_2-macroglobulin. *Manuscript in preparation.*
52. Dolmer, K., W. Huang, and P.G. Gettins. 1998. Characterization of the calcium site in two complement-like domains from the low-density lipoprotein receptor-related protein (LRP) and comparison with a repeat from the low-density lipoprotein receptor. *Biochemistry* 37:17016-17023.
53. Dolmer, K., W. Huang, and P.G. Gettins. 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. *J.Biol.Chem.* 275:3264-3269.
54. Fass, D., S. Blacklow, P.S. Kim, and J.M. Berger. 1997. Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module. *Nature* 388:691-693.
55. Obermoeller, L.M., Z. Chen, A.L. Schwartz, and G. Bu. 1998. Ca2+ and receptor-associated protein are independently required for proper folding and disulfide bond formation of the low density lipoprotein receptor-related protein. *J.Biol.Chem.* 273:22374-22381.
56. Bu, G. 1998. Receptor-associated protein: a specialized chaperone and antagonist for members of the LDL receptor gene family. *Curr.Opin.Lipidol.* 9:149-155.
57. Trommsdorff, M., M. Gotthardt, T. Hiesberger, J.Shelton, W.Stockinger, J.Nimpf, R.E.Hammer, J.A.Richardson, and J.Herz. 1999. Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell* 97:689-701.
58. Ishibashi, S., M.S. Brown, J.L. Goldstein, R.D. Gerard, R.E. Hammer, and J. Herz. 1993. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J.Clin.Invest.* 92:883-893.
59. Willnow, T.E., J. Hilpert, S.A. Armstrong, A. Rohlmann, R.E. Hammer, D.K. Burns, and J. Herz. 1996. Defective forebrain development in mice lacking gp330/megalin. *Proc.Natl.Acad.Sci.U.S.A.* 93:8460-8464.
60. Herz, J., D.E. Clouthier, and R.E. Hammer. 1992. LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. *Cell* 71:411-421.
61. Rohlmann, A., M. Gotthardt, R.E. Hammer, and J. Herz. 1998. Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *J.Clin.Invest.* 101 :689-695.
62. Bu, G., E.A. Maksymovitch, H. Geuze, and A.L. Schwartz. 1994. Subcellular localization and endocytic function of low density lipoprotein receptor-related protein in human glioblastoma cells. *J.Biol.Chem.* 269:29874-29882.

63. Li, Y., M.P. Marzolo, P. van Kerkhof, G.J. Strous, and G. Bu. 2000. The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. *J.Biol.Chem.* 275:17187-17194.
64. Trowbridge, I.S., J.F. Collawn, and C.R. Hopkins. 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu.Rev.Cell Biol.* 9:129-161.
65. Li, Y., P. van Kerkhof, M.P. Marzolo, G.J. Strous, and G. Bu. 2001. Identification of a major cyclic AMP-dependent protein kinase A phosphorylation site within the cytoplasmic tail of the low-density lipoprotein receptor-related protein: implication for receptor-mediated endocytosis. *Mol.Cell Biol.* 21:1185-1195.
66. Mukherjee, S., R.N. Ghosh, and F.R. Maxfield. 1997. Endocytosis. *Physiol.Rev.* 77:759-803.
67. Hussain, M.M., D.K. Strickland, and A. Bakillah. 1999. The mammalian low-density lipoprotein receptor family. *Annu.Rev.Nutr.* 19:141-72.
68. Melman, L., H.J. Geuze, Y. Li, L.M. McCormick, P. van Kerkhof, G.J. Strous , A.L. Schwartz, and G. Bu. 2002. Proteasome regulates the delivery of LDL receptor-related protein into the degradation pathway. *Mol.Biol.Cell* 13:3325-3335.
69. Bernfield, M., M. Gotte, P.W. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, and M. Zako. 1999. Functions of cell surface heparan sulfate proteoglycans. *Annu.Rev.Biochem.* 68:729-777.
70. Ji, Z.S., W.J. Brecht, R.D. Miranda, M.M. Hussain, T.L. Innerarity, and R.W. Mahley. 1993. Role of heparan sulfate proteoglycans in the binding and uptake of apolipoprotein E-enriched remnant lipoproteins by cultured cells. *J.Biol.Chem.* 268:10160-10167.
71. Ji, Z.S., H.L. Dichek, R.D. Miranda, and R.W. Mahley. 1997. Heparan sulfate proteoglycans participate in hepatic lipase and apolipoprotein E-mediated binding and uptake of plasma lipoproteins, including high density lipoproteins. *J.Biol.Chem.* 272:31285-31292.
72. Sarafanov, A.G., N.M. Ananyeva, M. Shima, and E.L. Saenko. 2001. Cell surface heparan sulfate proteoglycans participate in factor VIII catabolism mediated by low density lipoprotein receptor-related protein. *J.Biol.Chem.* 276:11970-11979.
73. Neels, J.G., B.M. van den Berg, K. Mertens, H. ter Maat, H. Pannekoek, A.J. van Zonneveld, and P.J. Lenting. 2000. Activation of factor IX zymogen results in exposure of a binding site for low-density lipoprotein receptor-related protein. *Blood* 96:3459-3465.
74. Chen, H., J. Sottile, D.K. Strickland, and D.F. Mosher. 1996. Binding and degradation of thrombospondin-1 mediated through heparan sulphate proteoglycans and low-density-lipoprotein receptor-related protein: localization of the functional activity to the trimeric N-terminal heparin-binding region of thrombospondin-1. *Biochem.J.* 318:959-963.
75. Patel, M., J. Morrow, F.R. Maxfield, D.K. Strickland, S. Greenberg, and I. Tabas. 2003. The cytoplasmic domain of the low density lipoprotein (LDL) receptor-related protein, but not that of the LDL receptor, triggers phagocytosis. *J.Biol.Chem.* 278:44799-44807.
76. Su, H.P., K. Nakada-Tsukui, A.C. Tosello-Trampont, Y. Li, G. Bu, P.M. Henson, and K.S. Ravichandran. 2002. Interaction of CED-6/GULP, an adapter protein involved in engulfment of apoptotic cells with CED-1 and CD91/low density lipoprotein receptor-related protein (LRP). *J.Biol.Chem.* 277:11772-11779.
77. Ogden, C.A., A. deCathelineau, P.R. Hoffmann, D. Bratton, B. Ghebrehiwet, V.A. Fadok, and P.M. Henson. 2001. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J.Exp.Med.* 194:781-795.
78. Demeule, M., J. Poirier, J. Jodoin, Y. Bertrand, R.R. Desrosiers, C. Dagenais, T. Nguyen, J. Lanthier, R. Gabathuler, M. Kennard, W.A. Jefferies, D. Karkan, S. Tsai, L. Fenart, R. Cecchelli, and R. Beliveau. 2002. High transcytosis of melanotransferrin (P97) across the blood-brain barrier. *J.Neurochem.* 83:924-933.
79. Pan, W., A.J. Kastin, T.C. Zankel, P. van Kerkhof, T. Terasaki, and G. Bu. 2004. Efficient transfer of receptor-associated protein (RAP) across the blood-brain barrier. *J.Cell Sci.* 117:5071-5078.

80. Imamura, T., K. Engleka, X. Zhan, Y. Tokita, R. Forough, D. Roeder, A. Jackson, J.A. Maier, T. Hla, and T. Maciag. 1990. Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence. *Science* 249:1567-1570.
81. Kimura, H. 1993. Schwannoma-derived growth factor must be transported into the nucleus to exert its mitogenic activity. *Proc.Natl.Acad.Sci.U.S.A.* 90:2165-2169.
82. Wiedlocha, A., P.O. Falnes, I.H. Madshus, K. Sandvig, and S. Olsnes. 1994. Dual mode of signal transduction by externally added acidic fibroblast growth factor. *Cell* 76:1039-1051.
83. Carpenter, G. 2003. Nuclear localization and possible functions of receptor tyrosine kinases. *Curr.Opin.Cell Biol.* 15 :143-148.
84. Muramatsu, T. 1993. Midkine (MK), the product of a retinoic acid responsive gene, and pleiotrophin constitute a new protein family regulating growth and differentiation. *Int.J.Dev.Biol.* 37:183-188.
85. Muramatsu, H., K. Zou, N. Sakaguchi, S. Ikematsu, S. Sakuma, and T. Muramatsu . 2000. LDL receptor-related protein as a component of the midkine receptor. *Biochem.Biophys.Res.Commun.* 270:936-941.
86. Shibata, Y., T. Muramatsu, M. Hirai, T. Inui , T. Kimura, H. Saito, L.M. McCormick, G. Bu, and K. Kadomatsu. 2002. Nuclear targeting by the growth factor midkine. *Mol.Cell Biol.* 22:6788-6796.
87. Lee, B.A., D.W. Maher, M. Hannink, and D.J. Donoghue. 1987. Identification of a signal for nuclear targeting in platelet-derived-growth-factor-related molecules. *Mol.Cell Biol.* 7:3527-3537.
88. Loukinova, E., S. Ranganathan, S. Kuznetsov, N. Gorlatova, M.M. Migliorini, D. Loukinov, P.G. Ulery, I. Mikhailenko, D.A. Lawrence, and D.K. Strickland. 2002. Platelet-derived growth factor (PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). Evidence for integrated co-receptor function between LRP and the PDGF receptor. *J.Biol.Chem.* 277:15499-15506.
89. Boucher, P., P. Liu, M. Gotthardt, T. Hiesberger, R.G. Anderson, and J. Herz. 2002. Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low Density lipoprotein receptor-related protein in caveolae. *J.Biol.Chem.* 277:15507-15513.
90. Gotthardt, M., M. Trommsdorff, M.F. Nevitt, J. Shelton, J.A. Richardson, W. Stockinger, J. Nimpf, and J. Herz. 2000. Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. *J.Biol.Chem.* 275:25616-25624.
91. Barnes, H., B. Larsen, M. Tyers, and P. van der Geer. 2001. Tyrosine-phosphorylated low density lipoprotein receptor-related protein 1 (Lrp1) associates with the adaptor protein SHC in SRC-transformed cells. *J.Biol.Chem.* 276:19119-19125.
92. Barnes, H., E.J. Ackermann, and P. van der Geer. 2003. v-Src induces Shc binding to tyrosine 63 in the cytoplasmic domain of the LDL receptor-related protein 1. *Oncogene* 22:3589-3597.
93. Ranganathan, S., C.X. Liu, M.M. Migliorini, C.A. Von Arnim, I.D. Peltan, I. Mikhailenko, B.T. Hyman, and D.K. Strickland. 2004. Serine and threonine phosphorylation of the low density lipoprotein receptor-related protein by protein kinase Calpha regulates endocytosis and association with adaptor molecules. *J.Biol.Chem.* 279:40536-40544.
94. Goretzki, L. and B.M. Mueller. 1998. Low-density-lipoprotein-receptor-related protein (LRP) interacts with a GTP-binding protein. *Biochem.J.* 336:381-386.
95. Swertfeger, D.K., G. Bu, and D.Y. Hui. 2002. Low density lipoprotein receptor-related protein mediates apolipoprotein E inhibition of smooth muscle cell migration. *J.Biol.Chem.* 277:4141-4146.
96. Boucher, P., M. Gotthardt, W.P. Li, R.G. Anderson, and J. Herz. 2003. LRP: Role in vascular wall integrity and protection from atherosclerosis. *Science* 300:329-332.
97. Boucher, P. and M. Gotthardt. 2004. LRP and PDGF signaling: a pathway to atherosclerosis. *Trends Cardiovasc.Med.* 14:55-60.

98. Qiu, Z., D.K. Strickland, B.T. Hyman, and G.W. Rebeck. 2002. Alpha 2-Macroglobulin exposure reduces calcium responses to N-methyl-D-aspartate via low density lipoprotein receptor-related protein in cultured hippocampal neurons. *J.Biol.Chem.* 277:14458-14466.
99. Bacskai, B.J., M.Q. Xia, D.K. Strickland, G.W. Rebeck, and B.T. Hyman. 2000. The endocytic receptor protein LRP also mediates neuronal calcium signaling via N-methyl-D-aspartate receptors. *Proc.Natl.Acad.Sci U.S.A.* 97:11551-11556.
100. May, P., A. Rohlmann, H.H. Bock, K. Zurhove , J.D. Marth, E.D. Schomburg, J.L. Noebels, U. Beffert, J.D. Sweatt, E.J. Weeber, and J. Herz. 2004. Neuronal LRP1 functionally associates with postsynaptic proteins and is required for normal motor function in mice. *Mol.Cell Biol.* 24:8872-8883.
101. McEver, R.P. 2001. Adhesive interactions of leukocytes, platelets, and the vessel wall during hemostasis and inflammation. *Thromb.Haemost.* 86:746-756.
102. von Andrian, U.H. and C.R. Mackay. 2000. T-cell function and migration. Two sides of the same coin. *N.Engl.J.Med.* 343:1020-1034.
103. Harris, E.S., T.M. McIntyre, S.M. Prescott, and G.A. Zimmerman. 2000. The leukocyte integrins. *J.Biol.Chem.* 275:23409-23412.
104. Springer, T.A. 1995. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu.Rev.Physiol.* 57:827-872.
105. Dickeson, S.K. and S.A. Santoro. 1998. Ligand recognition by the I domain-containing integrins. *Cell Mol.Life Sci.* 54:556-566.
106. Gahmberg, C.G., M. Tolvanen, and P. Kotovuori. 1997. Leukocyte adhesion - structure and function of human leukocyte beta2-integrins and their cellular ligands. *Eur.J.Biochem.* 245:215-232.
107. Hermand, P., M. Huet, I. Callebaut, P. Gane , E. Ihanus, C.G. Gahmberg, J.P. Cartron, and P. Bailly. 2000. Binding sites of leukocyte beta 2 integrins (LFA-1, Mac-1) on the human ICAM-4/LW blood group protein. *J.Biol.Chem.* 275:26002-26010.
108. Tian, L., Y. Yoshihara, T. Mizuno, K. Mori, and C.G. Gahmberg. 1997. The neuronal glycoprotein telencephalin is a cellular ligand for the CD11a/CD18 leukocyte integrin. *J.Immunol.* 158:928-936.
109. Altieri, D.C., R. Bader, P.M. Mannucci, and T.S. Edgington. 1988. Oligospecificity of the cellular adhesion receptor Mac-1 encompasses an inducible recognition specificity for fibrinogen. *J.Cell Biol.* 107:1893-1900.
110. Wright, S.D., J.I. Weitz, A.J. Huang, S.M. Levin, S.C. Silverstein, and J.D. Loike. 1988. Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. *Proc.Natl.Acad.Sci.U.S.A.* 85:7734-7738.
111. Loike, J.D., B. Sodeik, L. Cao, S. Leucona, J.I. Weitz, P.A. Detmers, S.D. Wright, and S.C. Silverstein. 1991. CD11c/CD18 on neutrophils recognizes a domain at the N terminus of the A alpha chain of fibrinogen. *Proc.Natl.Acad.Sci.U.S.A.* 88:1044-1048.
112. Altieri, D.C. and T.S. Edgington. 1988. The saturable high affinity association of factor X to ADP-stimulated monocytes defines a novel function of the Mac-1 receptor. *J.Biol.Chem.* 263:7007-7015.
113. Peter, K., M. Schwarz, C. Conradt, T. Nordt , M. Moser, W. Kubler, and C. Bode. 1999. Heparin inhibits ligand binding to the leukocyte integrin Mac-1 (CD11b/CD18). *Circulation* 100:1533-1539.
114. Moyle, M., D.L. Foster, D.E. McGrath, S.M. Brown, Y. Laroche, J. De Meutter, P. Stanssens, C.A. Bogowitz, V.A. Fried, J.A. Ely, and .H.R. Soule, and G.P. Vlasuk. 1994. A hookworm glycoprotein that inhibits neutrophil function is a ligand of the integrin CD11b/CD18. *J.Biol.Chem.* 269:10008-10015.
115. Malhotra, V., N. Hogg, and R.B. Sim. 1986. Ligand binding by the p150,95 antigen of U937 monocytic cells: properties in common with complement receptor type 3 (CR3). *Eur.J.Immunol.* 16:1117-1123.

116. Liu, S., D.A. Calderwood, and M.H. Ginsberg. 2000. Integrin cytoplasmic domain-binding proteins. *J.Cell Sci.* 113:3563-3571.
117. Ehirchiou, D., Y.M. Xiong, Y. Li, S. Brew, and L. Zhang. 2005. Dual function for a unique site within the beta 2I-domain of integrin alpha Mbeta 2. *J.Biol.Chem.* 280:8324-8331.
118. Sengelov, H., L. Kjeldsen, M.S. Diamond, T.A. Springer, and N. Borregaard. 1993. Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. *J.Clin.Invest.* 92:1467-1476.
119. Hogg, N. and B. Leitinger. 2001. Shape and shift changes related to the function of leukocyte integrins LFA-1 and Mac-1. *J.Leukoc.Biol.* 69:893-898.
120. Hughes, P.E., F. Diaz-Gonzalez, L. Leong, C. Wu, J.A. McDonald, S.J. Shattil, and M.H.Ginsberg. 1996. Breaking the integrin hinge. A defined structural constraint regulates integrin signaling. *J.Biol.Chem.* 271:6571-6574.
121. Constantin, G., M. Majeed, C. Giagulli, L. Piccio, J.Y. Kim, E.C. Butcher, and C. Laudanna. 2000. Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity.* 13:759-769.
122. Kucik, D.F., M.L. Dustin, J.M. Miller, and E.J. Brown. 1996. Adhesion-activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes. *J.Clin.Invest.* 97:2139-2144.
123. Xiong, J.P., T. Stehle, S.L. Goodman, and M.A. Arnaout. 2003. New insights into the structural basis of integrin activation. *Blood* 102:1155-1159.
124. Selkoe, D.J. 1998. The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends Cell Biol.* 8:447-453.
125. Ghiso, J. and B. Frangione. 2002. Amyloidosis and Alzheimer's disease. *Adv.Drug Deliv.Rev.* 54:1539-1551.
126. Kounnas, M.Z., R.D. Moir, G.W. Rebeck, A.I. Bush, W.S. Argraves, R.E. Tanzi, B.T. Hyman, and D.K. Strickland. 1995. LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell* 82:331-340.
127. Knauer, M.F., R.A. Orlando, and C.G. Glabe. 1996. Cell surface APP751 forms complexes with protease nexin 2 ligands and is internalized via the low density lipoprotein receptor-related protein (LRP). *Brain Res.* 740:6-14.
128. Shibata, M., S. Yamada, S.R. Kumar, M. Calero, J. Bading, B. Frangione, D.M. Holtzman, C.A. Miller, D.K. Strickland, J. Ghiso, and B.V. Zlokovic. 2000. Clearance of Alzheimer's amyloid-β(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. *J.Clin.Invest.* 106:1489-1499.
129. Zerbinatti, C.V., D.F. Wozniak, J. Cirrito, J.A. Cam, H. Osaka, K.R. Bales, M. Zhuo, S.M. Paul, D.M. Holtzman, and G.Bu. 2004. Increased soluble amyloid-beta peptide and memory deficits in amyloid model mice overexpressing the low-density lipoprotein receptor-related protein. *Proc.Natl.Acad.Sci.U.S.A.* 101:1075-1080.
130. Narita, M., D.M. Holtzman, A.L. Schwartz, and G. Bu. 1997. Alpha2-macroglobulin complexes with and mediates the endocytosis of beta-amyloid peptide via cell surface low-density lipoprotein receptor-related protein. *J.Neurochem.* 69:1904-1911.
131. Ulery, P.G. and D.K. Strickland. 2000. LRP in Alzheimer's disease: friend or foe? *J.Clin.Invest.* 106:1077-1079.
132. Kang, D.E., T. Saitoh, X. Chen, Y. Xia, E. Masliah, L.A. Hansen, R.G. Thomas, L.J. Thal, and R. Katzman. 1997. Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), an apolipoprotein E receptor, with late-onset Alzheimer's disease. *Neurology* 49:56-61.
133. Scharfstein, J., A. Ferreira, I. Gigli, and V. Nussenzweig. 1978. Human C4-binding protein. I. Isolation and characterization. *J.Exp.Med.* 148:207-222.
134. Griffin, J.H., A. Gruber, and J.A. Fernandez. 1992. Reevaluation of total, free, and bound protein S and C4b-binding protein levels in plasma anticoagulated with citrate or hirudin. *Blood* 79:3203-3211.

135. Kusada-Funakoshi, M., J. Sasaki, Y. Takada, T. Soji, and K. Arakawa. 1991. Evidence that C4b-binding protein (proline-rich protein) is synthesized by hepatocytes. *Biochem.Med.Metab.Biol.* 45:350-354.
136. Sanchez-Corral, P., G.O.Criado, and S. Rodriguez de Cordoba. 1995. Isoforms of human C4b-binding protein. I. Molecular basis for the C4BP isoform pattern and its variations in human plasma. *J.Immunol.* 155:4030-4036.
137. Blom, A.M. 2002. Structural and functional studies of complement inhibitor C4b-binding protein. *Biochem.Soc.Trans.* 30:978-982.
138. Dahlback, B., C.A. Smith, and H.J. Muller-Eberhard. 1983. Visualization of human C4b-binding protein and its complexes with vitamin K-dependent protein S and complement protein C4b. *Proc.Natl.Acad.Sci.U.S.A.* 80:3461-3465.
139. Fujita, T. and V. Nussenzweig. 1979. The role of C4-binding protein and beta 1H in proteolysis of C4b and C3b. *J.Exp.Med.* 150:267-276.
140. Blom, A.M., B.O. Villoutreix, and B. Dahlback. 2003. Mutations in alpha-chain of C4BP that selectively affect its factor I cofactor function. *J.Biol.Chem.* 278:43437-43442.
141. Gigli, I., T. Fujita, and V. Nussenzweig. 1979. Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. *Proc.Natl.Acad.Sci.U.S.A.* 76:6596-6600.
142. Hessing, M., C. van 't Veer, and B.N. Bouma. 1990. The binding site of human C4b-binding protein on complement C4 is localized in the alpha'-chain. *J.Immunol.* 144:2632-2637.
143. Hessing, M., D. Kanters, T.M. Hackeng, and B.N. Bouma. 1990. Identification of different forms of human C4b-binding protein lacking beta-chain and protein S binding ability. *Thromb.Haemost.* 64:245-250.
144. Schwalbe, R.A., B. Dahlback, and G.L. Nelsestuen. 1990. Independent association of serum amyloid P component, protein S, and complement C4b with complement C4b-binding protein and subsequent association of the complex with membranes. *J.Biol.Chem.* 265:21749-21757.
145. Blom, A.M., K. Berggard, J.H. Webb, G. Lindahl, B.O. Villoutreix, and B. Dahlback. 2000. Human C4b-binding protein has overlapping, but not identical, binding sites for C4b and streptococcal M proteins. *J.Immunol.* 164:5328-5336.
146. Dahlback, B. and J. Stenflo. 1981. High molecular weight complex in human plasma between vitamin K-dependent protein S and complement component C4b-binding protein. *Proc.Natl.Acad.Sci.U.S.A.* 78:2512-2516.
147. Hillarp,A. and B.Dahlback. 1988. Novel subunit in C4b-binding protein required for protein S binding. *J.Biol.Chem.* 263:12759-12764.
148. Garcia de Frutos, P., R.I. Alim, Y. Hardig, B. Zoller, and B. Dahlback. 1994. Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S. *Blood* 84:815-822.
149. Westein, E., C.V. Denis, B.N. Bouma, and P.J. Lenting. 2002. The alpha-chains of C4b-binding protein mediate complex formation with low density lipoprotein receptor-related protein. *J.Biol.Chem.* 277:2511-2516.

chapter 2

LDL receptor-related protein regulates β2-integrin-mediated leukocyte adhesion

Patricia P.E.M. Spijkers[1], Paula da Costa Martins[2], Erik Westein[1], Carl G. Gahmberg[3], Jaap J. Zwaginga[2] and Peter J. Lenting[1]

[1] Laboratory for Thrombosis and Haemostasis, Department of Haematology, University Medical Center Utrecht, Utrecht, The Netherlands, [2] Department of Experimental Immunohematology, Sanquin Research, Amsterdam, The Netherlands, [3] Department of Biological and Environmental Sciences, Division of Biochemistry, University of Helsinki, Finland

Blood, 2005, 105(1):170-177

Abstract

β2-Integrin clustering upon activation is a key event in leukocyte adhesion to the endothelium during the inflammatory response. In the search for molecular mechanisms leading to this clustering, we have identified low-density lipoprotein (LDL) receptor–related protein (LRP) as a new partner for β2-integrins at the leukocyte surface. Immobilized recombinant LRP fragments served as an adhesive surface for blood-derived leukocytes and the U937 cell line. This adhesion was decreased up to 95% in the presence of antibodies against β2-integrins, pointing to these integrins as potential partners for LRP. Using purified proteins, LRP indeed associated with the $\alpha_M\beta2$ complex and the α_M and α_L I-domains ($K_{d,app} \approx 0.5$ μM). Immunoprecipitation experiments and confocal microscopy revealed that endogenously expressed LRP and $\alpha_L\beta2$ colocalized in monocytes and U937 cells. Furthermore, activation of U937 cells resulted in clustering of $\alpha_L\beta2$ and LRP to similar regions at the cell surface, indicating potential cooperation between both proteins. This was confirmed by the lack of $\alpha_L\beta2$ clustering in U937 cells treated by antisense oligonucleotides to downregulate LRP. In addition, the absence of LRP resulted in complete abrogation of β2-integrin-dependent adhesion to endothelial cells in a perfusion system, demonstrating the presence of a previously unrecognized link between LRP and leukocyte function.

Introduction

Low-density lipoprotein (LDL) receptor–related protein (LRP), also known as α_2-macroglobulin receptor or CD91[1], is a member of the LDL-receptor family. It consists of an 85-kDa intracellular and transmembrane domain that is non-covalently linked to a 515-kDa extracellular domain[2]. The extracellular domain comprises 4 clusters of complement-type repeats, 2 of which (clusters II and IV) play a dominant role in ligand binding[3;4]. At present, more than 30 structurally and functionally unrelated ligands have been identified for this receptor (for a review, see Herz and Strickland[5]), suggesting that LRP is involved in a diverse range of (patho)physiological processes.

The intracellular domain of LRP harbors 2 NP*X*Y-motifs and 1 Y*XX*L motif; the latter is the main motif that controls internalization of LRP[6]. In its function as an endocytic receptor, LRP mediates the cellular uptake of both circulating and membrane-associated proteins, which are subsequently degraded in lysosomes[7]. Alternatively, ligands can be transcytosed[8] or transported to the nucleus[9]. Apart from its endocytic function, LRP has been shown to be involved in signaling pathways[10]. In this respect, various intracellular adaptor proteins have been identified that interact with the cytoplasmic tail of LRP through its NP*X*Y motif[11]. Moreover, binding of adaptor proteins to the cytoplasmic tail inhibits the internalization of LRP[11]. This allows LRP to form heterodimeric complexes with other receptors at the cell surface, such as N-methyl-D-aspartate receptor[12] and platelet-derived growth factor receptor[13;14]. In these cases, the presence of LRP is essential for appropriate function of the co-receptor.

LRP is expressed in a variety of cell types, including hepatocytes, fibroblasts, neurons and smooth muscle cells[15]. Among blood cells, LRP is expressed in leukocytes, including polymorphonuclear cells (PMNs) and monocytes, but not in erythrocytes or platelets[15;16]. PMNs and monocytes are essential in the inflammatory process. Leukocytes circulate in blood in a resting state and become activated in response to inflammatory stimuli. Leukocyte activation may induce adhesion to the vascular wall and subsequent migration to inflammatory sites.

Firm adhesion and subsequent transmigration involves adhesion molecules, such as β2-integrins. β2-Integrins are specifically expressed in leukocytes, and 4 isotypes are known[17]. β2-integrins consist of a common β2-chain (CD18), which is non-covalently linked to an α-chain, namely α_L (CD11a), α_M (CD11b), α_X (CD11c), or α_D (CD11d). The α-chains contain an I-domain, which harbors the main ligand-binding site[18-20]. Although all β2-integrin isoforms interact with a wide subset of proteins, ligand recognition appears to be specific among the isoforms. The main ligands of β2-integrins expressed at the cell surface of other cell types are the intercellular adhesion molecules (ICAMs). At present, 5 ICAMs

have been described with slightly different binding specificities[21]. For instance, ICAM-1, -2, and -4 associate with α_L and α_M, whereas ICAM-3 and -5 are only recognized by α_L[21-23]. Furthermore, α_L specifically binds to junction adhesion molecule-1[24] and α_M binds to junction adhesion molecule-3 and glycoprotein Ib[25-28]. Other β2-integrin ligands are fibrinogen, collagen type I, iC3b, and neutrophil inhibitory factor[20;29;30].

Whereas the role of β2-integrins in leukocyte function has been well studied, little is known about the role of LRP in this connection. The present study focused on the identification of leukocyte-surface proteins that associate with LRP. Our results show that LRP is able to interact with β2-integrins. Moreover, LRP appears to regulate α_Lβ2-integrin clustering and, as such, β2-integrin-mediated adhesion to endothelial cells. These observations identify a previously unrecognized link between LRP and the inflammatory system.

Experimental Procedures

Materials

Cell culture medium RPMI 1640, Dulbecco modified Eagle medium (DMEM)/F-12, penicillin, streptomycin, and L-glutamine were obtained from Gibco Life Technologies (Paisley, United Kingdom). Fetal bovine serum was from Cambrex Bio Science (Verviers, Belgium). Microtiter plates were from Costar (New York, NY) or Nunc (Roskilde, Denmark). The Biacore2000 system and required reagents were from Biacore AB (Uppsala, Sweden). Phorbol-12-myristate-13-acetate (PMA), methotrexate, P-nitrophenyl phosphate (PNP), polyvinylpyrrolidone-360 (PVP-360), and bovine serum albumin (BSA) fraction V were purchased from Sigma (St Louis, MO).

Antibodies and proteins

The following antibodies were used: anti-α_L clone 38 (R&D Systems, Minneapolis, MN), anti-α_M clone 44 (BD PharMingen, San Diego, CA) and M1/70 (R&D Systems), anti-β2 clone 68-5A5 (Cymbus, Hants, United Kingdom) and R2E7B[31], goat anti-LRP A-18 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-LRP clones 2-M-R-II2C7, 2-M-R-II4/8 and anti–LRP-fluorescein isothiocyanate (FITC) clone 2-M-R-I4C2 (Biomac, Leipzig, Germany), goat anti-mouse-FITC (Becton Dickinson, San Jose, CA), anti-urokinase-type plasminogen activator receptor (uPAR) clone 62022 (R&D Systems), anti-4 clone HP2/1 (Immunotech, Westbrook, ME), donkey anti-goat-FITC and donkey anti-mouse-TexasRed (both from Jackson ImmunoResearch, West Grove, PA). Receptor-associated protein[32] was purified as a glutathione-S-transferase fusion protein (GST-RAP), as described[33]. Purified

full-length LRP and stable cell lines expressing recombinant LRP fragments (LRP clusters II and IV) were kindly provided by Dr H. Pannekoek (Department of Biochemistry, University of Amsterdam, The Netherlands). Clusters II and IV were purified from the cell culture supernatant using a GST-RAP sepharose column, as reported previously[4]. The $\alpha_M\beta2$ complex and the I-domains of the α_M and α_L subunit fused to GST were purified as described[34]. Human multimeric vitronectin was purified as described[35].

Cell lines and culture conditions

Human umbilical vein endothelial cells (HUVECs) were isolated according to the method of Jaffe et al[36] with some modifications[37]. Only first and second passages were used for experiments. The monocytic line U937[38] was obtained from the American Type Culture Collection (Manassas, VA) (CRL-1593.2) and was maintained in RPMI 1640, 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 50 μM β-mercaptoethanol in a humidified CO_2 (5%) incubator at 37°C. To downregulate LRP protein expression, U937 cells (5.0×10^5 cells/mL) were incubated with phosphorothioate antisense oligodeoxynucleotides at a concentration of 20 μM for 2 days. Fresh oligonucleotides (10 μM) were added every 24 hours. The sequence of the antisense oligonucleotide was 5'-CGGCGGGGTCAGCAT-3', which is complementary to the initiation site on LRP mRNA[39]. As a control, oligonucleotides having the corresponding sense sequence 5'-ATGCTGACCCCGCCG-3' were used[39]. LRP expression was examined by confocal scanning fluorescence microscopy (see "Confocal scanning fluorescence microscopy"). PMNs were freshly isolated from blood obtained from healthy volunteers by Ficoll-Paque (Amersham-Pharmacia, Uppsala, Sweden) density centrifugation. Erythrocytes were removed from the granulocyte fraction by ice-cold erythrocyte lysis buffer (0.155 mM NH_4Cl, 7.4 mM $KHCO_3$, and 0.1 mM EDTA [ethylenediaminetetraacetic acid], pH 7.4). Peripheral blood monocytes were isolated from the mononuclear cell fraction using CD14 microbeads and AutoMACS (Miltenyi-Biotec, Bergisch-Gladbach, Germany). After isolation, cells were directly used for experiments. Cell purity was greater than 95% for PMNs and 90% for monocytes, as examined by CD15 and CD14 detection by flow cytometric analysis, respectively.

Static cell adhesion

In static cell adhesion experiments, LRP clusters II and IV (50 μg/mL) were immobilized in microtiter wells in Tris-buffered saline (pH 7.4) for 16 hours at 4°C. Alternatively, HUVECs were grown in microtiter wells until confluence. HUVECs were stimulated with tumor necrosis factor-α (TNF-α) (100 U/mL) for 4 hours and were subsequently fixed for 15 minutes with 4% paraformaldehyde. Wells

were blocked with either 5% BSA (U937 cells) or 0.5% PVP-360 (PMNs/monocytes) for 1 hour at 37°C. Cells (2 x 10^6/mL) were washed twice with phosphate-buffered saline (PBS) and were activated with 100 nM PMA in DMEM/F-12 supplemented with 0.1% BSA and 1 mM $MnCl_2$ for 15 minutes. Where indicated, cells were preincubated for 15 minutes with specific blocking antibodies (20 μg/mL) against different integrin subunits, or wells coated with LRP cluster II or IV were incubated with 50 μg/mL GST-RAP in the presence of 3 mM $CaCl_2$ for 15 minutes before the addition of cell suspensions. Cells (1.5 x 10^5/well) were incubated in the microtiter plates for either 60 minutes at 37°C (U937 cells to clusters II and IV) or 30 minutes at room temperature (PMNs/monocytes to clusters II and IV; U937 cells to HUVECs). Non-bound cells were removed by gently washing wells with PBS. Adherent cells were determined by endogenous alkalic phosphatase activity using PNP as a substrate (3 mg/mL in 1% Triton-X100/50 mM acetic acid (pH 5)). Optical density was measured at 405 nm. Alternatively, cells were visualized using light microscopy (Leitz Diaplan; Leica, Rijswijk, The Netherlands) and computer-assisted analysis with OPTIMAS 6.0 software (DVS, Breda, The Netherlands).

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) binding assays were performed using a Biacore2000 biosensor system. LRP was immobilized on a CM5-sensorchip at a density of 7.7 fmol/mm^2 using the amine-coupling kit, as described by the manufacturer. A control channel was routinely activated and blocked in the absence of protein. Binding of GST/I-domain fusion proteins to LRP-coated channels was corrected for binding to noncoated channels (less than 5% of binding to coated channels). SPR analysis was performed in 100 mM NaCl, 0.005% Tween-20, 2 mM $CaCl_2$, 2 mM $MnCl_2$, 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.4) at 25°C with a flow rate of 5 μL/min. Regeneration of the sensorchip surface was performed by incubating with 0.1 M H_3PO_4 for 2 minutes at a flow rate of 5 μL/min. Data obtained from steady state SPR analysis were used for the calculation of the apparent affinity constants ($K_{d,app}$), as described[40].

Immunoprecipitations

U937 cells (6 x 10^6) were stimulated for 15 minutes with PMA and were lysed in PBS containing 1% Nonidet-40 and 0.5% DOC at 4°C for 1 hour. Lysates were clarified by centrifugation and precleared by incubation with Protein G-Sepharose. The lysate was immunoprecipitated with anti-LRP or anti-α_M antibodies (1 μg/mL) and Protein A- or Protein G-Sepharose, respectively, for 16 hours at 4°C. Immunocomplexes were pelleted, washed, and resolved by sodium do-

decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Detergent-resistant membrane fractions were isolated as described[41] and immunocomplexes were prepared similarly.

Confocal scanning fluorescence microscopy

U937 cells or blood monocytes were collected on object glasses by cytospin centrifugation (10^5 cells/spin). Cells were fixed in 4% paraformaldehyde/PBS and were blocked in 2% BSA/0.1% saponin/PBS. Localization of the α_L subunit and LRP was detected using mouse anti-α_L (1:10) and goat anti-LRP (1:20) antibodies, followed by incubation with donkey anti-mouse (1:100) and donkey anti-goat (1:200) antibodies, which were labeled with TexasRed and FITC, respectively. Cells were mounted in mowiol containing 2.5% 1,4-diazabicyclo[2.2.2]octane. Cells were visualized using Leica DMIRB confocal scanning laser microscope equipped with a 63x/1.40 Plan APO objective lens and a TCS 4D system (Leica, Voorburg, The Netherlands).

Cell adhesion under flow conditions

HUVECs were coated on glass coverslips, grown until confluence, and stimulated with TNF-α (100 U/mL) for 4 hours before perfusion. Cells (2×10^6 cells/mL) were perfused over HUVECs for 10 minutes at a flow rate of 100 μL/min. Wall shear stress was calculated to be 0.8 dyne/cm^2. During perfusion, the flow chamber[42;43] was mounted on a microscope stage (Axiovert 25; Zeiss, Oberkochen, Germany), which was equipped with a black-and-white charge-coupled device videocamera (Sanyo, Osaka, Japan) and was coupled to a VHS videorecorder. Video images were evaluated for the number of adherent cells, with dedicated routines made in the image analysis software OPTIMAS 6. U937 cells (non-transfected and transfected with sense or antisense LRP-oligonucleotides) that were in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording.

Statistical analysis

All data are expressed as mean ± SD, unless stated otherwise. Between-group variations were examined using the Student's t test. A P value of less than .05 was considered statistically significant.

Results

Leukocytes adhere to immobilized LRP fragments

To investigate whether leukocytes express surface proteins that interact with LRP, adhesion of blood monocytes and PMNs, and of the monocytic line U937 to immobilized ligand-binding LRP fragments (ie, clusters II and IV) was determined. Freshly isolated monocytes were stimulated with PMA and were added to wells coated with cluster II or IV. Each recombinant fragment appeared to provide an adhesive surface for monocytes, as visualized by light microscopy (*Fig 1A*). Dose-dependence was subsequently tested by measuring endogenous phosphatase activity of adhered cells, which revealed that adhesion to clusters II and IV was cell number dependent (*Fig 1Bi*). In addition, adhesion to these LRP fragments was decreased by 74% and 71% in the presence of GST-RAP, respectively (*Fig 1Bii*). Similar data were obtained for PMNs (data not shown) and U937 cells (*Fig 1C*), indicating that leukocytes express LRP-binding elements at the surface.

Binding of U937 cells to LRP fragments involves β2-integrins

Binding of leukocytes to LRP cluster IV was examined in more detail using U937 cells. First, GST-RAP was observed to diminish the adhesion of U937 cells to cluster IV by 89% (*Fig 1C*). Activated leukocytes are characterized by the presence of active adhesion molecules, such as $\alpha_4\beta1$, $\alpha_V\beta3$, and β2-integrins. To assess the contribution of these integrins in the binding of U937 cells to LRP fragments, adhesion was examined in the presence of potential inhibitors. Anti–α_4-integrin antibodies and the $\alpha_V\beta3$-ligand vitronectin were unable to reduce adhesion (*Fig 1C*). In contrast, a β2-integrin-directed antibody inhibited the binding of stimulated U937 cells to cluster IV by 95% (*Fig 1C*). Given that β2-integrins consist of a heterodimeric complex with distinct α-subunits, we also examined the effect of antibodies directed against various α-subunits on cell adhesion. These antibodies decreased adhesion to cluster IV up to 77% (*Fig 1C*). Adhesion was also reduced in the presence of the β2-integrin ligand fibrinogen (*Fig 1C*). It should be noted that similar data were obtained when adhesion to cluster II was tested (data not shown). Thus, it appears that LRP provides a binding site for β2-integrins and that $\alpha_L\beta2$, $\alpha_M\beta2$, and $\alpha_X\beta2$ are able to associate with LRP.

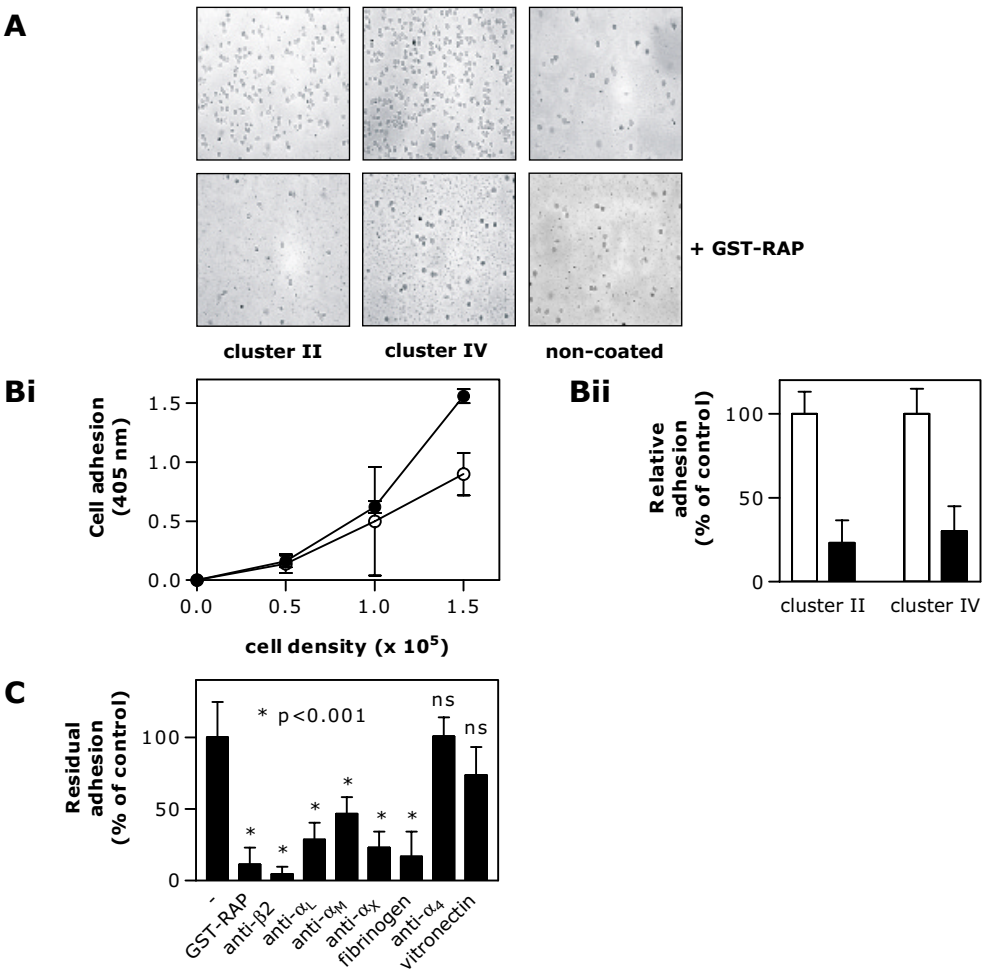


Figure 1. Adhesion of monocytes to LRP clusters II and IV. Freshly isolated monocytes were stimulated with 100 nM PMA for 15 minutes and added to immobilized LRP cluster II or IV for 30 minutes at room temperature in the presence or absence of LRP-antagonist GST-RAP (50 μg/mL). *A*. Typical experiment visualized by light microscopy, in which 1.5 x 10^5 cells were added to each well. Original magnification, 400x. *Bi*. To quantify cell adhesion, different amounts of cells (0-1.5 x 10^5 cells/well) were added. After incubation and subsequent washing, bound cells were lysed using 1% Triton-X100/50 mM acetic acid (pH 5.0), and endogenous alkalic phosphatase activity was determined using PNP as substrate. Open circles indicates LRP cluster II; closed circles LRP cluster IV. *Bii*. Relative adhesion in the presence (black bars) or absence (white bars) of GST-RAP. Data are corrected for adhesion to uncoated wells (less than 20% of cluster IV coated wells) and represent mean ± SD of 3 experiments performed in duplicate. *C*. PMA-stimulated U937 cells (1.5 x 10^6) were incubated with indicated antibodies (20 μg/mL), fibrinogen (50 μg/mL), or vitronectin (100 μg/mL) for 15 minutes, or wells were preincubated with GST-RAP (50 μg/mL) and added to immobilized IV for 60 minutes at 37°C. Adhered cells were detected as described in panel B. Presented is the percentage of adhesion relative to adhesion in the absence of antibodies or GST-RAP. Data represent the mean ± SD of 3 to 10 experiments performed in duplicate. ns indicates not significant ($p > .05$).

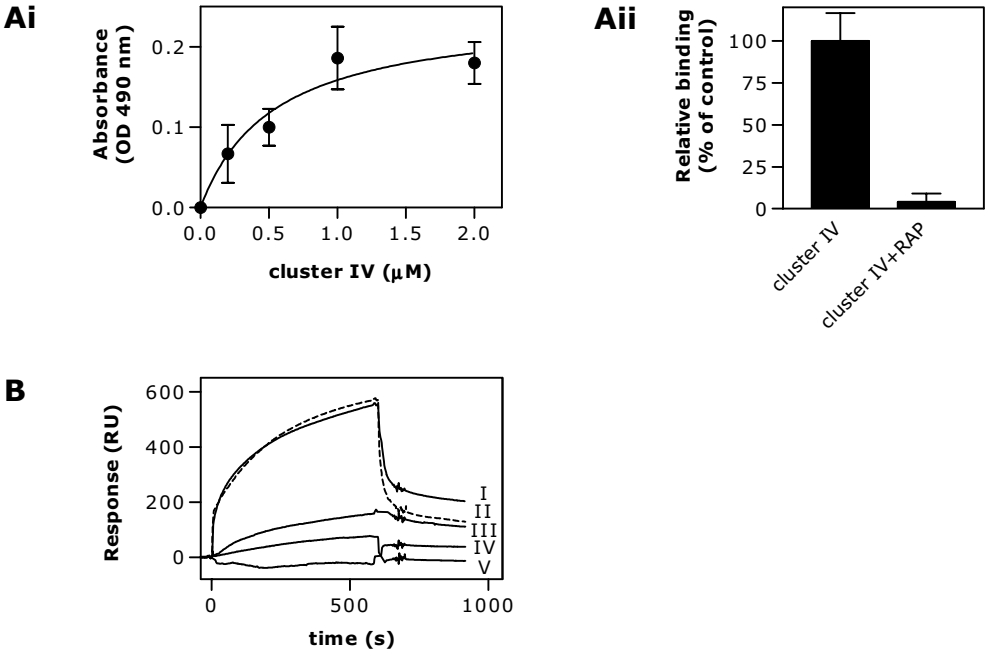


Figure 2. Complex formation between LRP and $\alpha_M\beta 2$. *Ai*. Purified recombinant cluster IV (0-2.0 μM) was incubated with immobilized $\alpha_M\beta 2$ complex (2.5 μg/well) in Tris-buffered saline/3 mM $CaCl_2$/1 mM $MnCl_2$ (pH 7.4) for 2 hours at 37°C. Bound cluster IV was subsequently determined using peroxidase-labeled polyclonal antibodies directed against cluster IV. Data represent mean ± SEM of 4 experiments. *Aii*. Binding of 200 nM cluster IV to immobilized $\alpha_M\beta 2$ in the presence or absence of a 10-fold excess of GST-RAP. *B*. 500 nM purified recombinant I-domain of the α_M- (line I) or the α_L-subunit (line II) were perfused over LRP immobilized onto a CM5-sensorchip (7.7 fmol/mm^2) in 100 mM NaCl, 0.005% Tween-20, 2 mM $CaCl_2$, 2 mM $MnCl_2$, 25 mM HEPES (pH 7.4) at a flow rate of 5 μL/min for 20 minutes at 25°C. Line III: Before perfusion, 500 nM α_M was preincubated with a 5-fold molar excess of fibrinogen for 30 minutes. Line IV: 5 μM fibrinogen. Line V: 500 nM GST. Ligand solution was replaced with buffer 10 minutes after injection to initiate dissociation. Depicted are sensorgrams corrected for aspecific binding, which was less than 5% of binding to LRP-coated channels.

LRP comprises a binding site for β2-integrins

To assess the interaction between LRP and β2-integrins at the level of purified proteins, binding of LRP cluster IV to the $\alpha_M\beta2$ complex was assessed in a qualitative manner using an immunosorbent assay. Various concentrations of LRP cluster IV (0-2 μM) were incubated with immobilized $\alpha_M\beta2$ (2.5 μg/well), and bound cluster IV was subsequently determined using polyclonal anti-cluster IV antibodies. Cluster IV bound to immobilized $\alpha_M\beta2$ in a dose-dependent and saturable manner, and half-maximum binding was observed at a concentration of 0.5 μM cluster IV (*Fig 2Ai*). Furthermore, binding of cluster IV to the immobilized complex could be blocked in the presence of GST-RAP (*Fig 2Aii*). In a second approach, we investigated whether the α-subunits of the $\alpha_L\beta2$ or $\alpha_M\beta2$ complex contribute to the interaction with LRP. Therefore, SPR analysis was performed using LRP and the recombinant I-domains of both subunits fused to GST. SPR analysis demonstrated that both I-domain/GST fusion proteins associate with immobilized full-length LRP in a reversible manner (*Fig 2B, lines I and II*), whereas GST alone did not bind to LRP (*Fig 2B, line* V). In addition, binding of the α_M I-domain to LRP was inhibited in the presence of the α_M ligand fibrinogen (*Fig 2B, line III*). Steady state analysis further indicated that the α_L and α_M I-domains both interact with LRP with an apparent affinity constant of 0.5 μM. These data demonstrate that β2-integrins directly interact with LRP and that binding is at least in part mediated by the α-subunits.

LRP and β2-integrins are targeted to detergent-insoluble membrane regions

To address the possibility that both receptors associate in the cellular environment, complex formation was assessed in immunoprecipitation experiments using U937 cells. With non-stimulated U937 cells, the β2-subunit was readily immunoprecipitated using an antibody against LRP but not an isotype-matched control antibody (*Fig 3A, lanes 1 and 2*). In turn, an anti-α_M antibody, but not a control antibody, was able to immunoprecipitate LRP (*Fig 3A, lanes 3 and 5*). LRP coprecipitated with an anti-α_M–integrin antibody to the same extent when cells were stimulated with PMA for 1 hour (*Fig 3A, lane 4*). Because leukocyte stimulation is associated with the redistribution of β2-integrins at the cell surface to cholesterol-enriched regions[44], we further examined the presence of LRP/β2-integrin complexes in such membrane fractions of resting and stimulated U937 cells. In detergent-insoluble fractions of non-stimulated cells, some coprecipitation of LRP with β2-integrins was observed (*Fig 3B, left 3 lanes*). An increase in coprecipitated LRP was found on stimulation of U937 cells with PMA (*Fig 3B, right 3 lanes*), suggesting that the LRP/β2-integrin complex is targeted to cholesterol-enriched membrane regions.

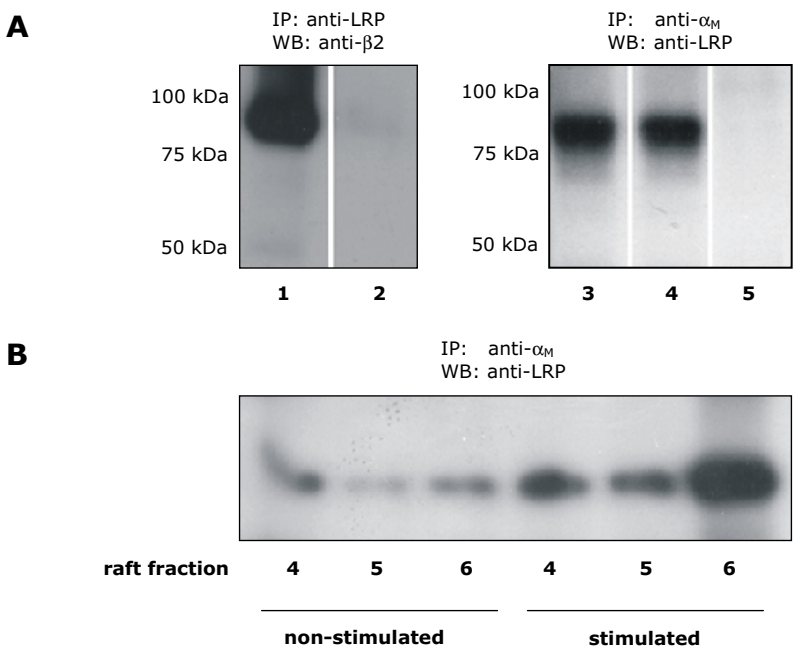


Figure 3. Coimmunoprecipitation of LRP and β2-integrins. *A*. U937 cells were lysed for 1 hour on ice and incubated with Protein A-Sepharose and anti-LRP clone α2-M-R-II2C7 (lane 1) or an isotype control (lane 2) at 4°C overnight. Beads were washed extensively and boiled to release bound proteins. Samples were analyzed by SDS-PAGE and Western blotting using anti-β2 (clone R2E7B) and peroxidase-conjugated rat anti-mouse. Non-stimulated (lane 3) or stimulated (lane 4) cells were lysed and incubated with protein G-Sepharose and an anti-α_M antibody (clone M1/70) (lanes 3 and 4) or an isotype control (lane 5). Precipitated proteins were analyzed by SDS-PAGE and Western blotting using anti-LRP antibody (clone α2-M-R-II4/8). *B*. Lipid rafts were isolated from non-stimulated or stimulated cells, as described elsewhere[41]. Immunoprecipitations were performed as described in the legend of Figure 3A.

Surface expression of $\alpha_L\beta 2$ is independent of LRP

The subcellular localization of endogenously expressed LRP and β2-integrins was further studied by confocal immunofluorescence microscopy. First, the presence of β2-integrins at the cell surface in blood-derived monocytes or the U937 cell line was confirmed using anti-α_L antibodies (*Fig 4A, D*). Similar surface staining was observed for LRP (*Fig 4B, E*), which overlapped with that of α_L to a significant extent (*Fig 4C, F*). To investigate whether the surface location of $\alpha_L\beta 2$-integrin was linked to that of LRP, U937 cells were prepared in which LRP expression was downregulated using LRP-specific phosphorothioate antisense oligodeoxynucleotides. LRP expression appeared to be unaffected when resting

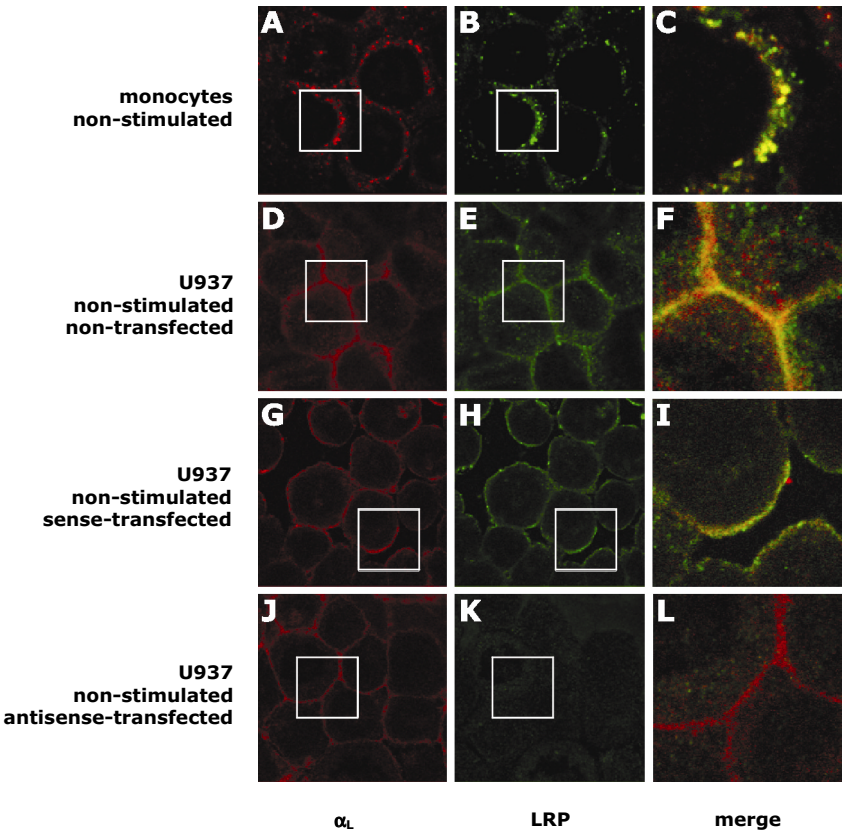


Figure 4. Surface expression of LRP and α_L-subunit in monocytes and U937 cells. Freshly isolated monocytes (*A-C*), non-transfected U937 cells (*D-F*), and U937 cells transfected with sense- (*G-I*) or antisense LRP oligonucleotides (*J-L*) were collected on object glasses by cytospin centrifugation (10^5 cells/spin). After fixation, α_L-subunit (*A, D, G, J*) was visualized using monoclonal antibodies and a TexasRed–labeled secondary antibody. LRP (*B, E, H, K*) was detected using polyclonal antibodies and a FITC-labeled secondary antibody. Original magnification, x 250. *C, F, I, L*. Enlarged merge images; original magnification, x 750. White boxes indicate the positions of the enlarged merge images.

cells were treated with sense oligodeoxynucleotides (*Fig 4H*). In contrast, a strong downregulation of LRP expression was observed in cells that were transfected with antisense LRP oligodeoxynucleotides (*Fig 4K*). Downregulation of LRP-surface expression was examined in a quantitative manner using flow cytometric analysis, which revealed that the amount of surface-exposed LRP was similar in non- and sense-transfected U937 cells, but reduced to background levels in antisense-transfected cells (*Fig 5A*). The expression of the α_L subunit at the cell surface remained unaffected in sense- and antisense-transfected cells (*Fig 4G, J*). Indeed, flow cytometric analysis showed similar mean fluorescence intensities for non-, sense-, and antisense-transfected U937 cells (*Fig 5B*). In addition, the surface expression of uPAR, which has the potential to associate with LRP and β2-integrins, remained unchanged on transfection of the U937 cells (*Fig 5C*). These data support the view that the amount of α_L expression at the cell surface is independent of the presence of LRP.

Reduced PMA-mediated clustering of α_L subunit in LRP-deficient cells

Because PMA-mediated stimulation results in the clustering of $\alpha_L\beta$2-integrins, we further addressed the effect of PMA stimulation on sense and antisense oligodeoxynucleotide-treated U937 cells. PMA stimulation was indeed associated with an increase in the density of the α_L subunit at the cell surface in sense-transfected cells (*Fig 6A*). A similar increase in density at indicated regions at the cell surface was observed for LRP (*Fig 6B and C*), suggesting that the translocation of α_L and LRP is linked to some extent. With regard to the antisense-transfected cells, no obvious increase in density was observed for the α_L subunit (*Fig 6D*), despite normal expression levels for this subunit. This suggests that clustering of β2-integrins is dependent on the presence of LRP.

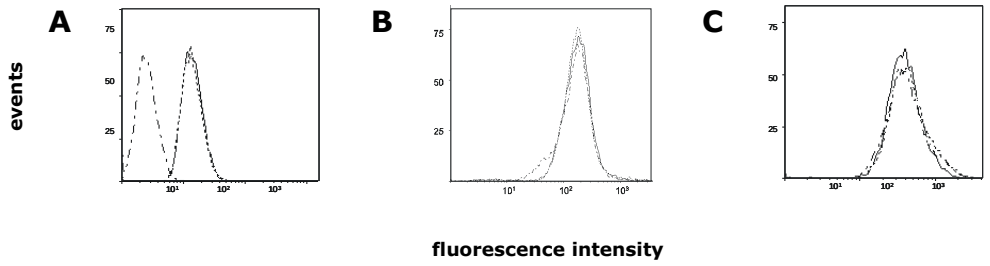


Figure 5. Cell surface expression of LRP, α_L, and uPAR. Flow cytometric analysis of the cell surface expression of LRP (*A*), α_L (*B*), and uPAR (*C*) in non- (—), sense- (....), and antisense- (._._.) transfected U937 cells.

Reduced LRP levels result in impaired β2-integrin-dependent adhesion to endothelial cells

β2-Integrin-dependent adhesion of leukocytes to the endothelium requires clustering of these integrins. Given that our microscopic analysis suggested that clustering of β2-integrins is LRP dependent, the effect of LRP deficiency on leukocyte adhesion to the endothelial surface was addressed. Therefore, the adhesion of normal U937 cells and U937 cells transfected with sense or antisense LRP oligos to HUVECs was compared. First, in a static adhesion assay using stimulated HUVECs as a surface, sense-transfected U937 cells were similar to non-transfected cells (*Fig 7A*). In contrast, a markedly reduced adhesion was observed for antisense-transfected cells (*Fig 7A*). This was further addressed in perfusion experiments. As shown in Figure 7B, U937 cells adhered efficiently to the HUVEC layer (474 ± 25 cells/mm^2; n=3). A similar amount of adhesion was detected for sense-transfected cells (463 ± 77 cells/mm^2; n=8; P > .05). Monoclonal antibodies directed against the β2-subunit or the LRP antagonist GST-RAP effectively interfered with the adhesion of sense-transfected cells (321 ± 6 cells/mm^2; n=3; P = .0005 and 287 ± 44 cells/mm^2; n=4; P = .0013, respectively). Thus, the adhesion of U937 cells to endothelial cells is a process that involves β2-integrins and LRP. With regard to antisense-transfected cells, lesser cells were observed to adhere to the endothelial surface compared with non- and sense-transfected cells (255 ± 66 cells/mm^2; n=8; P = .0004 compared with non-transfected cells). Moreover, no further decrease in adhesion was observed in the presence of either anti-β2 antibodies (270 ± 38 cells/mm^2; n=3; P = .0015 compared with non-transfected cells and P > .05 relative to antisense-transfected cells) or the LRP antagonist GST-RAP (241 ± 75 cells/mm^2; n=4; P = .0039 compared with non-transfected cells and P > .05 relative to antisense-transfected cells). In contrast, an additional decrease in adhesion was observed in the presence of an anti-α_4 antibody (153 ± 15 cells/mm^2; n=3; P = .003 compared with antisense-transfected cells). In conclusion, these data indicate that the absence of functional LRP is associated with reduced β2-integrin–dependent adhesion to the endothelial surface.

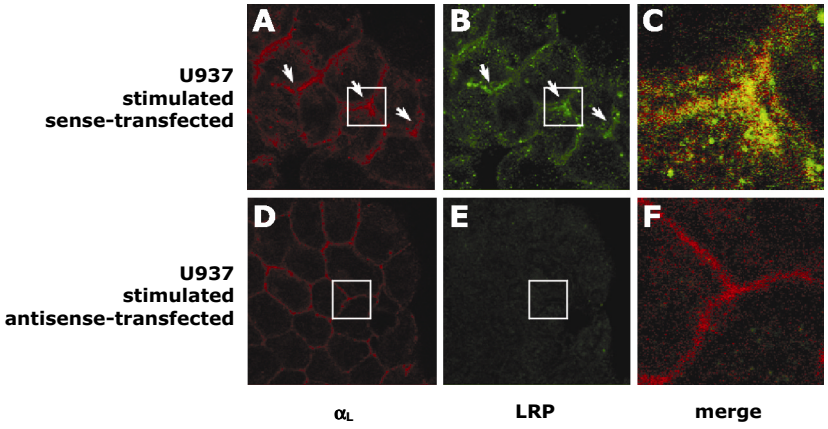


Figure 6. Effect of sense and antisense nucleotides on expression of LRP and α_L-subunit. U937 cells were transfected with sense (*A*-C) or antisense oligonucleotides (*D-F*) and were stimulated with 100 nM PMA for 15 minutes. Cells were collected on object glasses and stained for α_L and LRP, as described in the legend to Figure 4. Original magnification, x 250. *C and F*. Enlarged merge images; original magnification, x 1375. White boxes indicate the positions of the enlarged merge images.

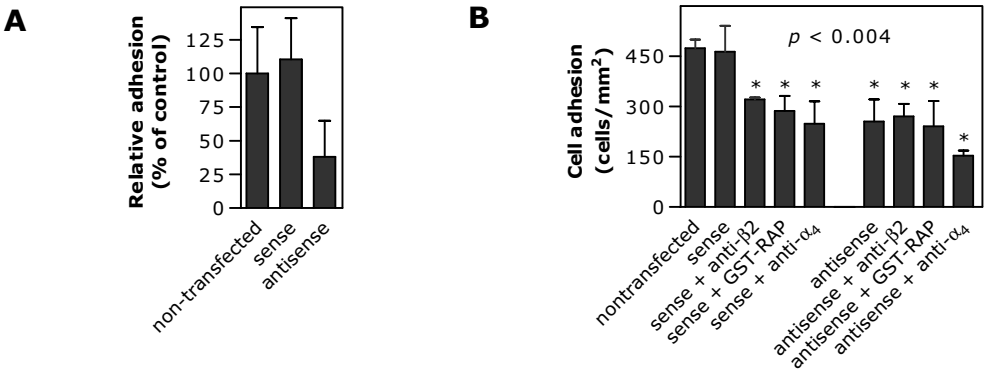


Figure 7. Adhesion of U937 cells to HUVECs under flow conditions. *A*. Non-, sense-, and antisense-transfected U937 cells were added to wells coated with HUVECs, which were stimulated with TNF-α (100 U/mL) for 4 hours and fixed afterward. Bound U937 cells were analyzed as described in the legend to Figure 1B. *B*. Non-, sense-, and antisense-transfected U937 cells (2 x 10^6 cells/mL) were perfused for 10 minutes with a wall shear stress of 0.8 dyne/cm^2 over a glass coverslip confluently covered with HUVECs, which were stimulated with TNF-α (100 U/mL) for 4 hours before perfusion. Where indicated, cells were preincubated with anti-β2-integrin or anti-α_4 antibodies (20 μg/mL) or GST-RAP (50 μg/mL) for 15 minutes. The number of firmly adhered cells per square millimeter was obtained from video-image analysis. Data represent the mean ± SD of 3 to 8 perfusions.

Discussion

Many receptors have evolved to fulfill one specific function, though a subset of receptors is known to display multispecificity and multifunctionality[45]. One such example is LRP, a member of the LDL-receptor family that, since its first description in 1988, has been classified as an endocytic receptor[1]. However, in the past few years, it has become evident that LRP function encompasses other processes as well. For instance, LRP expressed in brain vascular endothelial cells is involved in the regulation of the vascular tone and permeability of the blood-brain barrier[46], whereas LRP expressed in primary neurons mediates calcium signaling through N-methyl-D-aspartate receptors[12]. This suggests that the functionality of LRP is dependent on the cell type in which this receptor is expressed.

LRP is abundantly present in leukocytes[15;16], but its contribution to leukocyte function has remained poorly understood. In the present study, we obtained evidence that LRP is able to bind to leukocyte-specific β2-integrin complexes: 1) adhesion of the monocytic cell line U937 to recombinant fragments of LRP, i.e. clusters II and IV, could be inhibited by antibodies directed against β2-, α_L-, α_M-, or α_X-subunits (*Fig 1C*); 2) LRP and β2-integrins coprecipitated in immunoprecipitation experiments (*Fig 3*); 3) recombinant fragments of the α_M- and α_L-subunits interacted with full-length LRP (*Fig 2B*); 4) LRP cluster IV displayed binding to purified α_Mβ2-integrin (*Fig 2A*).

The integrin superfamily has been reported to comprise 18 different α-subunits and 8 different β-subunits, which can combine to make up to 24 different heterodimers. To the best of our knowledge, the β2-integrin subfamily is the first to be reported to bind LRP. It should be mentioned that LRP has recently been implicated to promote the maturation and intracellular trafficking of β1-integrins[47]. Furthermore, LRP has been reported to mediate endocytosis of complexes between plasminogen activator inhibitor-1 and the α_V-subunit[48]. However, no evidence of direct interaction between these integrin subunits and LRP could be obtained in these studies. Indeed, we could not detect any inhibition of cell adhesion to LRP fragments in the presence of inhibitors of α_4β1 or α_Vβ3 (*Fig 1C*).

β2-Integrins and LRP are transmembrane proteins containing cytoplasmic and extracellular domains. Our results obtained from cell adhesion and protein-interaction assays demonstrate that at least some of the interactive sites are located in the cluster II and IV regions of LRP and the I-domain of the α-subunits (*Fig 1 and 2*), both of which are part of the respective extracellular regions. Cell adhesion was also inhibited in the presence of the anti-β2 antibody. It seems reasonable to assume that this subunit is involved in complex assembly as well. Alternatively, antibody binding to the β2-subunit may prevent binding to

the complementary subunit by sterical hindrance. With regard to the cytoplasmic regions of the receptors, it is unclear whether they are involved in complex formation. This aspect is currently under investigation.

We considered the possibility that LRP is involved in the removal of β2-integrins from the cell surface. As such, the amount of β2-integrins expressed at the cell surface would be increased on the downregulation of LRP. However, examination of α_L (*Fig 5B*) or α_M (data not shown) surface expression using flow cytometric analysis revealed that sense- and antisense-transfected monocytic U937 cells display a fluorescence response similar to that of non-transfected cells. Apparently, the absence of LRP leaves the amount of α_L or α_M at the cell surface unaffected, suggesting another function for the interaction between LRP and β2-integrins. Data obtained from immunoprecipitation experiments (*Fig 3A*) and confocal microscopy (*Fig 4*) point to the possibility that β2-integrins and LRP form a complex at the cell surface. Indeed, we observed not only a cooperative clustering of both receptors (*Fig 6*), but also an increase of β2-integrin/LRP complex in detergent-insoluble membrane fractions (*Fig 3B*). The notion that both receptors have the potential to be present in detergent-resistant membrane fractions is in line with previous reports showing the presence of β2-integrins and LRP in lipid rafts of leukocytes and smooth muscle cells, respectively[13;44].

Recently, one of us reported that β2-integrins associate with matrix metalloproteases[49] and that this interaction is critical for β2-integrin-dependent leukocyte migration. This indicates that β2-integrins have the potential to form supramolecular complexes that are of functional importance. We have addressed this possibility for the LRP/β2-integrin complex by testing β2-dependent adhesion to stimulated HUVECs. Adhesion of U937 cells to HUVECs was reduced by almost 50% in the presence of anti-β2 subunit antibodies and to the same extent in the presence of GST-RAP (*Fig 7A*). Moreover, a similar reduction in adhesion was observed for LRP-deficient cells, and this reduced adhesion remained unchanged in the presence of anti-β2-integrin antibodies or GST-RAP. It should be noted that the residual adhesion observed could reflect adhesion mediated by selectins and $\alpha_4\beta1$-integrins. Indeed, the adhesion of LRP-expressing and -deficient cells was significantly decreased in the presence of anti-α_4-antibodies (*Fig 7B*). Nevertheless, our data demonstrate that the absence or inhibition of LRP abrogates β2-integrin-dependent cell adhesion. Thus, it seems conceivable that LRP and β2-integrins form a functionally important complex at the leukocyte surface.

Of importance in this regard is the notion that LRP and β2-integrins may form complexes with other receptors as well. For instance, LRP has been shown to be involved in the regulation of uPAR surface expression, whereas urokinase plas-

minogen activator receptor (uPAR) contributes to β2-integrin function. As such, some of our observations could be explained by a model in which the absence of LRP modulates the surface expression of uPAR, which in turn could affect β2-integrin-dependent adhesion. Thus, LRP and β2-integrins could be part of a larger complex in which uPAR acts as an intermediate between both receptors. However, several observations argue against such a model. First, it has been established that binding of uPAR to LRP requires the presence of the uPA/PAI-1 complex[50]. In agreement with this notion, we were unable to detect coprecipitation between LRP and uPAR in immunoprecipitation experiments (data not shown). Second, the surface expression of uPAR was unaffected in sense- or antisense-transfected cells (*Fig 5C*). In view of our experimental data, it seems reasonable to assume that complex formation between LRP and β2-integrins is independent of uPAR.

The β2-integrins are known to recognize a variety of ligands to facilitate leukocyte adhesion to a broad range of surfaces. The main ligands are the ICAM molecules, but also fibrinogen and the platelet glycoprotein Ib/IX/V receptor may provide adhesive surfaces for these integrins. The interaction with these counter-receptors involves the I-domain of α_M[34], the same domain that contains a binding site for LRP. Because β2-integrin-dependent leukocyte adhesion to these ligands is obviously not hampered by the presence of LRP, the possibility exists that the LRP-binding site is distinct from those of ICAMs, fibrinogen, and glycoprotein Ib. Alternatively, the relatively low affinity for LRP may allow easy dissociation by ICAMs, fibrinogen, or glycoprotein Ib, provided that they display higher affinity for α_M than LRP. This alternative explanation seems favorable in view of our observation that fibrinogen was found to interfere with the binding of the α_M I-domain to LRP (*Fig 2B*). Thus, it seems conceivable that LRP is required to position β2-integrins appropriately to facilitate the interaction with counter-receptors such as fibrinogen. This "cis" interaction, however, does not exclude the possibility that β2-integrins and LRP may interact in "trans" or, in other words, that β2-integrins exposed on the surface of one cell interact with LRP on the surface of another cell. This possibility is supported by our experiments that show effective leukocyte adhesion to purified LRP fragments (*Fig 1*).

In conclusion, the present study provides evidence for a previously unrecognized link between LRP and the inflammatory system. Our data point to a model in which LRP is a regulator of β2-integrin function, because LRP deficiency abrogates β2-dependent cell adhesion. The possibility that LRP also contributes to other β2-integrin-dependent functions, such as phagocytosis and migration, remains to be investigated.

Acknowledgements

This study was supported by a grant from the Dutch Organization of Scientific Research ZonMW (no. 902-26-236) to P.J.L.

References

1. Herz, J., U. Hamann, S. Rogne, O. Myklebost, H. Gausepohl, and K.K. Stanley. 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 J.* 7:4119-4127.
2. Herz, J., R.C. Kowal, J.L. Goldstein, and M.S. Brown. 1990. Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *EMBO J.* 9:1769-1776.
3. Willnow, T.E., K. Orth, and J. Herz. 1994. Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *J.Biol.Chem.* 269:15827-15832.
4. Neels, J.G., B.M. van den Berg, A. Lookene, G. Olivecrona, H. Pannekoek, and A.J. van Zonneveld. 1999. The second and fourth cluster of class A cysteine-rich repeats of the low density lipoprotein receptor-related protein share ligand-binding properties. *J.Biol.Chem.* 274:31305-31311.
5. Herz, J. and D.K. Strickland. 2001. LRP: a multifunctional scavenger and signaling receptor. *J.Clin.Invest.* 108:779-784.
6. Li, Y., M.P. Marzolo, P. van Kerkhof, G.J. Strous, and G. Bu. 2000. The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. *J.Biol.Chem.* 275:17187-17194.
7. Gliemann, J., A. Nykjaer, C.M. Petersen, K.E. Jorgensen, M. Nielsen, P.A. Andreasen, E.I. Christensen, A. Lookene, G. Olivecrona, and S.K. Moestrup. 1994. The multiligand alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein (alpha 2MR/LRP). Binding and endocytosis of fluid phase and membrane-associated ligands. *Ann.N.Y.Acad.Sci.* 737:20-38.
8. Demeule,M., J. Poirier, J. Jodoin, Y. Bertrand, R.R. Desrosiers, C. Dagenais, T. Nguyen, J. Lanthier, R. Gabathuler, M. Kennard, W.A. Jefferies, D. Karkan, S. Tsai, L. Fenart, R. Cecchelli, and R. Beliveau. 2002. High transcytosis of melanotransferrin (P97) across the blood-brain barrier. *J.Neurochem.* 83:924-933.
9. Shibata, Y., T. Muramatsu, M. Hirai, T. Inui, T. Kimura, H. Saito, L.M. McCormick, G. Bu, and K. Kadomatsu. 2002. Nuclear targeting by the growth factor midkine. *Mol.Cell Biol.* 22:6788-6796.
10. Willnow, T.E., A. Nykjaer, and J. Herz. 1999. Lipoprotein receptors: new roles for ancient proteins. *Nat.Cell Biol.* 1:E157-E162.
11. Gotthardt, M., M. Trommsdorff, M.F. Nevitt, J. Shelton, J.A. Richardson, W. Stockinger, J. Nimpf, and J. Herz. 2000. Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. *J.Biol.Chem.* 275:25616-25624.
12. Bacskai, B.J., M.Q. Xia, D.K. Strickland, G.W. Rebeck, and B.T. Hyman. 2000. The endocytic receptor protein LRP also mediates neuronal calcium signaling via N-methyl-D-aspartate receptors. *Proc.Natl.Acad.Sci.U.S.A* 97:11551-11556.
13. Boucher, P., P. Liu, M. Gotthardt, T. Hiesberger, R.G. Anderson, and J. Herz. 2002. Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low density lipoprotein receptor-related protein in caveolae. *J.Biol.Chem.* 277:15507-15513.
14. Loukinova, E., S. Ranganathan, S. Kuznetsov, N. Gorlatova, M.M. Migliorini, D. Loukinov, P.G. Ulery, I. Mikhailenko, D.A. Lawrence, and D.K. Strickland. 2002. Platelet-derived growth factor

(PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). Evidence for integrated co-receptor function between LRP and the PDGF receptor. *J.Biol.Chem.* 277:15499-15506.

15. Moestrup, S.K., J. Gliemann, and G. Pallesen. 1992. Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res.* 269:375-382.
16. Moestrup, S.K., K. Kaltoft, C.M. Petersen, S. Pedersen, J. Gliemann, and E.I. Christensen. 1990. Immunocytochemical identification of the human alpha 2-macroglobulin receptor in monocytes and fibroblasts: monoclonal antibodies define the receptor as a monocyte differentiation antigen. *Exp.Cell Res.* 190:195-203.
17. Gahmberg, C.G., M. Tolvanen, and P. Kotovuori. 1997. Leukocyte adhesion-structure and function of human leukocyte beta2-integrins and their cellular ligands. *Eur.J.Biochem.* 245:215-232.
18. Diamond, M.S., J. Garcia-Aguilar, J.K. Bickford, A.L. Corbi, and T.A. Springer. 1993. The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. *J.Cell Biol.* 120:1031-1043.
19. Shimaoka, M., J. Takagi, and T.A. Springer. 2002. Conformational regulation of integrin structure and function. *Annu.Rev.Biophys.Biomol.Struct.* 31:485-516.
20. Dickeson, S.K. and S.A. Santoro. 1998. Ligand recognition by the I domain-containing integrins. *Cell Mol.Life Sci.* 54:556-566.
21. Gahmberg, C.G. 1997. Leukocyte adhesion: CD11/CD18 integrins and intercellular adhesion molecules. *Curr.Opin.Cell Biol.* 9:643-650.
22. Hermand, P., M. Huet, I. Callebaut, P. Gane, E. Ihanus, C.G. Gahmberg, J.P. Cartron, and P. Bailly. 2000. Binding sites of leukocyte beta 2 integrins (LFA-1, Mac-1) on the human ICAM-4/LW blood group protein. *J.Biol.Chem.* 275:26002-26010.
23. Tian, L., Y. Yoshihara, T. Mizuno, K. Mori, and C.G. Gahmberg. 1997. The neuronal glycoprotein telencephalin is a cellular ligand for the CD11a/CD18 leukocyte integrin. *J.Immunol.* 158:928-936.
24. Ostermann,G., K.S.Weber, A.Zernecke, A.Schroder, and C.Weber. 2002. JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat.Immunol.* 3:151-158.
25. Santoso, S., U.J. Sachs, H. Kroll, M. Linder , A. Ruf, K.T. Preissner, and T. Chavakis. 2002. The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. *J.Exp.Med.* 196:679-691.
26. Ehlers, R., V. Ustinov, Z. Chen, X. Zhang, R. Rao, F.W. Luscinskas, J. Lopez, E. Plow, and D.I. Simon. 2003. Targeting platelet-leukocyte interactions: identification of the integrin Mac-1 binding site for the platelet counter receptor glycoprotein Ibalpha. *J.Exp.Med.* 198:1077-1088.
27. Simon, D.I., Z. Chen, H. Xu, C.Q. Li, J. Dong, L.V. McIntire, C.M. Ballantyne, L. Zhang, M.I. Furman, M.C. Berndt, and J.A. Lopez. 2000. Platelet glycoprotein Ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J.Exp.Med.* 192:193-204.
28. Hoffmeister, K.M., E.C. Josefsson, N.A. Isaac, H. Clausen, J.H. Hartwig, and T.P. Stossel. 2003. Glycosylation restores survival of chilled blood platelets. *Science* 301:1531-1534.
29. Harris, E.S., T.M. McIntyre, S.M. Prescott, and G.A. Zimmerman. 2000. The leukocyte integrins. *J.Biol.Chem.* 275:23409-23412.
30. Springer, T.A. 1995. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu.Rev.Physiol.* 57:827-872.
31. Nortamo, P., M. Patarroyo, C. Kantor, J. Suopanki, and C.G. Gahmberg. 1988. Immunological mapping of the human leucocyte adhesion glycoprotein gp90 (CD18) by monoclonal antibodies. *Scand.J.Immunol.* 28:537-546.
32. Herz, J., J.L. Goldstein, D.K. Strickland, Y.K. Ho, and M.S. Brown. 1991. 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *J.Biol.Chem.* 266:21232-21238.

33. Lenting, P.J., J.G. Neels, B.M. van den Berg, P.P. Clijsters, D.W. Meijerman, H. Pannekoek, J.A. van Mourik, K. Mertens, and A.J. van Zonneveld. 1999. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *J.Biol.Chem.* 274:23734-23739.
34. Ihanus, E., L. Uotila, A. Toivanen, M. Stefanidakis, P. Bailly, J.P. Cartron, and C.G. Gahmberg. 2003. Characterization of ICAM-4 binding to the I domains of the CD11a/CD18 and CD11b/CD18 leukocyte integrins. *Eur.J.Biochem.* 270:1710-1723.
35. Yatohgo, T., M. Izumi, H. Kashiwagi, and M. Hayashi. 1988. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct.Funct.* 13:281-292.
36. Jaffe, E.A., R.L. Nachman, C.G. Becker, and C.R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J.Clin.Invest.* 52:2745-2756.
37. Willems, C., G.C. Astaldi, P.G. de Groot, M.C. Janssen, M.D. Gonsalvez, W.P. Zeijlemaker, J.A. van Mourik, and W.G. van Aken. 1982. Media conditioned by cultured human vascular endothelial cells inhibit the growth of vascular smooth muscle cells. *Exp.Cell Res.* 139:191-197.
38. Sundstrom, C. and K. Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int.J.Cancer* 17:565-577.
39. Llorente-Cortes, V., J. Martinez-Gonzalez, and L. Badimon. 2000. LDL receptor-related protein mediates uptake of aggregated LDL in human vascular smooth muscle cells. *Arterioscler.Thromb.Vasc.Biol.* 20:1572-1579.
40. Lenting, P.J., E. Westein, V. Terraube, A.S. Ribba, E.G. Huizinga, D. Meyer, P.G. de Groot, and C.V. Denis. 2004. An experimental model to study the in vivo survival of von Willebrand factor. Basic aspects and application to the R1205H mutation. *J.Biol.Chem.* 279:12102-12109.
41. Heijnen, H.F., M. van Lier, S. Waaijenborg, Y. Ohno-Iwashita, A.A. Waheed, M. Inomata, G. Gorter, W. Mobius, J.W. Akkerman, and J.W. Slot. 2003. Concentration of rafts in platelet filopodia correlates with recruitment of c-Src and CD63 to these domains. *J.Thromb.Haemost.* 1:1161-1173.
42. Sakariassen, K.S., P.A. Aarts, P.G. de Groot, W.P. Houdijk, and J.J. Sixma. 1983. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. *J.Lab Clin.Med.* 102:522-535.
43. van Zanten, H., E.U. Saelman, K.M. Schut-Hese, Y.P. Wu, P.J. Slootweg, H.K. Nieuwenhuis, P.G. de Groot, and J.J. Sixma. 1996. Platelet adhesion to collagen type IV under flow conditions. *Blood* 88:3862-3871.
44. Krauss, K. and P. Altevogt. 1999. Integrin leukocyte function-associated antigen-1-mediated cell binding can be activated by clustering of membrane rafts. *J.Biol.Chem.* 274:36921-36927.
45. Krieger, M. and D.M. Stern. 2001. Series introduction: multiligand receptors and human disease. *J.Clin.Invest.* 108:645-647.
46. Yepes, M., M. Sandkvist, E.G. Moore, T.H. Bugge, D.K. Strickland, and D.A. Lawrence. 2003. Tissue-type plasminogen activator induces opening of the blood-brain barrier via the LDL receptor-related protein. *J.Clin.Invest.* 112:1533-1540.
47. Salicioni, A.M., A. Gaultier, C. Brownlee, M.K. Cheezum, and S.L. Gonias. 2004. Low density lipoprotein receptor-related protein-1 promotes beta 1 integrin maturation and transport to the cell surface. *J.Biol.Chem.* 279:10005-10012.
48. Czekay, R.P., K. Aertgeerts, S.A. Curriden, and D.J. Loskutoff. 2003. Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. *J.Cell Biol.* 160:781-791
49. Stefanidakis, M., M. Bjorklund, E. Ihanus, C.G. Gahmberg, and E. Koivunen. 2003. Identification of a negatively charged peptide motif within the catalytic domain of progelatinases that mediates binding to leukocyte beta 2 integrins. *J.Biol.Chem.* 278:34674-34684.
50. Czekay, R.P., T.A. Kuemmel, R.A. Orlando, and M.G. Farquhar. 2001. Direct binding of occupied urokinase receptor (uPAR) to LDL receptor-related protein is required for endocytosis of uPAR and regulation of cell surface urokinase activity. *Mol.Biol.Cell* 12:1467-1479.

chapter 3

Differential localization of LDL receptor-related protein / CD91 in lymphocytic subsets

Patricia P.E.M. Spijkers, Alexander M. de Bruin, Lucy M. Goossens-Leverink and Peter J. Lenting

Laboratory for Thrombosis and Haemostasis, Department of Haematology, University Medical Center Utrecht, Utrecht, The Netherlands

Submitted

Abstract

LDL receptor-related protein (LRP), also known as CD91, is a multifunctional protein that is expressed in a broad range of cells, including leukocytes. For instance, we recently demonstrated the requirement of LRP for β2-integrin function in leukocytes [Spijkers et al, 2005]. Whereas LRP expression and function in monocytes has been well established, little is known about LRP expression in lymphocytes. The present study was aimed to elucidate LRP expression patterns in various lymphocytic subsets. The presence of LRP mRNA and protein could be detected in B-lymphocytes, natural killer (NK) cells and T-lymphocytes as analyzed by RT-PCR, Western blotting, flow cytometry and confocal microscopy. LRP is predominantly present at the cell surface of B-lymphocytes and NK cells, but it was found selectively inside the cell in >95% of the CD4+ and CD8+ T-lymphocytes. This let us to hypothesize that LRP may become redistributed to the cell surface upon T-lymphocyte activation. This was first tested by stimulating T-lymphocytes using interleukin-2 and phytohemagglutinin. Interleukin-2/phytohemagglutinin stimulation resulted in a transient increase of cell-surface-located LRP protein. After 48 h, the number of T-lymphocytes containing LRP at the cellular surface was increased from 2% till 12%, which was followed by a gradual decline to baseline levels in the subsequent 5 days. In a second approach, changes in LRP-surface expression were monitored by mixing peripheral blood mononuclear cells derived from two different donors. This resulted in a near immediate translocation of LRP to the cell-surface in 60-80% of the T-lymphocytes. By using isolated cells, we found that this translocation occurred exclusively in the presence of CD14+ monocytes. The observation that LRP can be rapidly recruited to the cellular surface in T-lymphocytes upon interaction with non-autologous monocytes may point to a role of LRP in the pathophysiology of transfusion-related complications, such as graft-versus-host disease.

Introduction

The LDL-receptor related protein (LRP), also known as α_2-macroglobulin receptor or CD91, is a multiligand receptor of the LDL receptor family[1]. It is a heterodimeric protein consisting of an 85-kDa cytoplasmic and transmembrane domain, and a non-covalently linked 515-kDa extracellular domain[2]. The extracellular domain comprises four clusters of complement-type repeats[3;4] that mediate the binding of over 35 structurally and functionally unrelated ligands (for review see Herz and Strickland[5]). The cytoplasmic tail contains motifs for binding of numerous adaptor proteins implicated in endocytosis and signaling pathways[6;7].

The physiological function of LRP has initially been reported to be related to the endocytic process in order to mediate the cellular uptake of its ligands from the circulation or from the cell membrane[8;9]. LRP is also implicated in various other transport pathways, such as transcytosis across the blood-brain barrier[10;11] and nuclear localization of the growth factor midkine[12]. Furthermore, LRP is involved in cellular signal transduction pathways leading to cell proliferation, migration, neurotransmission and others[13]. One intriguing aspect of LRP is that it exerts functions that are highly cell-type restricted. For example, LRP regulates platelet-derived growth factor-induced proliferation and migration specifically in smooth muscle cells through complex formation with the platelet-derived growth factor receptor[14]. In neurons, LRP associates with N-methyl-D-aspartate receptor, leading to calcium influx[15]. Thus, LRP appears to form heterologous receptor-complexes that control cellular processes.

LRP is expressed in a large spectrum of cell types, including hepatocytes, fibroblasts, adipocytes and astrocytes[16]. Concerning leukocytes, LRP is known to be present in polymorphonuclear cells (PMNs), monocytes and a subset of dendritic cells[16-19]. Several studies point to essential physiological functions of LRP in these leukocytes: 1) the cytoplasmic tail of LRP is an essential element in the phagocytosis in macrophages[20;21]; 2) LRP acts as a cofactor in 12/15 lipoxygenase-dependent LDL oxidation in macrophages[22]; 3) LRP mediates uptake of heat shock protein / peptide complexes in dendritic cells[19]; 4) LRP is required for β2-integrin-dependent leukocyte adhesion[23].

In view of its versatility, it is conceivable that LRP also contributes to functions in lymphocytes. However, conflicting information exists with regard to LRP expression in these cells. Moestrup et al were unable to detect LRP in T-lymphocytes when assessed by immunohistochemistry[16]. In contrast, Gläser and coworkers reported the presence of LRP in these cells based on *in situ* hybridization and immunohistochemistry[24]. Apparently, whether or not LRP is present in T-lymphocytes is still inconclusive. With regard to other lymphocytic cells, like B-

lymphocytes and natural killer (NK) cells, no information is available to the best of our knowledge.

Therefore, the aim of the present study was to elucidate the LRP expression pattern in lymphocytes. Our data show that LRP mRNA and protein is present in B-lymphocytes, NK cells and T-lymphocytes. LRP protein is predominantly located at the cell surface of B-lymphocytes and NK cells, whereas it is stored intracellularly in T-lymphocytes. In these cells, near immediate translocation of LRP to the cell surface occurs in a mixed lymphocyte reaction. Our data show that LRP is differentially expressed at the cell membrane of lymphocytes, suggesting distinct functions for LRP in these leukocytes.

Experimental Procedures

Materials

Ficoll-Paque was from Amersham Biosciences (Uppsala, Sweden). MicroBeads-conjugated antibodies and MiniMACS were from Miltenyi Biotec (Bergisch Gladbach, Germany). All antibodies against CD-markers were obtained from BD Biosciences (San Jose, CA), anti-LRP 85-kDa from RDI (Flanders, NJ), fluorescein isothiocyanate (FITC)-conjugated mouse anti-LRP clone α2-M-R-I4C2 from Biomac (Leipzig, Germany), peroxidase-conjugated rabbit anti-mouse IgG from DAKO (Glostrup, Denmark). RPMI 1640 and penicillin/streptomycin were from Gibco Life Technologies (Paisley, UK), fetal bovine serum from Cambrex (Verviers, Belgium), interleukin-2 (IL-2) from Chiron (Emeryville, CA) and phytohaemagglutinin (PHA) from Remel (Lenexa, KS).

Lymphocyte isolation

Anticoagulated blood was collected from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density centrifugation as instructed by the manufacturer. The following cells were isolated: total T-lymphocytes (CD3+), T-helper cells (CD3+CD4+), cytotoxic T-lymphocytes (CD3+CD8+), NK cells (CD16+CD56+) and B-lymphocytes (CD19+). CD3+ T-lymphocytes were isolated by magnetic cell sorting using the Pan T-cell isolation kit. Other lymphocyte subsets were incubated with an antibody against a specific CD-marker and subsequently with MicroBeads-conjugated anti-mouse IgG, and isolated using magnetic cell sorting. Peripheral blood monocytes were isolated using CD14-MicroBeads. For all cell isolations, a MiniMACS cell separator was used. Cell purity appeared to be greater than 95% for all isolations, as examined by flow cytometry.

Culturing of T-lymphocytes

PBMCs were isolated using Ficoll-Paque and respuspended at 1 x 10^6 cells/ml in RPMI, 10% FCS, 1% penicillin/streptomycin and 50 μM β-mercapto-ethanol. 300 U/ml IL-2 and 1 μg/ml PHA were added to the cell suspension and cells were cultured in a humidified CO_2 (5%) incubator at 37ºC for 7 days. Cells were kept in culture at a density of 0.5 x 10^6 cells/ml. Activated T-lymphocytes were characterized by their CD25 expression. LRP levels were measured at day 0, 1, 2, 4 and 7.

RT-PCR analysis

Total RNA was purified from isolated leukocytes by means of the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After a cDNA synthesis step, a PCR was performed using Amplitaq DNA polymerase (Roche, Basel, Switzerland) and the following primers: forward primer 5' GAACGAGCCAGTAGACCG 3' and reverse primer 5' CAATCTTGCTGTCGACGA 3'. The forward primer spans exon 5 nucleotide 125 to exon 6 nucleotide 13; the reverse primers spans exon 8 nucleotide 78-85. 30 cycles of cDNA amplification were performed with annealing temperature of 62ºC. This reaction resulted in a specific LRP product of 527 bp. To confirm the identity of the product, the cDNA product was cloned into a TOPO vector (Invitrogen, Carlsbad, CA) and subsequently sequenced, or digested using BglI. This resulted in cleavage of the 527 bp band into two bands of 305 and 222 bp.

Confocal scanning laser microscopy

Purified lymphocytes were collected on glass coverslips by cytospin centrifugation (10^5 cells/spin). Cells were fixed by 4% paraformaldehyde (PFA) in PBS. Cells were washed extensively and free aldehyde groups were quenched using 50 mM NH_4Cl in PBS. Cells were optionally permeabilized with 0.1% saponin. Subsequently, cells were stained with FITC-conjugated mouse anti-LRP (Biomac) and mounted in mowiol containing 2.5% 1,4-diazabicyclo[2.2.2]octane. Cells were visualized using a Leica confocal scanning laser microscope and a TCS 4D system.

Flow cytometric analysis

PBMCs were stained with FITC-conjugated anti-LRP and antibodies against different cell markers. For total cell staining, cells were first fixed in 4% PFA and permeabilized using 0.1% saponin. Lymphocytes were identified in the scatter plot by their CD-marker staining and analyzed for their LRP expression at the cell surface and/or intracellular.

Mixed lymphocyte reaction

Either PBMCs or isolated T-lymphocytes and monocytes from 2 different donors were mixed during 30 minutes (0.5 x 10^6 cells from donor 1 + 0.5 x 10^6 cells from donor 2) and fixed with 4% PFA. Cells were stained for their specific CD-marker and LRP and subsequently analyzed by flow cytometry. Using isolated cells, cells were selected by their CD-marker and passed twice over MS columns to ensure ultrapure populations.

Results

LRP mRNA is present in lymphocytic subsets

In order to investigate LRP expression by lymphocytes, various lymphocytic subsets were analyzed for the presence of LRP mRNA and protein. First, RNA was purified from isolated CD4+ T-helper cells, CD8+ cytotoxic T-lymphocytes, CD16+CD56+ NK cells and CD19+ B-lymphocytes. Primers specifically detecting LRP mRNA were subsequently used in an RT-PCR reaction to generate a 527 bp product. This LRP-specific product could indeed be obtained from RNA isolated from the monocytic cell line THP-1 and freshly isolated monocytes (*Fig 1A*). Analysis of mRNA content in CD3+ lymphocytes showed an identical 527 bp band. Further purification of the CD3+ T-lymphocytes obtaining CD4+ and CD8+ cells revealed that both T-lymphocytic subsets expresses LRP mRNA. Two other lymphocytic cell types, B-lymphocytes (CD19+) and NK cells (CD16+CD56+) also appeared to contain LRP mRNA. To confirm the identity of the 527 bp band, restriction site analysis using BglI results in specific digestion of the 527 bp into 2 fragments of 305 and 222 bp (*Fig 1B*). Also, sequence analysis showed that the obtained PCR products encoded for LRP cDNA (data not shown). Thus, LRP mRNA seems to be produced in each of these lymphocyte subsets.

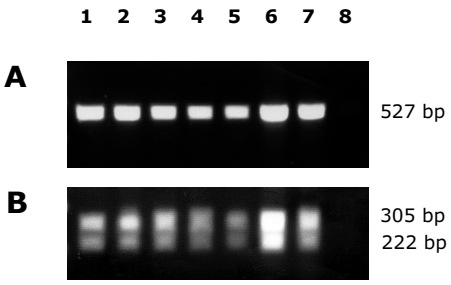


Figure 1. LRP mRNA expression in lymphocytes. *A.* Lymphocytes were isolated from the PBMCs fraction after Ficoll-Paque density centrifugation. After total RNA isolation, an RT-PCR was performed using equal amounts of RNA and specific primers for LRP. PCR products were visualized on an ethidium-bromide stained agarose gel. The length of the expected LRP fragment was 527 bp. *B.* The obtained PCR product was digested using Bgl1, resulting in cleavage of the 527 bp product in a 305 bp and a 222 bp fragment. 1: CD3, 2: CD4, 3: CD8, 4: CD16, 5: CD19, 6: CD14, 7: THP-1, 8: no template.

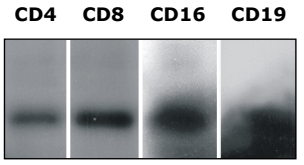


Figure 2. Western blot analysis of LRP expression. Lymphocytes were isolated and lysed in 1% NP-40 and 0.5% DOC in PBS. Samples were resolved on SDS-PAGE and subsequently transferred onto a PVDF membrane. Blots were incubated with anti-LRP 85-kDa (RDI, 1:50) and peroxidase-conjugated rabbit anti-mouse IgG (DAKO, 1:1000).

LRP protein is differentially expressed at the cell surface lymphocytes

To test whether these cells also contain LRP protein, two experimental approaches were employed to detect LRP protein in lymphocytes: 1) Western blotting using an antibody directed against the 85-kDa intracellular tail of LRP, and 2) confocal microscopy using an antibody against the extracellular domain. First, total cell lysates of isolated lymphocytes were subjected to Western blot analysis. The presence of the 85-kDa band could be detected in both T-lymphocytic subsets (*Fig 2*). In addition, LRP protein was also detected in lysates of B-lymphocytes and NK cells. In a second approach, purified lymphocytes were collected on glass coverslips for confocal microscopy studies, permeabilized with saponin, and subsequently stained using a FITC-conjugated antibody against the extracellular domain of LRP (*Fig 3*). T-lymphocytes (CD4+ (*Fig 3A*) and CD8+ (*Fig 3C*)), NK cells (*Fig 3E*) and B-lymphocytes (*Fig 3G*) were all positive for LRP. Thus, both experimental approaches point to the presence of LRP protein in these lymphocytes.

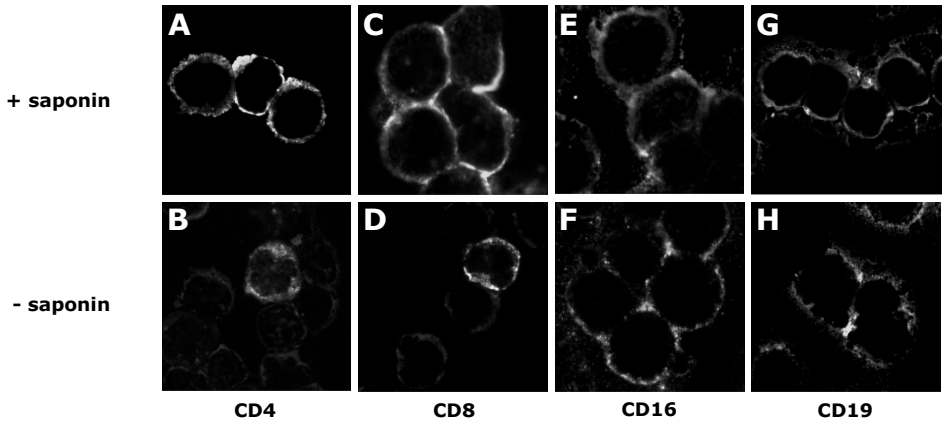


Figure 3. Localization of LRP in lymphocytes. PBMCs were isolated using Ficoll-Paque density centrifugation and collected on glass coverslips (10^5 cells/spin). After fixation, cells were treated with (*A, C, E, G*) or without saponin (*B, D, F, H*). LRP was stained with a FITC-conjugated antibody and cells were visualized by confocal microscopy**.** *A, B.* CD4+ T-helper cells. *C, D*. CD8+ cytotoxic T-lymphocytes. *E, F*. CD16+ NK cells. *G, H*. CD19+ B-lymphocytes.

To distinguish between LRP located at the cell surface or intracellularly, LRP staining was also examined in non-permeabilized lymphocytes. LRP staining was detected in NK cells (*Fig 3F*) and B-lymphocytes (*Fig 3H*). Interestingly, most CD4+ (*Fig 3B*) and CD8+ cells (*Fig 3D*) were negative for LRP cell surface staining. Apparently, CD3+ T-lymphocytes differ from NK and B-lymphocytes in that LRP seems to be maintained within the cell.

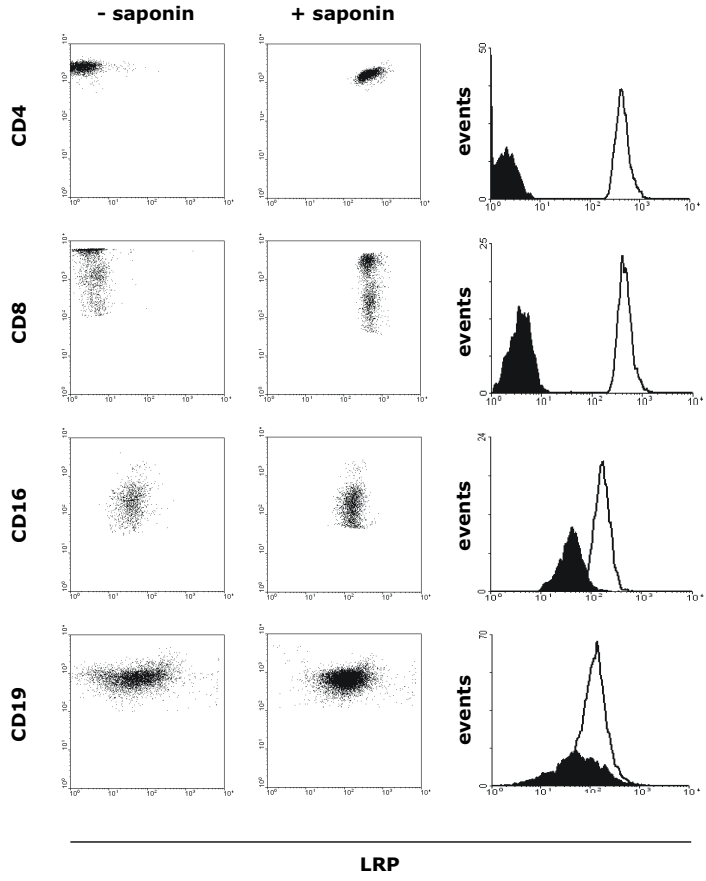


Figure 4. Quantification of intracellular and cell surface located LRP in lymphocytes. PMBCs were isolated using Ficoll-Paque density centrifugation. Cells were fixed and treated with 0.1% saponin. Cells were stained for LRP and CD4, CD8, CD16 or CD19, respectively and analyzed by FACS measurements. Right panels: histogram of LRP levels in lymphocytes. Black: without saponin, white: with saponin.

LRP is present in intracellular compartments in T-lymphocytes

The apparent dissimilar expression of LRP in NK cells and B-lymphocytes versus T-lymphocytes was further addressed in flow cytometric experiments. To quantify the ratio of intracellularly and cell-surface distributed LRP, permeabilized and non-permeabilized lymphocytes were compared for LRP staining in FACS experiments (*Fig 4*). In the non-permeabilized condition, high numbers of NK cells and B-lymphocytes expressed LRP at the cell surface (91.4 ± 4.4%, and 93.0 ± 3.9%, respectively). Saponin treatment increased the percentage of LRP positive cells up to 100% and the MFI was 2-3-fold higher than in non-permeabilized cells. Thus, these results confirm that LRP is located at the cell surface of most NK cells and B-lymphocytes. In contrast, LRP cell-surface expression was as low as background levels in both CD4+ and CD8+ T-lymphocytes, and only a small number of T-lymphocytes were LRP positive (< 5% of the CD4+ and CD8+ population). To exclude the possibility that the epitope for the antibody was not available at the cell surface of T-lymphocytes, also 3 other monoclonal and polyclonal antibodies against the extracellular domain of LRP were tested. However, none of these antibodies were able to detect LRP on non-permeabilized T-lymphocytes (data not shown), supporting the view that LRP is absent from the T-lymphocytic cell surface.

Under permeabilized conditions the number of LRP-positive T-lymphocytes increased from 2% to 100% for both CD4+ and CD8+ T-lymphocytes. The intensity of staining increased over 50-fold upon permeabilization for both cell types. CD4 and CD8 staining remained unaffected upon permeabilization, indicating that saponin treatment is not associated with non-specific binding of antibodies (data not shown). In conclusion, our data indicate that LRP is located at the cell surface of virtually all B-lymphocytes and NK cells, whereas LRP is mainly distributed intracellularly in T-lymphocytes.

Activation of T-lymphocytes in a mixed lymphocyte reaction results in redistribution of LRP

We hypothesized that LRP may become exposed at the cell surface during activation of circulating T-lymphocytes upon an immune response. Therefore, two activation methods were applied to investigate their effect on LRP cell surface expression. First, activation of the immune system may lead to proliferation of naïve T-lymphocytes, which can be mimicked *in vitro* by IL-2 and PHA stimulation. Indeed, a marker for activation (*ie* CD25) increased after 1 day and sustained during 7 day follow-up (*Fig 5B*). LRP was present at the cell surface of 2.0 ± 0.7% of the T-lymphocytes prior to activation (*Fig 5A*). Stimulation caused a 6-fold increase in the number of LRP positive T-lymphocytes, reaching its maxi-

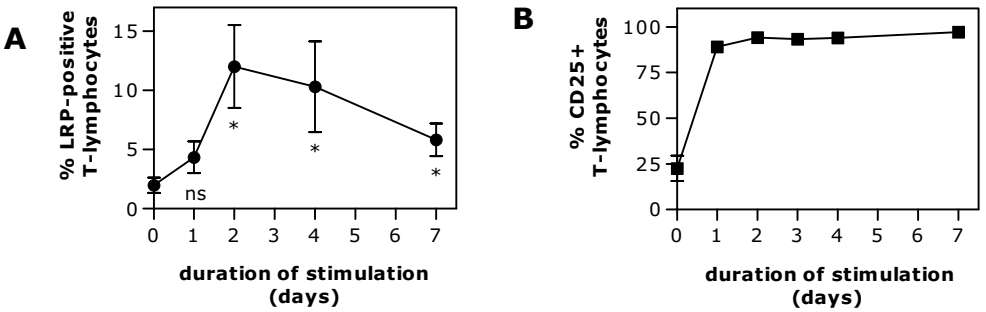


Figure 5. Stimulation of T-lymphocytes with IL-2 and PHA. *A.* T-lymphocytes were cultured in medium containing IL-2 and PHA during 7 days. At day 0, 1, 2, 4 and 7, LRP expression was measured in activated T-lymphocytes by FACS analysis. *B.* Activated cells were determined by their CD3 and CD25 cell surface expression. Data represent mean ± SEM. ns = not significant, * = $P < 0.02$, as compared to t=0.

mum after 2 days (12.0 ± 3.5% of the total vital T-lymphocytes). After 7 days, the number of LRP expressing cells gradually declined to 5.8 ± 1.4%.

In a second approach, PBMCs from one donor were mixed with PBMCs from a second donor. Only a few non-mixed T-lymphocytes from various donors were positive for LRP at the cell surface before mixing (3.2 ± 1.1% and 5.5 ± 2.9% of CD4+ and CD8+ T-lymphocytes, respectively) (*Fig 6*). Remarkably, combining PBMCs from 2 donors caused cell surface LRP expression in 68.5 ± 6.6% and 79.9 ± 1.9% of the CD4+ and CD8+ T-lymphocytes, respectively. Compared to non-mixed T-lymphocytes, cell surface LRP levels were increased up to 40.0 ± 18.6 and 26.6 ± 14.1 fold in the combined CD4+ and CD8+ T-lymphocytes, respectively (*Fig 6*). The number of T-lymphocytes positive for LRP at their cellular surface increased in time, reaching maximum and steady-state levels after 30 minutes (*Fig 7*). Under permeabilizing conditions, similar total LRP levels were observed for the non-mixed and mixed T-lymphocytes, indicating that appearance of LRP is independent of *de novo* synthesis. Cell surface and intracellular staining using a non-relevant antibody was not increased upon permeabilization or activation (data not shown). Thus, in T-lymphocytes intracellularly positioned LRP may be translocated to the cell surface in an MLR.

Monocytes are required for the induction of LRP cell surface expression in T-lymphocytes

Next, we investigated which cells were responsible for the upregulation of cell surface LRP in an MLR. To this end, monocytes, CD4+ and CD8+ T-lymphocytes were purified from various donors. Cells were mixed in different combinations for 30 minutes and subsequently fixed (*Fig 8*). In the resting condition, only a

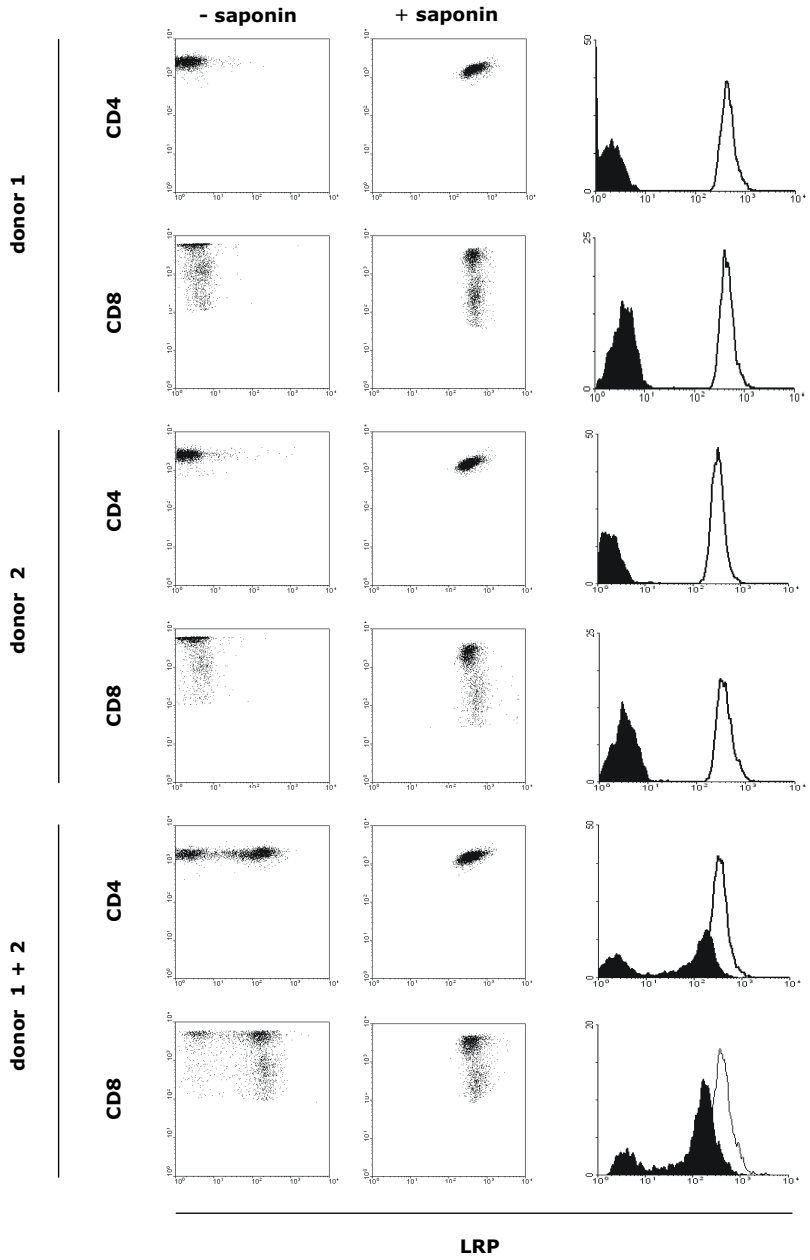


Figure 6. LRP expression in T-lymphocytes in a mixed lymphocyte reaction. 0.5 x 10^6 cells from donor 1 were mixed with 0.5 x 10^6 cells from donor 2 for 30 min. Cells were subsequently fixed and optionally permeabilized with 0.1% saponin. Cells were stained for LRP and CD4 or CD8 and analyzed by flow cytometry. Right panels: histogram of LRP levels in lymphocytes. Black: without saponin, white: with saponin.

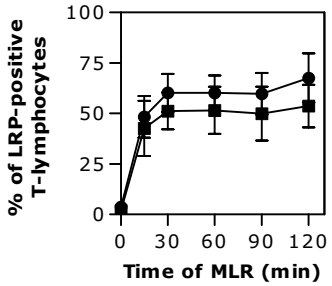


Figure 7. Time-dependent upregulation of LRP cell surface levels in an MLR. 0.5 x 10^6 cells from donor 1 were mixed with 0.5 x 10^6 cells from donor 2 for indicated time. Cells were subsequently fixed, stained for LRP and CD4 (squares) or CD8 (circles) and analyzed by flow cytometry.

few T-lymphocytes showed LRP at the cellular surface, and mixing T-lymphocytes from one donor with T-lymphocytes from a second donor did not increase these cell surface levels (*Fig 8*). Interestingly, monocytes from one donor with CD4+ or CD8+ T-lymphocytes from a second donor resulted in LRP cell surface expression of 62.6 ± 2.8% and 60.9 ± 3.4% of the CD4+ and CD8+ T-lymphocytes, respectively. Thus, these results indicate that the interaction between monocytes as antigen presenting cells from one donor and T-lymphocytes from a second donor is responsible for the observed upregulation of cell-surface-exposed LRP in T-lymphocytes.

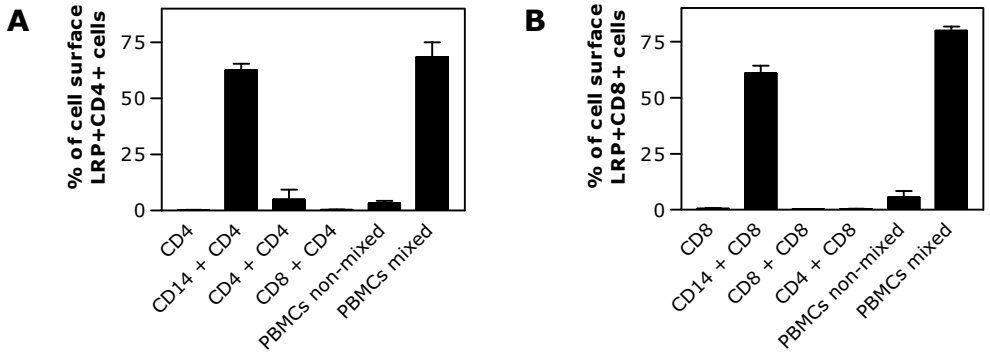


Figure 8. LRP expression in T-lymphocytes in a mixed lymphocyte reaction using purified cells. Monocytes (CD14+), CD4+ and CD8+ T-lymphocytes were purified from 2 donors. 0.5 x 10^6 cells from donor 1 were mixed with 0.5 x 10^6 cells from donor 2 for 30 min and subsequently fixed. Cells were stained for LRP and CD4 or CD8 and analyzed by flow cytometry. The x-axis represents the investigated combinations of cells from the 2 donors. The y-axis represents the percentage of CD4+ (*A*) or CD8+ T-lymphocytes (*B*) that express LRP at their cell surface. Data are represented as mean ± SEM.

Discussion

The multifunctional receptor LRP is expressed in a wide spectrum of cell types, including adipocytes, neurons, hepatocytes and smooth muscle cells[16]. Whereas initial studies have identified LRP as an endocytic receptor[8;9], more recent studies have revealed that LRP is involved in other cellular processes as well, like transcytosis and cellular signaling[10;11;13]. However, these latter functions seem to be dependent on the cell-type in which LRP is expressed, suggesting that cell-specific components regulate the functionality of LRP. This is for instance exemplified in monocytes and granulocytes, for which we recently found that LRP forms a functional active complex with β2-integrins[23] that are selectively expressed in leukocytes. Thus, LRP may contribute to leukocyte function by interacting with leukocyte-specific components.

Although the expression pattern of LRP in myeloid cells has been well established[16;21;23;25;26], data regarding LRP expression in lymphoid cells have been inconclusive. In the present study we have clearly demonstrated the presence of LRP mRNA and protein in various lymphocytic cells: B-lymphocytes, NK cells and CD4+ and CD8+ T-lymphocytes (*Fig 1-4*). Surprisingly, B-lymphocytes and NK cells differ from both types of T-lymphocytes in that the former two cell types expressed LRP abundantly at the cellular surface, whereas T-lymphocytes were characterized by a virtual absence of LRP at their surface (*Fig 3 and 4*). Confocal and flow cytometric analysis demonstrated that only a small number of individual cells were positive for LRP surface expression (*Fig 3 and 4*). Dual staining using anti-CD4 or anti-CD8 antibodies confirmed the identity of these cells, excluding the possibility that this small number of cells represent contamination of other LRP positive leukocytes.

Despite the lack of cell surface LRP in these cells, various experimental approaches to detect total LRP protein (confocal and flow cytometric analysis in the presence of saponin, and western blotting using total cell lysates) demonstrated that LRP is expressed by T-lymphocytes. In addition, expression levels roughly equal the levels in B-lymphocytes and NK cells. Thus, LRP seems to be maintained inside T-lymphocytes, possibly within storage pools. It is of interest to consider in this respect that B-lymphocytes, NK cells and T-lymphocytes derive from the same lymphoid precursor cell. The B-lymphocyte pathway branches from the T-lymphocyte/NK cell pathway, the latter two cell types sharing a bipotential precursor. During further maturation this precursor cell diverges in either T-lymphocytes or NK cells[27-30]. At some point during this differentiation process, regulatory mechanisms should occur that determine the cellular location of LRP, either by forcing surface expression in B-lymphocytes and NK cells, or by keeping LRP intracellularly in T-lymphocytes. So far, no other LRP-expressing cells

have been identified that are able to maintain the complete LRP population intracellularly. Therefore, we feel the second possibility to be favorable.

It should be noted that based on the MFI values, part of LRP (~ 50-60%) is present intracellularly in B-lymphocytes and NK cells. Such a distribution is also reported for other cells. For instance, LRP is present at the cellular surface of adipocytes. However, insulin treatment transiently increased uptake of LRP ligands α_2-macroglobulin and cholesteryl esters 2-3 fold within minutes, suggesting translocation of intracellularly stored LRP to the cell surface[31]. A similar upregulation of LRP surface expression was noticed in nerve growth factor-stimulated neurons[32]. Of importance, LRP is already present at the surface in both resting adipocytes and neurons, whereas this is untrue for LRP in T-lymphocytes. Such a dramatic increase in LRP cell surface expression as observed in T-lymphocytes, has not been detected in other stimulated cells.

The notion that LRP is maintained within T-lymphocytes prompted us to explore conditions that are associated with the translocation of LRP from the inside to the surface of these cells. In response to IL-2 and PHA, a transient raise in LRP surface expression was observed in 12% of the T-lymphocytes after 2 days (*Fig 5*). The observation that increased levels of LRP develop during T-lymphocyte activation is in line with a previous report by Banerjee et al[33], who described that differentiation of naïve T-lymphocytes into specific T-helper subsets parallels increased binding (approximately 15 fold) of the LRP ligand heat shock protein 96.

A more pronounced effect on the presence of LRP at the T-lymphocyte cell surface was observed upon the incubation of these cells with allogenic antigen-presenting cells (APCs) (*Fig 6-8*). In these MLRs, LRP cell surface expression is rapidly upregulated, suggesting the existence of a readily available storage pool. Indeed, our confocal microscopy studies using permeabilizing conditions demonstrated that LRP is located intracellularly just beneath the cell surface, ready for redistribution upon a trigger such as allogenic antigen recognition.

Activation of T-lymphocytes by allogenic APCs is a serious problem in graft-versus-host disease (GVHD)[34]. Many receptors on T-lymphocytes and their counterreceptors on APCs are involved in this process. A major group are the B7/CD28 family of receptors and their counterreceptors[35-37]. Recognition of foreign MHC/peptide complexes by T-lymphocytes induces several stimulatory and inhibitory pathways. For example, the CD28/B7 interactions lead to activation of the T-lymphocyte, whereas CTLA-4 on the T-lymphocyte turns activation down. This latter protein may be of special relevance, since it is also stored intracellularly in naïve T-lymphocytes and becomes exposed at the cell surface upon activation[38].

Although from our data it is tempting to speculate that LRP is involved in the complex T-lymphocyte response, further studies should be performed concerning the role of LRP in GVHD. Recently, we showed that LRP is required for proper function of β2-integrins at the cell surface of leukocytes[23]. β2-Integrins are another family of receptors that support interaction between a T-lymphocyte and an APC and evoke stimulatory signals towards the T-lymphocyte[39]. Therefore, it would be of interest to examine a possible role for LRP in modifying this interaction by β2-integrins.

In conclusion, our study focused on the elucidation of LRP expression patterns in lymphocyte subsets. Whereas NK cells, B-lymphocytes and T-lymphocytes all express LRP mRNA and protein, they show a distinct LRP protein localization. LRP is mainly present at the cell surface of NK cells and B-lymphocytes and intracellularly in T-lymphocytes. In T-lymphocytes, LRP cell surface expression is rapidly upregulated in an experimental set-up in which T-lymphocytes were incubated with allogenic monocytes. This suggests that LRP may play a role in GVHD. Future experiments must obtain more evidence on the precise function of LRP in this disease.

Acknowledgements

We gratefully acknowledge T. Mutis, A. Martens, S. Ebeling and F. Miedema (University Medical Center Utrecht, Utrecht, The Netherlands) for helpful discussions. This study was supported by a grant from the Dutch Organization of Scientific Research ZonMW (no. 902-26-236) to P.J.L.

References

1. Herz, J., U. Hamann, S. Rogne, O. Myklebost, H. Gausepohl, and K.K. Stanley. 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 J.* 7:4119-4127.
2. Herz, J., R.C. Kowal, J.L. Goldstein, and M.S. Brown. 1990. Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *EMBO J.* 9:1769-1776.
3. Willnow, T.E., K. Orth, and J. Herz. 1994. Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *J.Biol.Chem.* 269:15827-15832.
4. Neels, J.G., B.M. van den Berg, A. Lookene, G. Olivecrona, H. Pannekoek, and A.J. van Zonneveld. 1999. The second and fourth cluster of class A cysteine-rich repeats of the low density lipoprotein receptor-related protein share ligand-binding properties. *J.Biol.Chem.* 274:31305-31311.
5. Herz, J. and D.K. Strickland. 2001. LRP: a multifunctional scavenger and signaling receptor. *J.Clin.Invest.* 108:779-784.

6. Hussain, M.M., D.K. Strickland, and A. Bakillah. 1999. The mammalian low-density lipoprotein receptor family. *Annu.Rev.Nutr.* 19:141-172.
7. Li, Y., J. Cam, and G. Bu. 2001. Low-density lipoprotein receptor family: endocytosis and signal transduction. *Mol.Neurobiol.* 23:53-67.
8. Gliemann, J., A. Nykjaer, C.M. Petersen, K.E. Jorgensen, M. Nielsen, P.A. Andreasen, E.I. Christensen, A. Lookene, G. Olivecrona, and S.K. Moestrup. 1994. The multiligand alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein (alpha 2MR/LRP). Binding and endocytosis of fluid phase and membrane-associated ligands. *Ann.N.Y.Acad.Sci.* 737:20-38.
9. Gonias, S.L., L. Wu, and A.M. Salicioni. 2004. Low density lipoprotein receptor-related protein: regulation of the plasma membrane proteome. *Thromb.Haemost.* 91:1056-1064.
10. Demeule, M., J. Poirier, J. Jodoin, Y. Bertrand, R.R. Desrosiers, C. Dagenais, T. Nguyen, J. Lanthier, R. Gabathuler, M. Kennard, W.A. Jefferies, D. Karkan, S. Tsai, L. Fenart, R. Cecchelli, and R. Beliveau. 2002. High transcytosis of melanotransferrin (P97) across the blood-brain barrier. *J.Neurochem.* 83:924-933.
11. Deane, R., Z. Wu, A. Sagare, J. Davis, Y.S. Du, K. Hamm, F. Xu, M. Parisi, B. LaRue, H.W. Hu, P. Spijkers, H. Guo, X. Song, P.J. Lenting, W.E. van Nostrand, and B.V. Zlokovic. 2004. LRP/amyloid beta-peptide interaction mediates differential brain efflux of Abeta isoforms. *Neuron* 43:333-344.
12. Shibata, Y., T. Muramatsu, M. Hirai, T. Inui , T. Kimura, H. Saito, L.M. McCormick, G. Bu, and K. Kadomatsu. 2002. Nuclear targeting by the growth factor midkine. *Mol.Cell Biol.* 22:6788-6796.
13. Willnow, T.E., A. Nykjaer, and J. Herz. 1999. Lipoprotein receptors: new roles for ancient proteins. *Nat.Cell Biol.* 1:E157-E162.
14. Boucher, P. and M. Gotthardt. 2004. LRP and PDGF signaling: a pathway to atherosclerosis. *Trends Cardiovasc.Med.* 14:55-60.
15. Bacskai, B.J., M.Q. Xia, D.K. Strickland, G.W. Rebeck, and B.T. Hyman. 2000. The endocytic receptor protein LRP also mediates neuronal calcium signaling via N-methyl-D-aspartate receptors. *Proc.Natl.Acad.Sci.U.S.A.* 97:11551-11556.
16. Moestrup, S.K., J. Gliemann, and G. Pallesen. 1992. Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res.* 269:375-382.
17. Moestrup, S.K., K. Kaltoft, C.M. Petersen, S. Pedersen, J. Gliemann, and E.I. Christensen. 1990. Immunocytochemical identification of the human alpha 2-macroglobulin receptor in monocytes and fibroblasts: monoclonal antibodies define the receptor as a monocyte differentiation antigen. *Exp.Cell Res.* 190:195-203.
18. Jardi, M., J. Ingles-Esteve, M. Burgal, C. Azqueta, F. Velasco, C. Lopez-Pedrera, L.A. Miles, and J. Felez. 1996. Distinct patterns of urokinase receptor (uPAR) expression by leukemic cells and peripheral blood cells. *Thromb.Haemost.* 76:1009-1019.
19. Hart, J.P., M.D. Gunn, and S.V. Pizzo. 2004. A CD91-positive subset of CD11c+ blood dendritic cells: characterization of the APC that functions to enhance adaptive immune responses against CD91-targeted antigens. *J.Immunol.* 172:70-78.
20. Patel, M., J. Morrow, F.R. Maxfield, D.K. Strickland, S. Greenberg, and I. Tabas. 2003. The cytoplasmic domain of the low density lipoprotein (LDL) receptor-related protein, but not that of the LDL receptor, triggers phagocytosis. *J.Biol.Chem.* 278:44799-44807.
21. Su, H.P., K. Nakada-Tsukui, A.C. Tosello-Trampont, Y. Li, G. Bu, P.M. Henson, and K.S. Ravichandran. 2002. Interaction of CED-6/GULP, an adapter protein involved in engulfment of apoptotic cells with CED-1 and CD91/low density lipoprotein receptor-related protein (LRP). *J.Biol.Chem.* 277:11772-11779.
22. Xu, W., Y. Takahashi, T. Sakashita, T. Iwasaki, H. Hattori, and T. Yoshimoto. 2001. Low density lipoprotein receptor-related protein is required for macrophage-mediated oxidation of low density lipoprotein by 12/15-lipoxygenase. *J.Biol.Chem.* 276:36454-36459.

23. Spijkers, P.P., P. da Costa Martins, E. Westein, C.G. Gahmberg, J.J. Zwaginga, and P.J. Lenting. 2005. LDL-receptor-related protein regulates beta2-integrin-mediated leukocyte adhesion. *Blood* 105:170-177.
24. Glaser, C., K. Handschug, S. Ludvik, U. Schell, A. Hehr, A. Kuchenhoff, and H. Robenek. 1994. The importance of the alpha 2MR/LRP for human genetics. Investigations of the receptor expression in different human blood cells. *Ann.N.Y.Acad.Sci.* 737:447-450.
25. Watanabe, Y., T. Inaba, H. Shimano, T. Gotoda, K. Yamamoto, H. Mokuno, H. Sato, Y. Yazaki, and N. Yamada. 1994. Induction of LDL receptor-related protein during the differentiation of monocyte-macrophages. Possible involvement in the atherosclerotic process. *Arterioscler.Thromb.* 14:1000-1006.
26. Ogden, C.A., A. deCathelineau, P.R. Hoffmann, D. Bratton, B. Ghebrehiwet, V.A. Fadok, and P.M. Henson. 2001. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J.Exp.Med.* 194:781-795.
27. Plum, J., M. de Smedt, B. Verhasselt, T. Kerre, D. Vanhecke, B. Vandekerckhove, and G. Leclercq. 2000. Human T lymphopoiesis. In vitro and in vivo study models. *Ann.N.Y.Acad.Sci.* 917:724-731.
28. Yokoyama, W.M., S. Kim, and A.R. French. 2004. The dynamic life of natural killer cells. *Annu.Rev.Immunol.* 22:405-429.
29. Galy, A., M. Travis, D. Cen, and B. Chen. 1995. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* 3:459-473.
30. Lanier, L.L., H. Spits, and J.H. Phillips. 1992. The developmental relationship between NK cells and T cells. *Immunol.Today* 13:392-395.
31. Descamps, O., D. Bilheimer, and J. Herz. 1993. Insulin stimulates receptor-mediated uptake of apoE-enriched lipoproteins and activated alpha 2-macroglobulin in adipocytes. *J.Biol.Chem.* 268:974-981.
32. Bu, G., Y. Sun, A.L. Schwartz, and D.M. Holtzman. 1998. Nerve growth factor induces rapid increases in functional cell surface low density lipoprotein receptor-related protein. *J.Biol.Chem.* 273:13359-13365.
33. Banerjee, P.P., D.S. Vinay, A. Mathew, M. Raje, V. Parekh, D.V. Prasad, A. Kumar, D. Mitra, and G.C. Mishra. 2002. Evidence that glycoprotein 96 (B2), a stress protein, functions as a Th2-specific costimulatory molecule. *J.Immunol.* 169:3507-3518.
34. Ferrara, J.L. and H.J. Deeg. 1991. Graft-versus-host disease. *N.Engl.J.Med.* 324:667-674.
35. Sharpe, A.H. and G.J. Freeman. 2002. The B7-CD28 superfamily. *Nat.Rev.Immunol.* 2:116-126.
36. Riley, J.L. and C.H. June. 2005. The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation. *Blood* 105:13-21.
37. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe. 2005. The B7 Family Revisited. *Annu.Rev.Immunol.* 23:515-548.
38. Linsley, P.S., J. Bradshaw, J. Greene, R. Peach, K.L. Bennett, and R.S. Mittler. 1996. Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. *Immunity* 4:535-543.
39. Springer, T.A. 1990. Adhesion receptors of the immune system. *Nature* 346:425-434.

chapter 4

LRP / amyloid β-peptide interaction mediates differential brain efflux of Aβ isoforms

Rashid Deane[1*], Zhenhua Wu[1*], Abhay Sagare[1], Judianne Davis[2], Shi Du Yan[3], Katie Hamm[1], Feng Xu[2], Margaret Parisi[1], Barbra LaRue[1], Hong Wei Hu[3], Patricia Spijkers[4], Huang Guo[1], Xiaomei Song[1], Peter J. Lenting[4], William E. Van Nostrand[2] and Berislav V. Zlokovic[1]

[1] Frank P. Smith Laboratories for Neuroscience and Neurosurgical Research, Department of Neurosurgery and Division of Neurovascular Biology, University of Rochester Medical Center, Rochester, New York, [2] Department of Medicine, Health Sciences Center, Stony Brook University, Stony Brook, New York, [3] Departments of Pathology and Surgery, College of Physicians and Surgeons of Columbia University, New York, New York, [4] Laboratory for Thrombosis and Haemostasis, Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands
* contributed equally to this work

Neuron, 2004, 43(3):333-344

Abstract

LRP (low-density lipoprotein receptor-related protein) is linked to Alzheimer's disease (AD). Here, we report that amyloid β-peptide, Aβ40, binds to immobilized LRP clusters II and IV with high affinity (K_d = 0.6-1.2 nM) compared to Aβ42 and mutant Aβ, and LRP-mediated Aβ brain capillary binding, endocytosis and transcytosis across the mouse blood-brain barrier are substantially reduced by the high β-sheet content in Aβ and deletion of the receptor-associated protein gene. Despite low Aβ production in the brain, transgenic mice expressing low LRP-clearance mutant Aβ develop robust Aβ cerebral accumulations much earlier than Tg-2576 Aβ-overproducing mice. While Aβ does not affect LRP internalization and synthesis, it promotes proteasome-dependent LRP degradation in endothelium at concentrations > 1 μM consistent with reduced brain capillary LRP levels in Aβ-accumulating transgenic mice, AD and patients with cerebrovascular β-amyloidosis. Thus, low affinity LRP/Aβ interaction and/or Aβ-induced LRP loss at the BBB mediate brain accumulation of neurotoxic Aβ.

Introduction

The low density lipoprotein receptor-related protein (LRP), a member of the low-density lipoprotein (LDL) receptor family, is a multiligand receptor whose physiological functions are carried out by endocytosis of ligands and activation of multiple signal transduction pathways[1;2]. The precursor form of LRP, a 600-kDa transmembrane glycoprotein, is cleaved in *trans*-Golgi network by furin to generate a 515-kDa α-subunit and an 85-kDa β-subunit that remain non-covalently associated during LRP transport to the cell membrane[3]. A 39-kDa receptor-associated protein (RAP) is a specialized chaperone molecule which binds to LRP and regulates its proper folding[4]. LRP interacts with a broad range of secreted proteins and resident cell surface molecules in the brain (> 30 structurally diverse ligands) mediating their endocytosis and/or activating signaling pathways through multiple cytosolic adaptor and scaffold proteins[5]. Phosphorylation of LRP's tail regulates ligand internalization and signal transduction[6;7].

LRP is linked to Alzheimer's disease (AD) by genetic and biochemical evidence. Genetic polymorphism in *LRP* gene is associated with late-onset AD[8-11]. In addition, LRP binds amyloid β-peptide (Aβ) precursor protein (APP), Apolipoprotein E (ApoE) and α_2-macroglobulin (α_2M), which have been genetically linked to AD[1]. The exact pathogenic mechanism(s) by which LRP contributes to neurotoxic Aβ accumulations is unclear. LRP binds secreted APP and influences its degradation[12] and processing[13] leading to increased Aβ production[14]. It may be involved in α_2M-mediated slow reuptake of secreted Aβ by neurons derived from transgenic APP mice and in mouse fibroblasts with no degradation of Aβ[15;16]. Slow degradation of Aβ via α_2M in mouse fibroblasts and U97 human glioma cells has also been suggested[17]. ApoE3 may prevent Aβ neurotoxicity by forming complexes with Aβ, and its protective effect possibly requires LRP[18], although the role of other lipoprotein receptors, e.g., LDLR, megalin, cannot be ruled out. However, overexpression of functional LRP mini-receptors in neurons of Alzheimer's PDAPP mice results in age-dependent increases of soluble Aβ in the brain[19], suggesting LRP on neurons *in vivo* does not mediate Aβ clearance.

LRP is expressed in brain capillary endothelium[20;21]. The idea that LRP along the brain capillary membranes clears Aβ *in vivo* has been supported by brain efflux studies with exogenous soluble Aβ40 suggesting the blood-brain barrier (BBB) is a primary clearance site for Aβ[20]. However, neither this nor other studies have clarified at the molecular and cellular level how LRP at the BBB works as a cargo/clearance receptor for Aβ, and whether LRP on brain capillaries clears neurotoxic Aβ42[22] and vasculotropic Aβ mutants[23]. Reduced levels of LRP in AD have been associated with Aβ brain accumulations[8;16;20], but whether Aβ directly

interferes with LRP's internalization, degradation and/or synthesis at the clearance site(s) in brain, is not known.

Here, we provide evidence for a direct LRP/Aβ protein-protein interaction and demonstrate this interaction on brain capillaries regulates in an isoform-specific manner binding of Aβ to LRP followed by Aβ endocytosis and transcytosis across the BBB. We report LRP at the BBB favors clearance of Aβ40 relative to a high β-sheet content Aβ42 and vasculotropic mutant Aβ, while deletion of the RAP gene/depletion of LRP[24], but not of the LDLR and VLDLR genes, precludes rapid Aβ brain capillary clearance and efflux at the BBB. Transgenic mice producing low LRP-clearance mutant Aβ in the brain develop robust Aβ accumulations compared to Tg-2576 wild-type Aβ-overproducing mice[25], despite > 20-fold lower brain APP expression and > 30-fold reduced Aβ neuronal production. Finally, we show Aβ does not affect LRP internalization or synthesis, but promotes proteasome-dependent LRP degradation at pathological levels consistent with low LRP activity in brain microvessels in Aβ-accumulating mice and in patients with AD and familial cerebrovascular β-amyloidosis.

Experimental Procedures

Reagents

Wild-type and mutant Aβ (Dutch40, Dutch42, Dutch/Iowa40) peptides were synthesized by solid-phase F-moc (9-fluorenylmethoxycarbonyl) amino acid chemistry, purified by reverse phase-HPLC and structurally characterized, as described[26;27]. Recombinant LRP fragments encompassing clusters II and IV were produced using stable transfected baby hamster kidney cell lines[28]. We used human recombinant RAP (EMD Biosciences, Inc. San Diego, CA), polyclonal goat anti-human LRP N20 antibody which cross reacts with mouse LRP (1:200, Santa Cruz Biotech. Inc., Santa Cruz, CA), monoclonal mouse antibody against C-terminal domain of human LRP β-chain which cross reacts with mouse LRP (5A6, 1:350, 5 μg/ml; EMD Biosciences, Inc. San Diego, CA), monoclonal mouse antibody against human LRP α-chain (8G1,1:240, 5 μg/ml; EMD Biosciences, Inc. San Diego, CA), monoclonal mouse antibody P2-1 specific for human APP (1:1000, 1 mg/ml), 22C11 which recognizes mouse and human APP (1:100, 0.5 mg/ml; Chemicon International, Temecula, CA), 66.1 to residues 1-8 of human Aβ (1:1000, 1 mg/ml)[29], rat anti-mouse CD31 antibody (1:200, BD Pharmigen, Lexington, KY), polyclonal rabbit antibody to human Von Willebrand Factor (VWF) (1:200, DAKO, Glostrup, Denmark), RAGE-specific IgG (1:500, 1 mg/ml)[29], goat anti-human VLDLR (1:50, 200 μg/ml, Santa Cruz Biotech Inc.,

Santa Cruz, CA) and monoclonal mouse human LDLR-specific IgG C7 (1:50, 200 μg/ml, Research Diagnostics, Flanders, NJ).

Surface plasmon resonance analysis

LRP clusters II and IV were immobilized at CM5 chips at a density of 10-20 fmol/mm^2 and incubated with Aβ40, Aβ42, and mutant Aβ (Dutch/Iowa40) (0-50 nM) in 150 mM NaCl, 0.005% (v/v) Tween-20 and 25 mM Hepes (pH 7.4) at a flow rate of 5 μl/min for 2 min at 25ºC, as described[28]. RAP was used at 500 nM. The data were analyzed to calculate apparent association rate constants $K_{on,app}$ and apparent dissociation rate constants $K_{off,app}$ using a single-site binding model[28]. Apparent affinity constants $K_{d,app}$ were inferred from the ratio $K_{off,app}/K_{on,app}$. Data are based on 3-5 measurements using 6-9 different concentrations for each measurement and presented as the average (± SEM). Analysis was performed using Biacore X biosensor system (Uppsala, Sweden) and BIA evaluation 3.0 software (Biacore, Sweden).

Secondary structure analysis

Secondary structure of peptides was analyzed by circular dichroism[30;31]. Briefly, 20-25 μg of hexafluoroisopropanol treated seedless peptide was dissolved in 980 μL of 10 mM phosphate buffer, pH 7.4, and centrifuged to remove precipitated/undissolved material. The CD spectrum was recorded within 24 h using a 1 mm path length cell, on an Aviv 202 CD spectropolarimeter (Proterion, Piscataway, NJ). Results are expressed as molar ellipticity and the percentage of α-helix, β-sheet, β-turn and random coil determined.

Radio-iodination of Aβ

Radioiodination of Aβ was carried out by mild "lactoperoxidase" method (Thorell and Johansson, 1971). Typically, 10 μg of Aβ was labeled for 18 min at room temperature with 2 mCi of Na[^{125}I]. After radiolabeling, the preparations were subjected to reverse-phase HPLC separation using a Vydac C4 column and a 30 minute linear gradient of 25% to 40% acetonitrile in 0.059% trifluoroacetic acid to separate the monoiodinated non-oxidized form of Aβ (which is the tracer we are using) from diiodinated Aβ, non-labeled non-oxidized Aβ, and oxidized Aβ species, as we reported[29]. The content of material in the peaks eluted from HPLC was determined by MALDI-TOF mass-spectrometry to ensure the purity of the radiolabeled species. For MALDI-TOF mass spectrometry Aβ peptides were labeled under identical conditions using Na[^{127}I] instead of the radioactive nuclide. The specific activities were in the range of 45 to 65 μCi/μg of peptide. For brain capillary uptake studies and animal clearance studies, we used in most experiments the preparations within 24 h of labeling that were $\geq$ 99% TCA-

precipitable. If used within 72 h of labeling, the radiolabeled peptides were stabilized in ethanol as a quenching agent. The HPLC/SDS-PAGE analysis was used to confirm the monomeric state of infused radiolabeled Aβ.

Brain capillary binding, endocytosis and uptake of ^{125}I-Aβ

Brain capillaries from wild type, RAP null, LDLR null and VLDLR null mice were isolated using a modified procedure, as we described[32].

First, we determined ^{125}I-labeled Aβ40, Aβ42, Dutch/Iowa Aβ40 and reverse 40-1 Aβ binding to endogenous LRP at the abluminal side of brain capillaries. Capillaries were incubated in 0.6 ml Eppendorf tubes in the assay buffer (mock CSF containing 1 mM sodium perchlorate to prevent free iodide binding/uptake) with 2 nM ^{125}I-Aβ ligand at 4ºC for 30 min in the absence and presence of RAP (500 nM), LRP-specific IgG (Fab_2) and unlabeled Aβ (500 nM). After 30 min, incubation buffer containing unbound ligand was removed and capillaries washed, lysed in low SDS lysis buffer and counted.

Second, we determined internalization of ^{125}I-labeled Aβ40, Aβ42 and Dutch/Iowa Aβ40 at the abluminal surface of brain capillaries and the rate of LRP endocytosis in the presence of each isoform. Capillaries were incubated in 0.6 ml Eppendorf tubes in cold assay buffer with 2 nM ^{125}I-Aβ ligand at 4ºC for 30 min in the absence and presence of RAP (500 nM), LRP-specific IgG (Fab_2) and unlabeled Aβ (500 nM), as above. After 30 min, unbound ligand was removed by washing capillaries with cold assay buffer. Ice-cold stop/strip solution (0.2 M acetic acid, pH 2.6, 0.1 M NaCl) was added to one Eppendorf tube that was kept on ice. The remaining Eppendorf tubes were placed in a 37ºC water bath, and the assay buffer at 37ºC added quickly to capillaries to initiate ligand internalization. At predetermined times at 30 s, 1, 2 and 5 min, Eppendorf tubes were quickly placed on ice, and incubated for 12 min with the ice-cold stop/strip solution (0.2 M acetic acid, pH 2.6, 0.1 M NaCl) to remove ligand from the capillary abluminal cell surface. Capillaries were solubilized with low SDS lysis buffer and counted. The sum of internalized ligand plus the ligand associated with the abluminal capillary cell surface represented the amount of ligand available for internalization. The fraction of ligand internalized at each time point was plotted, and the half-time for internalization ($t_{1/2}$) calculated, as described[33;34].

^{125}I-Aβ ligand degradation was determined in brain capillary endothelial cell lysates and in the incubation medium at the end of internalization studies using the TCA precipitation assay, as reported[29].

To determine kinetics of ^{125}I-Aβ brain capillary unidirectional uptake, capillaries were incubated with ^{125}I-labeled Aβ40, Aβ42 and mutant Aβ (Dutch/Iowa40) at 37ºC for 1 min. Self- and cross-inhibition studies were performed with unlabeled Aβ40 from 1 to 120 nM, and unlabeled Aβ42 and mutant Aβ (Dutch40, Dutch42,

Dutch/Iowa40) at 40 nM, RAP (500 nM) and LRP-specific polyclonal N20 antibody (60 μg/ml).

Brain clearance studies

Studies were performed according to the National Institutes of Health guidelines using an approved institutional protocol. CNS clearance of ^{125}I-labeled Aβ ligands was determined simultaneously with ^{14}C-inulin (reference marker) in male C57Bl/6, RAP null, LDLR null and VLDLR null mice 8-10 weeks old, using a procedure as described[20]. Briefly, a stainless steel guide cannula was implanted stereotaxically into the right caudate-putamen of anesthetized mice (0.5 mg/kg ketamine and 5 mg/kg xylazine I.P.). Coordinates for tip of the cannula were 0.9 mm anterior and 1.9 mm lateral to the bregma and 2.9 mm below the surface of the brain. Animals were allowed to recover after surgery prior to radiotracer studies. The experiments were performed before substantial chronic processes have occurred, as assessed by histological analysis of tissue, *ie*, negative staining for astrocytes (glial fibrillar acidic protein) and activated microglia (anti-phosphotyrosine), but allowing time for BBB repair to large molecules[35], typically 4–6 h after the cannula insertion, as reported[36]. Tracer fluid (0.5 μL) containing [^{125}I]-Aβ and ^{14}C-inulin was injected into brain ISF over 5 min via an ultra micropump with a micro4-controller (World Precision Instruments, Sarasota, FL). When the effects of different unlabeled molecular reagents are tested, these were injected simultaneously with radiolabeled ligands. For self-inhibition studies, the uptake of ^{125}I-Aβ40 and ^{125}I-mutant Aβ (Dutch/Iowa40) was studied over a range of carrier concentrations from 0.5-120 nM. For cross-inhibition studies, efflux of ^{125}I-test-peptides was studied at a carrier concentration of 40 nM and the inhibitory concentration of unlabeled Aβ peptides at 120 nM. Brain and blood were sampled 30 min after tracers injection and prepared for radioactivity analysis by TCA, HPLC and SDS-PAGE/immunoprecipitation analysis to determine the molecular forms of test-tracers, as described[20;29;30]. Gamma counting was performed using Wallac Vizard Gamma Counter (Perkin Elmer, Meriden, CT) and beta-counting using Tri-carb 2100 Liquid Scintillation Counter (Perkin Elmer, CT). Previous studies with ^{125}I-labeled Aβ demonstrated an excellent correlation between TCA and HPLC methods. The intactness of ^{125}I-labeled Aβ40, Aβ42 and mutant Aβ (Dutch/Iowa40) injected into the brain ISF was > 99% by TCA/HPLC analysis. The Aβ standards eluted between 29.1 and 31.2 min for different Aβ peptides. For SDS-PAGE analysis, TCA precipitated samples were resuspended in 1% SDS, vortexed and incubated at 55^{o}C for 5 min, then neutralized, boiled for 3 min, homogenized and analyzed by electrophoresis in 10% Tris-tricine gels followed by fluorography. Methodological details were as we reported[20;29;30].

Calculations

^{125}I-Aβ brain capillary uptake was corrected for the distribution of ^{14}C-inulin (extracellular space marker) and determined as the tissue to medium ratio as: c.p.m. for TCA-precipitable ^{125}I-radioactivity (mg capillary protein)/c.p.m. for TCA-precipitable ^{125}I-radioactivity (ml medium) (1). All calculations of clearance parameters were as reported[20]. Briefly, the percentage of radioactivity remaining in the brain after microinjection was determined as % recovery in brain = 100 x (N_b/N_i) (2), where, N_b is the radioactivity remaining in the brain at the end of the experiment and N_i is the radioactivity injected into the brain ISF, *ie*, the d.p.m. for ^{14}C-inulin and the c.p.m. for TCA-precipitable ^{125}I-radioactivity (intact Aβ). The percentage of Aβ cleared through the BBB was calculated as [(1 - $N_{b(A\beta)}/N_{i(A\beta)}$) - (1 - $N_{b(inulin)}/N_{i(inulin)}$)] x 100, using a standard time of 30 min (3). Efflux of Aβ from brain ISF via transport across the BBB at different concentrations of peptides, J_{out}, was calculated as [(1 - $N_{b(A\beta)}/N_{i(A\beta)}$) - (1 - $N_{b(inulin)}/N_{i(inulin)}$)]/T x $C_{A\beta}$ (4) where $C_{A\beta}$ is Aβ concentration in the infusate. The half-saturation concentration for Aβ elimination via BBB transport, K_m, was calculated from $J_{out} = Cl_{max}/(K_m + C_{A\beta})$ (4), where Cl_{max} (pmol/s/L ISF) represents the maximal efflux capacity for the saturable Aβ efflux across the BBB corrected for the rate of ISF flow, as we reported[20]. The K_m value for Aβ40 uptake by isolated brain capillaries was calculated using Michaelis-Menten analysis. The inhibitory constants, K_i, were calculated from the velocity ratios[30] as $K_i = (J_i \times K_m \times C_i)/(J_{out} - J_i)(K_m + C_{A\beta40})$, where C_i and $C_{A\beta40}$ were the inhibitory concentrations of test-Aβ peptide and Aβ40 in the infusate *in vivo* or incubation medium *in vitro*. Kinetic constants were obtained by a non-linear regression curve fitting (Prism 3.0).

Transgenic mice

We used Tg-2576 mice in a C57Bl6/SJL background[25] and Tg-DI (Dutch/Iowa) mice in C57Bl6 background[37]. Human APP (770 isoform) cDNA harboring the Swedish (KM670/671NL), Dutch (E693Q), and Iowa (D694N) mutations was subcloned between exons II and IV of a Thy-1.2 expression cassette (gift from Dr. F. LaFerla, University of California, Irvine). The 9 kb transgene was liberated by NotI/PvuI digestion, purified, and microinjected into pronuclei of C57BL/6 single-cell embryos at the Stony Brook Transgenic Mouse Facility. Founder transgenic mice were identified by Southern blot analysis of tail DNA. Transgenic offspring were determined by PCR analysis of tail DNA using the following primers for human APP: 5'-CCTGATTGATACCAAGGAAGGCATCCTG-3':
5'-GTCATCATCGGCTTCTTCTTCTTCCACC-3' (generating 500 base pair product).

RAP null, VLDLR null and LDLR null mice were purchased from Jackson Laboratories, Bar Harber, ME.

Quantification of Aβ

Soluble and insoluble pools of Aβ were determined by ELISA of carbonate extracted forebrain tissue and of guanidine lysates of the insoluble pellets resulting from the carbonate extracted brain tissue, respectively[38]. Levels of total Aβ were compared between Tg-2576 and Tg-DI mice.

Histological analysis

For neuropathological analysis on mouse brain tissue in Tg-2576 and Tg-DI mice, tissue sections were cut from mouse brain hemispheres in the sagittal plane either at 5 μm (paraffin embedded fixed tissue) or 14 μm (fresh frozen tissue). Aβ immunoreactive deposits were identified with human specific monoclonal mouse antibody 66.1 to Aβ[29]. For LRP staining on brain microvessels in RAP null, Tg-2576, Tg-DI and wild-type mice, we used 14 μm frozen acetone fixed tissue sections and double immunostaining for LRP and CD31 (endothelial marker). LRP-specific IgG (5A6) was used as a primary antibody. Biotinylated anti-mouse IgG was used as a secondary antibody and was detected with fluorescein streptavidin (1:1000, Vector Laboratories, Inc., Burlingame, CA). M.O.M. kit (Vector Laboratories, Inc., Burlingame, CA) was used to block endogenous IgG, as described[39]. For CD31 staining, mouse CD31-specific IgG was used as a primary antibody, and Alexa Fluor 594 donkey anti-rat IgG (1:500, Molecular Probes, Inc. Eugene, OR) as a secondary antibody. For immunocytochemical analysis of LRP on brain microvessels in tissue from AD patients, patients with cerebrovascular β-amyloidosis Dutch-type and age-matched controls, we used cryostat sections (10 μm) of frontal cortex (Brodmann area 9/10) that were air dried. LRP was detected with 8G1 human LRP-specific IgG as a primary antibody and rhodamine red goat anti-mouse IgG (1:150, Molecular Probes, Eugene, OR) as a secondary antibody. The anti-vWF (endothelial marker) primary antibody was detected with fluorescein goat anti-rabbit IgG (1:150, Molecular Probes, Eugene, OR). Image analysis was performed using Olympus AX70 microscope equipped with the SPOT digital camera. Ten randomly selected fields in each region from ten sections spanning the entire hemisphere from 4 mice/group or from Brodman A9/10 areas in humans was performed.

Human brain endothelial cells

Human BEC were isolated from rapid autopsies of neurologically normal young individuals after trauma. BEC were characterized and cultured as we described[40] and incubated with different Aβ isoforms at concentrations ranging from 1 nM to 20 μM within 48 h. Cells were lysed and equal amounts of proteins electrophoresed (10 μg/ml) on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane and probed with 5A6 (β-chain) or 8G1 (α-chain) human LRP-specific

IgGs, RAGE-specific IgG[29] or transferrin-specific IgG. The relative density of each protein was determined by scanning densitometry using β-actin as an internal control.

LRP endocytosis rates were studied with ^{125}I-α_2M*, an LRP-specific ligand. BEC were incubated with 20 μM Aβ40, Aβ42 and Dutch/Iowa Aβ40 at 37ºC for 48 h in 12-well plates, and than switched to Aβ-free medium containing 5 mM $CaCl_2$ and 5 nM ^{125}I-α_2M* at 4ºC for 30 min followed by incubations with α_2M*-free medium at 37ºC to initiate internalization, as reported[33].

Metabolic labeling

Human BEC (4 x 10^5) were pulsed for 1 h at 37ºC with 400 μCi of [^{35}S]-methionine (> 1000 Ci/mmol; Perkin Elmer Life Sci. Inc., Boston, MA) in methionine-free Dulbecco modified Eagle medium (GIBCO BRL, New York, NY), as described[41]. Cells were chased at indicated times within 48 h. Cell lysates were immunoprecipitated with LRP-515-kDa α-chain specific IgG (8G1) on SDS-PAGE. The intensity of signal was quantified in pixels using Storm 860 Phosphoimager (Amersham Biosciences Corp., Piscataway, NJ).

Neuronal cultures and Aβ production

Primary mouse neuronal cultures were prepared as we described[42]. Briefly, cerebral cortices were dissected from fetal heterozygous Tg-DI mice[37] and Tg-2576 mice[25] at 16 days of gestation, treated with trypsin for 10 min at 37ºC, and dissociated with trituration. Cell suspensions were plated at 5 x 10^5 cells per well on 12-well plates in serum-free Neurobasal medium plus B27 supplement (GIBCO-BRL, Rockville, MA). The absence of astrocytes was confirmed by the lack of glial fibrillar acidic protein. Cultures were maintained in a humidified 5% CO_2 incubator at 37ºC for 5 days and the levels of Aβ in the culture medium determined by a quantitative immunoblot analysis using 4G8 antibody (1:1,000, 1 μg/ml, Signet, Dedham. MA), similar as described[15].

Statistical Analysis

Data were analyzed by multifactorial analysis of variance, Student's t test and Dunnett's t test.

Results

Fig. 1A-B show high affinity binding of monomeric Aβ40 to immobilized LRP clusters II and IV with K_d values of 0.57 ± 0.12 and 1.24 ± 0.01 nM, respectively, determined by the surface plasmon resonance (SPR) analysis. In contrast, Aβ42 and vasculotropic mutant Aβ (Dutch/Iowa Aβ40 double mutant model peptide[27]) exhibit greatly reduced binding affinity for LRP clusters II and IV by 6 and 9-fold and 28 and 12-fold, respectively, compared to Aβ40. The K_d values for Aβ42 binding to LRP II and IV clusters were 3.00 ± 0.11 and 10.10 ± 0.03 nM, respectively, and for mutant Aβ 15.10 ± 0.10 and 15.30 ± 0.07 nM, respectively. Binding of Aβ to LRP was abolished by RAP, an LRP antagonist[1] (*Fig 1A-B*). Its affinity to immobilized LRP fragments was greatly reduced by the high content of β-sheets (*Fig 1C*).

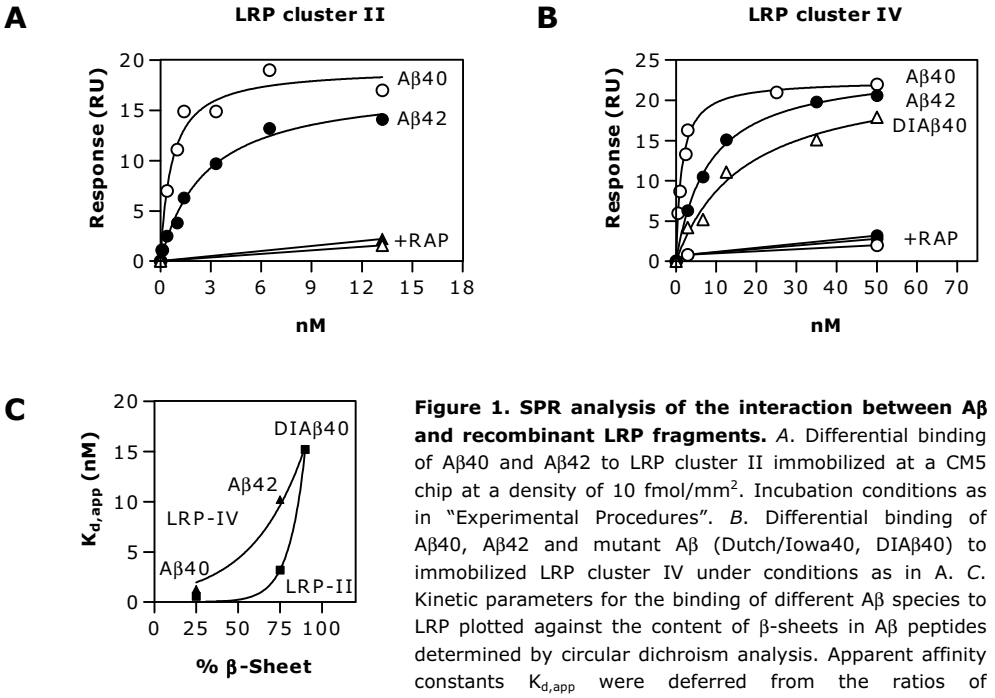


Figure 1. SPR analysis of the interaction between Aβ and recombinant LRP fragments. *A*. Differential binding of Aβ40 and Aβ42 to LRP cluster II immobilized at a CM5 chip at a density of 10 fmol/mm^2. Incubation conditions as in "Experimental Procedures". *B*. Differential binding of Aβ40, Aβ42 and mutant Aβ (Dutch/Iowa40, DIAβ40) to immobilized LRP cluster IV under conditions as in A. *C*. Kinetic parameters for the binding of different Aβ species to LRP plotted against the content of β-sheets in Aβ peptides determined by circular dichroism analysis. Apparent affinity constants $K_{d,app}$ were deferred from the ratios of $K_{off,app}/K_{on,app}$ as described in "Experimental Procedures". Means ± SEM (n=3-5); SEM $\leq$ 5% of the mean in A and B. $K_{d,app}$ (mean ± SD) values were determined from 6-9 different concentrations of Aβ and 3-5 independent measurements at each concentration. RAP, receptor-associated protein (500 nM); RU, resonance units.

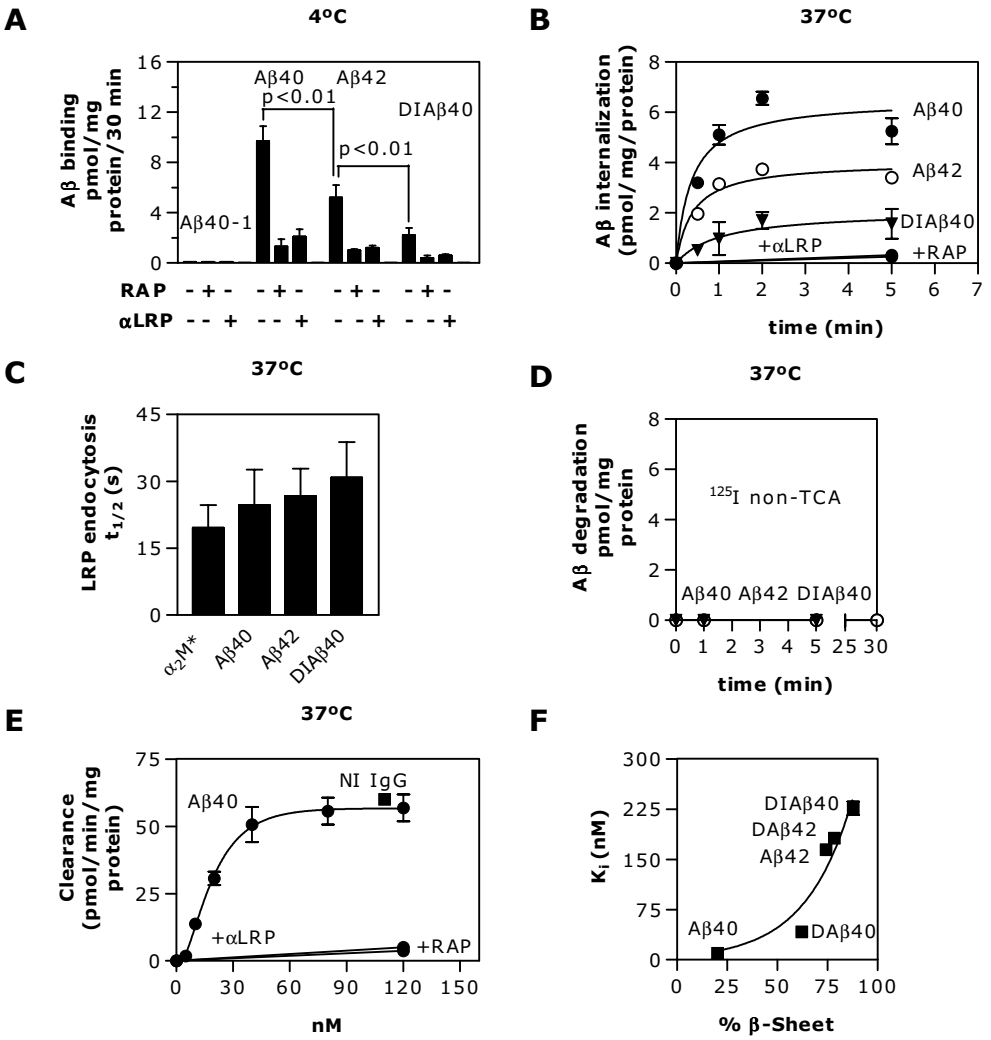


Figure 2. LRP-mediated Aβ binding, internalization and clearance on mouse brain capillaries. *A*. Binding of ^{125}I-Aβ ligands (2 nM) to the abluminal brain capillary surface was determined at 4ºC for 30 min. RAP (500 nM); αLRP, LRP-specific IgG (Fab_2, 60 μg/ml); Aβ40-1, reverse Aβ40 peptide. *B*. Internalization of Aβ peptides at the abluminal brain capillary surface. Capillaries were incubated with ^{125}I-Aβ ligands (2 nM) at 4ºC for 30 min and then at 37°C for the indicated times. The fraction of internalized ligand was calculated as in the "Experimental Procedures" and plotted against the internalization time. RAP (500 nM); αLRP (60 μg/ml). *C*. The half-time ($t_{1/2}$) of LRP endocytosis determined with α_2M* and different Aβ ligands from Fig. 2B. *D*. Undetectable ^{125}I non-TCA precipitable counts (^{125}I non-TCA) in lysed BEC during internalization of ^{125}I-Aβ ligands (2 nM). *E*. Rapid saturable Aβ40 uptake by brain capillaries determined at 37ºC within 1 min using ^{125}I-Aβ40 (1 nM) and increasing concentrations of unlabeled Aβ (1-120 nM). RAP (1 μM); αLRP and a non-immune IgG, NI IgG (60 μg/ml). *F*. K_i (inhibitory constant) for LRP-mediated brain capillary clearance of Aβ isoforms plotted against Aβ β-sheet content. K_i values were determined with ^{125}I-Aβ40 (2 nM) and 40 nM unlabeled Aβ40, Aβ42 and mutant Aβ (Dutch40, DAβ40; Dutch42, DAβ42; Dutch/Iowa40, DIAβ40). Means ± SEM (n=3-5).

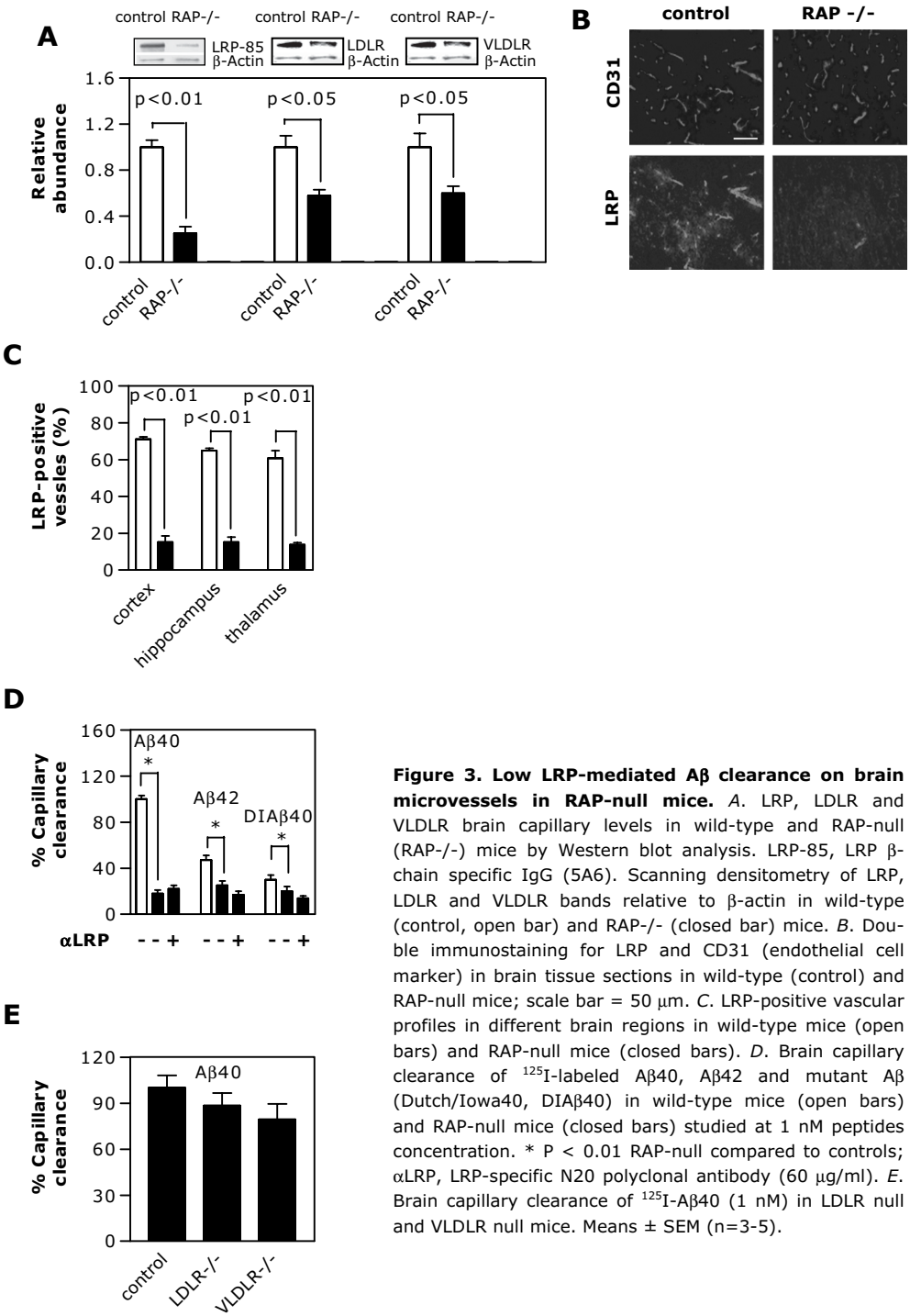


Figure 3. Low LRP-mediated Aβ clearance on brain microvessels in RAP-null mice. *A*. LRP, LDLR and VLDLR brain capillary levels in wild-type and RAP-null (RAP-/-) mice by Western blot analysis. LRP-85, LRP β-chain specific IgG (5A6). Scanning densitometry of LRP, LDLR and VLDLR bands relative to β-actin in wild-type (control, open bar) and RAP-/- (closed bar) mice. *B*. Double immunostaining for LRP and CD31 (endothelial cell marker) in brain tissue sections in wild-type (control) and RAP-null mice; scale bar = 50 μm. *C*. LRP-positive vascular profiles in different brain regions in wild-type mice (open bars) and RAP-null mice (closed bars). *D*. Brain capillary clearance of ^{125}I-labeled Aβ40, Aβ42 and mutant Aβ (Dutch/Iowa40, DIAβ40) in wild-type mice (open bars) and RAP-null mice (closed bars) studied at 1 nM peptides concentration. * P < 0.01 RAP-null compared to controls; αLRP, LRP-specific N20 polyclonal antibody (60 μg/ml). *E*. Brain capillary clearance of ^{125}I-Aβ40 (1 nM) in LDLR null and VLDLR null mice. Means ± SEM (n=3-5).

Figure 2A shows differential LRP-dependent binding of Aβ isoforms at the abluminal side of brain capillaries, *ie*, Aβ40 > Aβ42 > Dutch/Iowa Aβ40, and no binding of the reverse 40-1 peptide, consistent with the SPR findings (*Fig 1*). Binding of Aβ was displaced by RAP and LRP-specific IgG (*Fig 2A*), and excess of unlabeled ligand (not shown). Fig 2B illustrates differential LRP-mediated internalization of ^{125}I-labeled Aβ isoforms into brain endothelium, *ie*, Aβ40 > Aβ42 > DIAβ40, and inhibition (> 85%) with RAP and LRP-specific IgG. The half-time ($t_{1/2}$) of LRP internalization in the presence of different Aβ isoforms was similar between 26 and 30 s (*Fig 1C*). During internalization, there was no significant degradation of Aβ by brain endothelium as indicated by negligible non-trichloracetic acid (TCA)-precipitable counts in brain capillary cell lysates (*Fig 1D*) and in the medium (not shown). The short-term kinetic uptake studies at 37ºC confirmed a saturable LRP-dependent clearance of Aβ40 by brain capillaries with the Michaelis constant K_m of 10 ± 2 nM, and > 85% inhibition of uptake by RAP and LRP-specific IgG (*Fig 2D*). The kinetic inhibitory constants, K_i, confirmed that Dutch Aβ40, wild-type Aβ42, Dutch Aβ42 and Dutch/Iowa Aβ40 exhibit 6, 14, 18 and 22-fold lower affinities for LRP-mediated brain capillary clearance than Aβ40, respectively (*Fig 2E*).

Next, we compared ^{125}I-Aβ clearance by brain microvessels derived from RAP null, LDLR null, VLDLR null and control mice. The Western blot analysis indicated that LRP in brain capillaries of RAP null mice was decreased by > 75% compared to controls (*Fig 3A*), while VLDLR and LDLR levels were reduced approximately by 40%. LRP-positive vascular profiles in different brain regions in RAP null mice were reduced to 14%-16% vs. 60%-70% in controls, as indicated by double staining for LRP and endothelial cell marker CD31 (*Fig 3B and C*). Deletion of the RAP gene resulted in about 80% reduction in brain capillary clearance of Aβ40, Aβ42 and DIAβ40 (*Fig 3D*), while deletion of the VLDLR and LDLR genes did not affect significantly Aβ clearance (*Fig 3E*).

Fig 4A compares efflux across the BBB of [^{125}I]-labeled Aβ40 and mutant Aβ (Dutch/Iowa Aβ40) microinfused simultaneously with ^{14}C-inulin (reference marker) into brain ISF, as described[20]. *In vivo* transcytosis rates of ^{125}I-labeled Aβ at the BBB were obtained after correcting for the tracer's passive diffusion via the ISF bulk flow by subtracting the elimination rate of ^{14}C-inulin, as explained in the "Experimental Procedures". At concentrations comparable to physiological levels of soluble Aβ in brain ISF (*ie*, $\leq$ 1 nM)[36], Aβ40 was cleared rapidly across the BBB within few seconds. In contrast, only 40% of mutant Aβ was cleared across the BBB over a much longer time period of 30 min. At higher concentrations, mutant Aβ was almost devoid of clearance at the BBB, while Aβ40 still exhibited a substantial clearance. RAP and an anti-LRP antibody, but not a non-

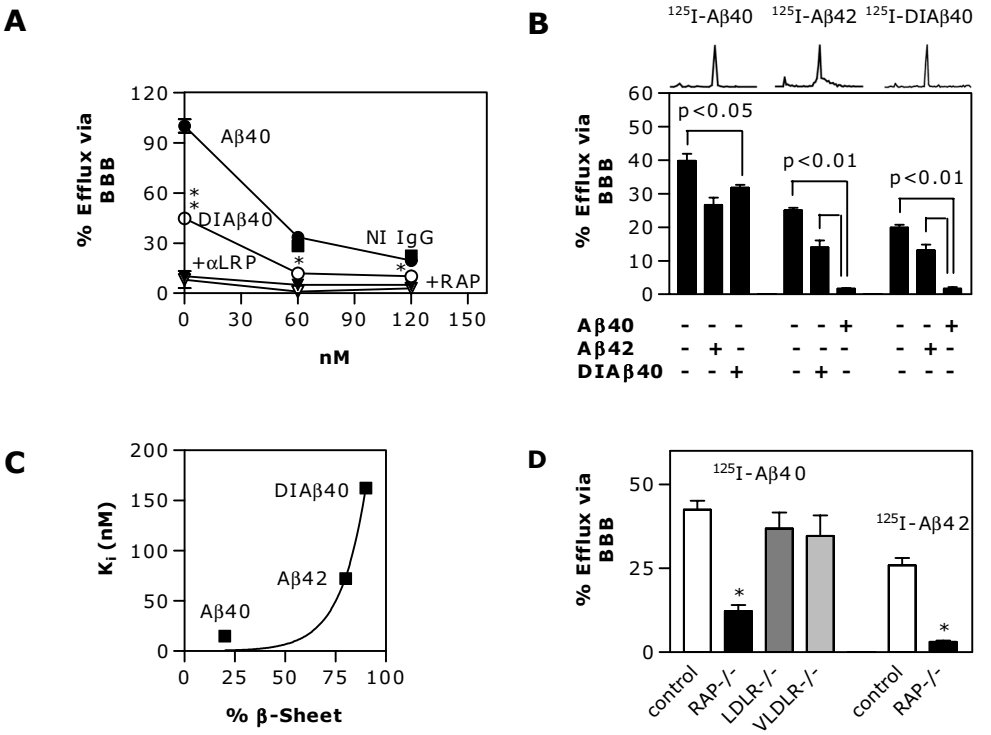


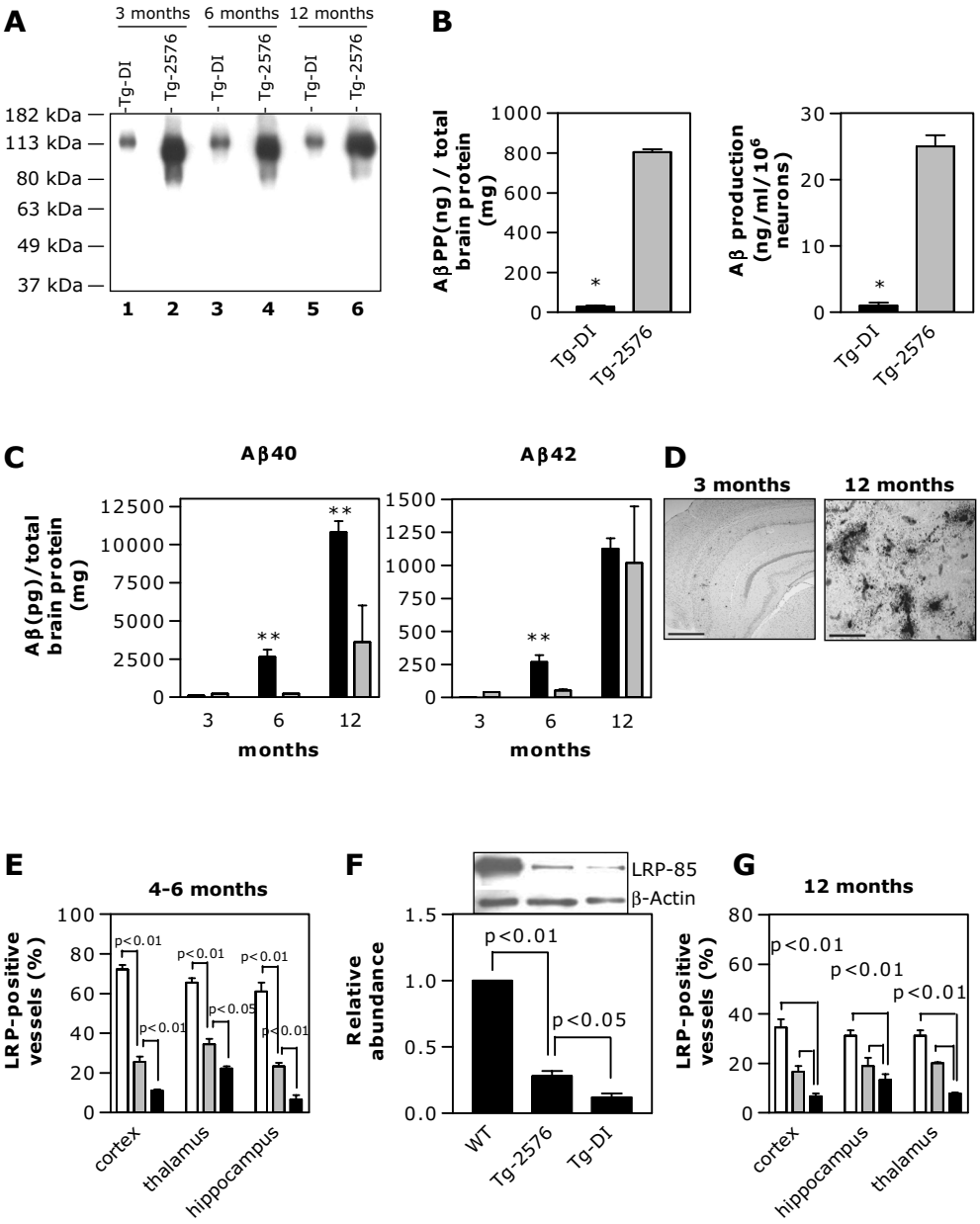
Figure 4. LRP-mediated transport of Aβ across the mouse BBB *in vivo*. *A*. Efflux of mutant Aβ (Dutch/Iowa40, DIAβ40; open circles) vs. wild-type Aβ40 (closed circles) across the BBB within 30 min of brain ISF ^{125}I-Aβ microinjections at 1-120 nM carrier concentration. RAP (1 μM); αLRP, LRP-specific IgG (60 μg/ml); NI IgG, non-immune IgG (60 μg/ml; closed square). ** P < 0.01 and * P < 0.05 for DIAβ40 vs. Aβ40. *B*. Efflux across the BBB of ^{125}I-labeled Aβ40, Aβ42, and mutant Aβ (Dutch/Iowa40, DIAβ40) at 40 nM in the absence and presence of unlabeled Aβ (120 nM). ^{125}I-Aβ monomers (HPLC analysis) in brain homogenates 30 min after microinfusion of ^{125}I/unlabeled Aβ mixture into brain ISF (insets above bars). *C*. K_i values for LRP-mediated efflux at the BBB of Aβ40, Aβ42 and mutant Aβ (Dutch/Iowa40; DIAβ40) plotted against Aβ β-sheets content. K_i values were determined with ^{125}I-Aβ40 at 40 nM and unlabeled Aβ at 120 nM. *D*. Aβ40 and Aβ42 efflux across the BBB in RAP null mice (RAP-/- ; closed bars) vs. wild-type mice (control, open bars), and Aβ40 efflux in LDLR null and VLDLR null mice. Means ± SEM (n=3-8).

immune immunoglobulin G (NI IgG) (*Fig 4A*), almost abolished Aβ elimination across the BBB.

A significant ($p < 0.05$) cross-inhibition of [^{125}I]-Aβ40 efflux at the BBB by unlabeled Aβ42 and mutant Aβ, and a pronounced > 95% inhibition of [^{125}I]-labeled Aβ42 and mutant Aβ clearance by unlabeled wild-type Aβ40 (*Fig 4B*), indicated all Aβ peptides compete for the same LRP-mediated efflux system to exit the brain, and Aβ40 exerts a significant retention effect on Aβ42 and mutant Aβ *in vivo*. All Aβ test-ligands microinfused into brain ISF in the presence of unlabeled Aβ remained > 97% in their monomeric forms[20;35], as confirmed by the HPLC (*Fig 4B, insets*) and SDS-PAGE analysis (not shown) of brain homogenates. During relatively short incubation time and low nM levels of ^{125}I-Aβ ligand and the cold peptide, ^{125}I-Aβ did not bind to unlabeled Aβ, consistent with reports suggesting significantly higher concentrations of Aβ and longer times are required for Aβ oligomerization or aggregation[43;44].

The K_i values indicated Aβ42 and mutant Aβ exhibit 8 and 15-fold lower affinity for LRP-mediated efflux at the BBB *in vivo* than Aβ40 consistent with their high β-sheet content (*Fig 4C*). There was 75% to 85% inhibition of Aβ40 and Aβ42 rapid efflux across the BBB in RAP null/severely depleted LRP mice (*Fig 4D*), while deletion of the LDLR and VLDR genes did not affect significantly Aβ clearance at the BBB (*Fig 4D*). Since LDLR and VLDLR do not contribute to rapid Aβ clearance in RAP null mice (*Fig 3E, 4D*), and LRP on neurons *in vivo* does not mediate Aβ clearance[19], one can speculate that increased amount of amyloid in double crossed RAP null/APP overexpressing mice[45] could be related to inefficient LRP-mediated Aβ clearance across the BBB.

(Page 95) **Figure 5. Aβ accumulation in transgenic mice expressing low LRP-clearance mutant Aβ vs. wild-type Aβ.** *A*. Immunoblot analysis of human APP in the brain of transgenic Tg-DI mice expressing mutant APP harboring Dutch and Iowa mutations vs. Tg-2576 mice. *B*. APP levels in the brain of Tg-DI mice vs. Tg-2576 mice at 6 months of age (left) and Aβ production from isolated mouse neurons into culture medium (right) determined by quantitative immunoblot analyses. *C*. Brain accumulation of low LRP-clearance mutant Aβ40 and Aβ42 (Dutch/Iowa, black bars) in Tg-DI mice vs. wild-type Aβ40 and Aβ42 (gray bars) in Tg-2576 mice. *D*. Early deposits of mutant Aβ (Dutch/Iowa) in the brain of Tg-DI mice at 3 months of age (scale bar = 200 μm), and abundant diffuse deposits with significant intracerebral microvascular Aβ deposits in Tg-DI mice at 12 months of age (scale bar = 50 μm) detected by immunostaining for Aβ. *E*. LRP-positive brain microvessels in Tg-DI mice (black bars), Tg-2576 mice (gray bars) and controls (open bars) at 4-6 months of age. *F*. Western blot analysis of LRP levels in mice in E. *G*. LRP-positive microvessels in Tg-DI mice (black bars), Tg-2576 mice (gray bars) and controls (open bars) at 12 months of age. Mean ± SEM (n = 4 mice). * $P < 0.001$; ** $P < 0.01$ in B and C for APP and Aβ levels in Tg-DI mice compared to Tg-2576 mice.


A
3 months
6 months
12 months
Tg-DI
Tg-2576
182 kDa
113 kDa
80 kDa
63 kDa
49 kDa
37 kDa
1 2 3 4 5 6
B
AβPP(ng) / total brain protein (mg)
Aβ production (ng/ml/10⁶ neurons)
C
Aβ40
Aβ42
Aβ(pg)/total brain protein (mg)
months
D
3 months
12 months
E
4-6 months
LRP-positive vessels (%)
p<0.01
p<0.05
cortex
thalamus
hippocampus
F
LRP-85
β-Actin
Relative abundance
WT
Tg-2576
Tg-DI
G
12 months
LRP-positive vessels (%)
cortex
hippocampus
thalamus

To validate our clearance hypothesis for endogenous Aβ, we compared Aβ accumulation in transgenic Dutch/Iowa (Tg-DI) mice expressing low levels of human APP under control of Thy 1.2 neuronal promoter harboring the Dutch[46] and Iowa[47] vasculotropic mutations[37] vs. Tg-2576 APP overexpressing mice[25]. Tg-DI mice produce mainly mutant Aβ40(Dutch/Iowa) (*Fig 5C*) that compared to the wild-type Aβ40 binds to LRP with significantly lower affinity (*Fig 2B and C*) and exhibits low LRP-clearance on brain capillaries (*Fig 2B, 3D*) and across the BBB (*Fig 4A and C*). At 3, 6 and 12 months of age APP levels in the brain of Tg-DI mice vs. Tg-2576 mice were considerably lower by 24-fold (*Fig 5A and B, left*). In fact, the Tg-DI mice express human APP at < 50% the levels of endogenous mouse APP, as reported in three independent lines[37]. Despite substantially lower levels of human APP (*Fig 5B, left*) and > 30-fold lower neuronal production of Aβ compared to Tg-2576 mice (*Fig 5B, right*), the Tg-DI mice exhibited earlier onset and more robust brain accumulations of Aβ than Tg-2576 mice (*Fig 5C*), *ie*, by 15- and 5-fold higher for the 40 and 42 isoforms, respectively, at 6 months of age.

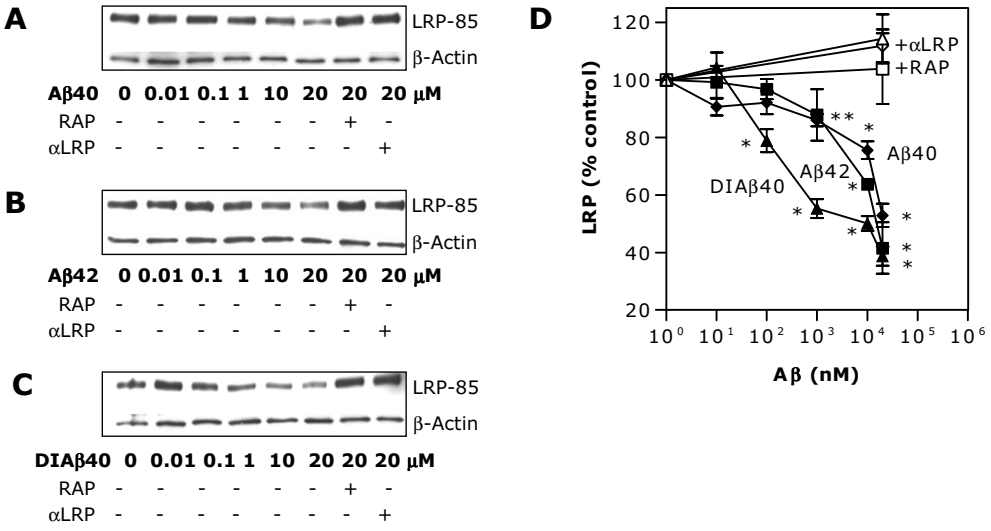


Figure 6. LRP downregulation in human brain endothelium by excess Aβ. *A-C.* Western blot analysis of LRP β-subunit (LRP-85) in brain endothelium exposed for 48 h to different concentrations of Aβ40, Aβ42 and mutant Aβ (Dutch/Iowa40; DIAβ40) from 1 nM to 20 μM. RAP (1.25 μM) and αLRP, LRP β-chain-specific IgG F(ab)$_2$ (25 μg/ml) were incubated 1 h prior to the addition of Aβ peptides and than for 48 h simultaneously with different Aβ peptides. *D*. Relative intensity of LRP bands in brain endothelium exposed to different Aβ peptides was determined by scanning densitometry and normalized for β-actin. Mean ± SEM (n=3-5 independent experiments); * P < 0.01; ** P < 0.05 for the relative expression of LRP levels in the presence of a given Aβ concentration compared to the absence Aβ.

Tg-DI mice developed early Aβ plaque-like deposits in the cortex and hippocampus at 3 months of age (*Fig 5D, left*), while the Tg-2576 mice[25;48] initially present Aβ deposits at ≈ 9 months of age (not shown). Similarly, the APP/PS-1 knock in mice homozygous for both the humanized APP and the PS-1 knock in mutation, which causes an increase in the amount of Aβ42, only develop Aβ deposits at ≈ 6 months of age[49]. Abundant Aβ plaque-like diffuse deposits with significant microvascular intracerebral association were found in Tg-DI mice at 12 months (*Fig 5D, right*). Plasma levels of mutant Aβ in Tg-DI mice were extremely low, *ie*, < 25 pM, consistent with the low clearance across the BBB (*Fig 4A*). Double immunostaining for LRP and endothelial cell marker CD31 indicated substantial reduction of LRP-positive vascular profiles in several brain regions in Tg-DI and Tg-2576 mice at 4-6 months of age, *ie*, only 5-20% and 25-30% of microvessels were positive for LRP, respectively, compared to 65-75% of LRP-positive vessels in age-matched littermate controls (*Fig 5E*). Significant LRP brain capillary reduction in transgenic mice was confirmed by Western blot analysis (*Fig 5F*). The decrease in LRP vascular profiles at 12 months of age was also more pronounced in Aβ-accumulating transgenic mice than in controls (*Fig 5G*).

Fig 6A-D indicate that Aβ40, Aβ42 and/or mutant Aβ (Dutch/Iowa Aβ40) at lower concentrations ($\leq$ 10 nM) do not alter LRP expression in primary human brain endothelial cells (BEC). However, at levels $\geq$ 1 μM over longer periods of time (*ie*, 48 h), all Aβ species down regulated LRP in BEC in a concentration-dependent manner, as demonstrated for LRP 85-kDa β-subunit (*Fig 6A-D*) and 515-kDa α-subunit (not shown). RAP and LRP-specific IgG, but not a control non-immune IgG (not shown), blocked Aβ-induced LRP down regulation. Aβ treatment did not reduce the expression of other cell surface receptors in BEC, as for example, the levels of the receptor for advanced glycation end products (RAGE) were increased (not shown) as reported[29] and the levels transferrin receptor remain unchanged (not shown). TUNEL and Hoechst staining in BEC were negative, and the levels of lactic acid dehydrogenase in the medium did not increase from control values (not shown).

Aβ40, Aβ42 and mutant Aβ at 20 μM over 48 h significantly reduced (> 60%) the amount of internalized ^{125}I-α_2M* into human BEC (*Fig 7A*) consistent with significantly reduced LRP levels (*Fig 6D*). However, this treatment did not alter significantly the LRP endocytosis rate, and the $t_{1/2}$ for LRP endocytosis in the presence of different Aβ isoforms remained unchanged compared to controls, *ie*, about 15 s (*Fig 7B*). On the other hand, the pulse-chase studies revealed that Aβ (20 μM) decreases the half-life ($t_{1/2}$) for LRP from 9.6 h to 6.4 h (*Fig 7C and D*). MG132, an inhibitor of the proteasome-dependent LRP degradation[50], increased

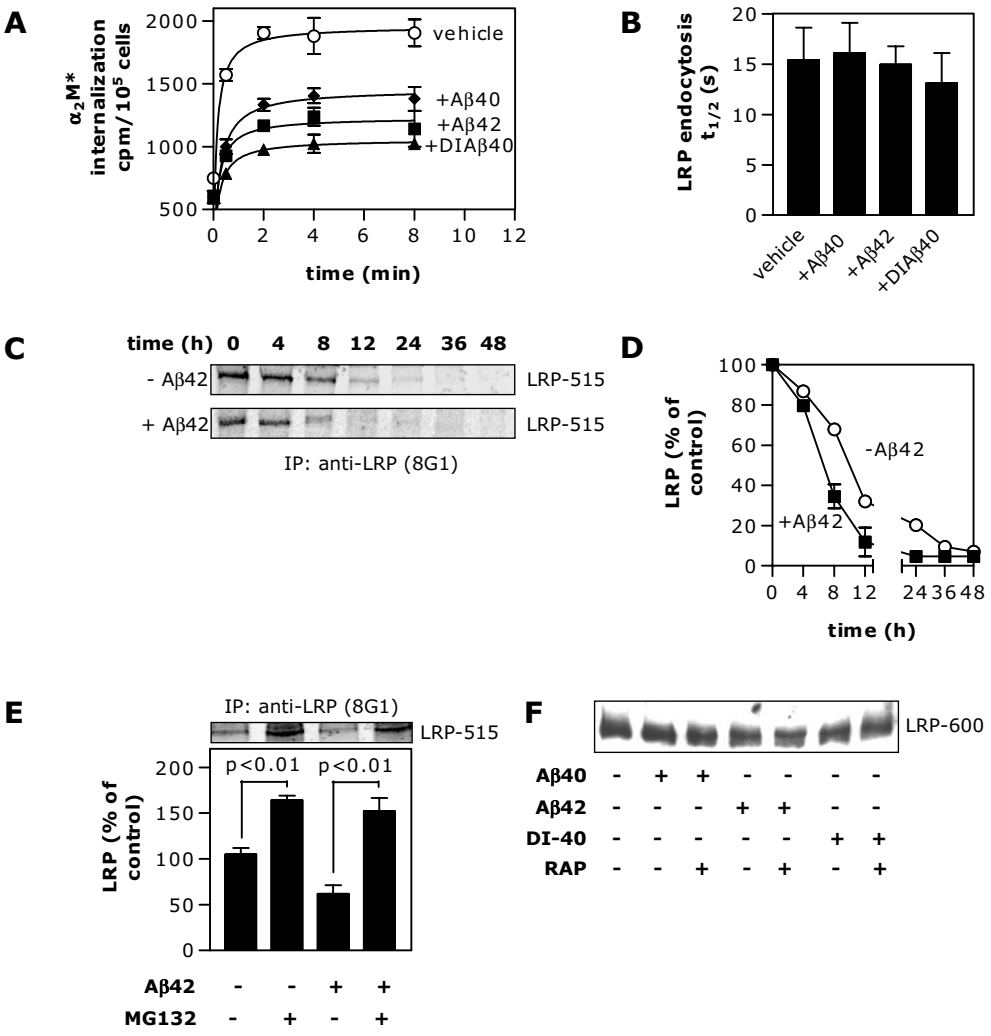


Figure 7. High Aβ levels reduce LRP's half-life in human brain endothelium. *A*. Internalization of ^{125}I-α_2M* by human BEC treated with 20 μM of Aβ40, Aβ42 and mutant Aβ (DIAβ40) for 48 h. *B*. The half-life ($t_{1/2}$) for rapid LRP endocytosis in human BEC in A. determined with ^{125}I-α_2M*. *C*. Cells were labeled by [^{35}S]-methionine for 1 h and chased for the indicated times. Autoradiograph of [^{35}S]-methionine labeled LRP immunoprecipitated with LRP 515 kDa α-subunit-specific IgG in the presence and absence of Aβ42 (20 μM). *D*. Graph of LRP levels from three independent experiments as in A. * P < 0.05; ** P < 0.01 in the presence of Aβ compared to the corresponding control value. *E*. Effects of the proteasomal inhibitor MG132 (20 μM) on LRP levels in the presence and absence of Aβ42 (20 μM) determined at 8 h. Autoradiograph of [^{35}S]-methionine labeled LRP immunoprecipitated with LRP 515 kDa α-subunit specific IgG. *F*. Graph of LRP levels from three independent experiments as in C. *G.* Western blot analysis for immature 600 kDa LRP (LRP-600) in the presence of Aβ40, Aβ42 or mutant Aβ (Dutch/Iowa40, DIAβ40) at 20 μM within 48 h, as demonstrated with LRP-specific IgG directed to the C-terminal region of LRP (5A6). RAP was used at 1.2 μM. Mean ± SEM (n=3) in B and D.

the $t_{1/2}$ for LRP in control and Aβ-treated cells (*Fig 7E and F*). Immunoblot analysis with an anti-LRP C-terminal antibody that recognizes immature 600-kDa form of LRP confirmed the levels of immature LRP are not affected by Aβ treatment (*Fig 7G*), suggesting no effect on LRP synthesis.

LRP-positive vascular profiles in human brain tissue revealed greatly reduced LRP expression in patients with AD and cerebrovascular β-amyloidosis Dutch-type compared to age-matched controls (*Fig 8A and B*). The number of LRP-positive cerebral vessels dropped from 45% in age-matched controls to ≈ 12% in AD, and was barely detectable in patients with Dutch-type cerebrovascular β-amyloidosis.

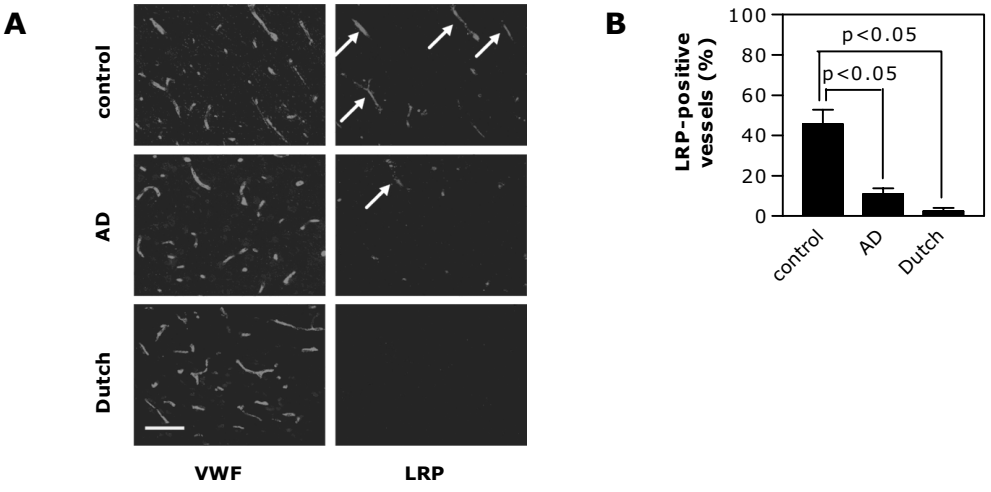


Figure 8. LRP expression in brain microvessels in Alzheimer's and Dutch patients. *A*. Immunostaining for Von Willebrand Factor (VWF, endothelial cell marker) and LRP in cortical sections (Brodman area 10) in age-matched controls and AD and Dutch patients with cerebrovasular β-amyloidosis. Scale bar = 50 μm. *B*. LRP-positive vessels in controls, AD and Dutch patients. Mean ± SEM (n=4 cases/group).

Discussion

Aβ accumulation in the brain is a chief pathogenic event[22]. Increased Aβ42 levels lead to formation of neurotoxic Aβ oligomers resulting in progressive synaptic, neuritic and neuronal dysfunction[43;44;51;52]. Missense mutations inside the Aβ sequence associate with vascular deposits in patients with the Dutch mutation (G to C at codon 693, Glu to Gln at position 22[46]) and Iowa mutation (G to A at codon 694, Asp to Asn at position 23[47]). How Aβ accumulates in the brain is unclear. Increased Aβ production can explain a small fraction of early onset AD familial cases bearing inherited mutations in the APP gene flanking the Aβ coding region (*ie*, Swedish mutation) or PS-1 or 2 genes. This leaves inefficient Aβ clearance as a major mechanism mediating Aβ cerebral accumulation in late-onset AD[53;54] and familial forms of cerebrovascular β-amyloidoses[55].

Here, we report LRP/Aβ direct interaction at the BBB critically influences Aβ brain accumulation by promoting retention of high β-sheet neurotoxic Aβ42 and vasculotropic mutant Aβ, while clearing soluble Aβ40 from brain ISF to blood. The SPR analysis (*Fig 1*) used as a first step to characterize Aβ interaction with the LRP ligand-binding domains[1] revealed Aβ40 binds to LRP with high affinity compared to Aβ42 and mutant Aβ. Consistent with the SPR findings, we next showed that LRP-mediated Aβ brain capillary binding and endocytosis *in vitro* and transcytosis across the BBB *in vivo* in mice are substantially reduced by the high β-sheet content in Aβ (*Fig 2-4*), and deletion of the RAP gene, but not the LDLR and VLDLR genes (*Fig 3 and 4*).

In contrast to remarkable LRP-mediated Aβ40 binding and endocytosis at the abluminal side of brain microvessels (*Fig 2 and 3*) and abluminal to luminal transcytosis across the BBB *in vivo* (*Fig 4*), our earlier study failed to detect significant receptor-mediated Aβ40 basolateral to apical transport in cultured brain endothelial monolayers[56]. It is possible that the density of LRP receptors at the basolateral membrane in the monolayer cultures[56] was much lower than at the abluminal membrane of freshly isolated brain capillaries and at the BBB *in vivo* (*Fig 2-4*), which could account for the loss of LRP-dependent Aβ40 basolateral to apical flux in monolayers[56] and may explain the discrepancies between results obtained in the monolayer transport model vs. present and previous study[20].

The present data show that rapid interaction of Aβ with LRP does not require chaperone molecules. However, this does not rule out the possibility that Aβ may also interact with LRP via chaperone molecules. Thus, both direct and indirect interaction of Aβ with LRP may exist in parallel. For example, α_2M is implicated in slow and/or delayed reuptake of secreted Aβ into neurons isolated from transgenic APP overexpressing mice which might require LRP[15], and may also influ-

ence slow uptake of Aβ into mouse fibroblasts via LRP[16] and/or degradation of Aβ in U97 human glioblastoma cells and mouse fibroblasts via α_2M requires LRP[17].

Despite extremely low human APP expression/Aβ production, *ie*, 25-30-fold lower than Tg-2576 mice, Tg-DI mice[37] expressing low LRP-clearance mutant Aβ develop robust Aβ brain accumulations much earlier than Tg-2576 Aβ-overproducing mice[25;48] (*Fig 5*). Significant intra-parenchymal microvascular Aβ association points to brain's blood vessels as a site of deficient clearance in Tg-DI mice consistent with prominent cerebrovascular pathology in Dutch and Iowa patients[23]. Increased fibrillogenicity of mutant Dutch/Iowa Aβ[27] may contribute to its decreased clearance from brain, but the presence of mainly non-fibrillar Aβ parenchymal deposits in Tg-DI mice would argue against this possibility. In addition to reduced LRP-mediated Aβ efflux, an increased formation of non-fibrillar Aβ species might favor Aβ deposition in this model.

The present data show that Aβ at pathological concentrations reduces LRP levels in brain endothelium by accelerating proteasome-dependent LRP degradation[50], while the receptor's internalization and synthesis are not affected (*Fig 6 and 7*). In contrast to LRP downregulation by simultaneous overexpression of secreted ligands such as ApoE, resulting in retention and degradation of LRP in the endoplasmic reticulum (ER)[2;57], binding of internalized Aβ to immature LRP in the ER compartment is not implicated in LRP degradation by extracellular Aβ. One can speculate that Aβ accumulations adjacent to brain vasculature may down regulate LRP at the BBB *in vivo* consistent with reduced brain capillary LRP levels in transgenic Aβ-accumulating mice (*Fig 5E-G*), AD and patients with the Dutch-type cerebrovascular β-amyloidosis (*Fig 8*), and reduced total brain LRP in AD[16;20].

In contrast to LRP, RAGE mediates a continuous influx of circulating Aβ into the brain and is overexpressed in brain vasculature in transgenic APP models and in AD[29]. Thus, an imbalance between LRP-mediated and RAGE-mediated Aβ transport at the BBB would create a positive feedback amplification mechanism for Aβ accumulation in AD and related familial cerebrovascular disorders. The present findings raise a possibility that efficacy of Aβ lowering therapies with peripheral Aβ sequestering agents[29;58;59] may depend at least in part on preserved LRP activity at the BBB. Whether therapeutic modifications of Aβ/LRP interaction at the BBB will have a major impact on controlling dementia remains to be seen in future.

References

1. Herz, J. and D.K. Strickland. 2001. LRP: a multifunctional scavenger and signaling receptor. *J.Clin.Invest.* 108:779-784.
2. Herz, J. and P. Marschang. 2003. Coaxing the LDL receptor family into the fold. *Cell* 112:289-292.
3. Herz, J., R.C. Kowal, J.L. Goldstein, and M.S. Brown. 1990. Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *EMBO J.* 9:1769-1776.
4. Bu, G. 2001. The roles of receptor-associated protein (RAP) as a molecular chaperone for members of the LDL receptor family. *Int.Rev.Cytol.* 209:79-116.
5. Herz, J. 2001. The LDL receptor gene family: (un)expected signal transducers in the brain. *Neuron* 29:571-581.
6. van der Geer, P. 2002. Phosphorylation of LRP1: regulation of transport and signal transduction. *Trends Cardiovasc.Med.* 12:160-165.
7. Green, P.S. and G. Bu. 2002. Genetics and molecular biology: phosphorylation of low-density lipoprotein receptor-related protein by platelet-derived growth factor. *Curr.Opin.Lipidol.* 13:569-572.
8. Kang, D.E., T. Saitoh, X. Chen, Y. Xia, E. Masliah, L.A. Hansen, R.G. Thomas, L.J. Thal, and R. Katzman. 1997. Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), an apolipoprotein E receptor, with late-onset Alzheimer's disease. *Neurology* 49:56-61.
9. Hollenbach, E., S. Ackermann, B.T. Hyman, and G.W. Rebeck. 1998. Confirmation of an association between a polymorphism in exon 3 of the low-density lipoprotein receptor-related protein gene and Alzheimer's disease. *Neurology* 50:1905-1907.
10. Lambert, J.C., F. Wavrant-De Vrieze, P. Amouyel, and M.C. Chartier-Harlin. 1998. Association at LRP gene locus with sporadic late-onset Alzheimer's disease. *Lancet* 351:1787-1788.
11. Wavrant-DeVrieze, F., J.C. Lambert, L. Stas, R. Crook, D. Cottel, F. Pasquier, B. Frigard, M. Lambrechts, E. Thiry, P. Amouyel, J.P. Tur, M.C. Chartier-Harlin, J. Hardy, and F. van Leuven. 1999. Association between coding variability in the LRP gene and the risk of late-onset Alzheimer's disease. *Hum.Genet.* 104:432-434.
12. Kounnas, M.Z., R.D. Moir, G.W. Rebeck, A.I. Bush, W.S. Argraves, R.E. Tanzi, B.T. Hyman, and D.K. Strickland. 1995. LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell* 82:331-340.
13. Pietrzik, C.U., T. Busse, D.E. Merriam, S. Weggen, and E.H. Koo. 2002. The cytoplasmic domain of the LDL receptor-related protein regulates multiple steps in APP processing. *EMBO J.* 21:5691-5700.
14. Ulery, P.G., J. Beers, I. Mikhailenko, R.E. Tanzi, G.W. Rebeck, B.T. Hyman, and D.K. Strickland. 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. *J.Biol.Chem.* 275:7410-7415.
15. Qiu, Z., D.K. Strickland, B.T. Hyman, and G.W. Rebeck. 1999. Alpha2-macroglobulin enhances the clearance of endogenous soluble beta-amyloid peptide via low-density lipoprotein receptor-related protein in cortical neurons. *J.Neurochem.* 73:1393-1398.
16. Kang, D.E., C.U. Pietrzik, L. Baum, N. Chevallier, D.E. Merriam, M.Z. Kounnas, S.L. Wagner, J.C. Troncoso, C.H. Kawas, R. Katzman, and E.H. Koo. 2000. Modulation of amyloid β-protein clearance and Alzheimer's disease susceptibility by the LDL receptor-related protein pathway. *J.Clin.Invest.* 106:1159-1166.
17. Narita, M., D.M. Holtzman, A.L. Schwartz, and G. Bu. 1997. Alpha2-macroglobulin complexes with and mediates the endocytosis of beta-amyloid peptide via cell surface low-density lipoprotein receptor-related protein. *J.Neurochem.* 69:1904-1911.

18. Jordan, J., M.F. Galindo, R.J. Miller, C.A. Reardon, G.S. Getz, and M.J. LaDu. 1998. Isoform-specific effect of apolipoprotein E on cell survival and beta-amyloid-induced toxicity in rat hippocampal pyramidal neuronal cultures. *J.Neurosci.* 18:195-204.
19. Zerbinatti, C.V., D.F. Wozniak, J. Cirrito, J.A. Cam, H. Osaka, K.R. Bales, M. Zhuo, S.M. Paul, D.M. Holtzman, and G. Bu. 2004. Increased soluble amyloid-beta peptide and memory deficits in amyloid model mice overexpressing the low-density lipoprotein receptor-related protein. *Proc.Natl.Acad.Sci.U.S.A.* 101:1075-1080.
20. Shibata, M., S. Yamada, S.R. Kumar, M. Calero, J. Bading, B. Frangione, D.M. Holtzman, C.A. Miller, D.K. Strickland, J. Ghiso, and B.V. Zlokovic. 2000. Clearance of Alzheimer's amyloid-ss(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. *J.Clin.Invest.* 106:1489-1499.
21. Wang, X., S.R. Lee, K. Arai, S.R. Lee, K. Tsuji, G.W. Rebeck, and E.H. Lo. 2003. Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator. *Nat.Med.* 9:1313-1317.
22. Hardy, J. and D.J. Selkoe. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297:353-356.
23. Vinters, H.V. and E.S. Farag. 2003. Amyloidosis of cerebral arteries. *Adv.Neurol.* 92:105-112.
24. Veinbergs, I., E. Van Uden, M. Mallory, M. Alford, C. McGiffert, R. DeTeresa, R. Orlando, and E. Masliah. 2001. Role of apolipoprotein E receptors in regulating the differential in vivo neurotrophic effects of apolipoprotein E. *Exp.Neurol.* 170:15-26.
25. Hsiao, K., P. Chapman, S. Nilsen, C. Eckman, Y. Harigaya, S. Younkin, F. Yang, and G. Cole. 1996. Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science* 274:99-102.
26. Burdick, D., B. Soreghan, M. Kwon, J. Kosmoski, M. Knauer, A. Henschen, J. Yates, C. Cotman, and C. Glabe. 1992. Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. *J.Biol.Chem.* 267:546-554.
27. Van Nostrand, W.E., J.P. Melchor, H.S. Cho, S.M. Greenberg, and G.W. Rebeck. 2001. Pathogenic effects of D23N Iowa mutant amyloid beta-protein. *J.Biol.Chem.* 276:32860-32866.
28. Westein, E., C.V. Denis, B.N. Bouma, and P.J. Lenting. 2002. The alpha-chains of C4b-binding protein mediate complex formation with low density lipoprotein receptor-related protein. *J.Biol.Chem.* 277:2511-2516.
29. Deane, R., Y.S. Du, R.K. Submamaryan, B. LaRue, S. Jovanovic, E. Hogg, D. Welch, L. Manness, C. Lin, J. Yu, H. Zhu, J. Ghiso, B. Frangione, A. Stern, A.M. Schmidt, D.L. Armstrong, B. Arnold, B. Liliensiek, P. Nawroth, F. Hofman, M. Kindy, D. Stern, and B. Zlokovic. 2003. RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain. *Nat.Med.* 9:907-913.
30. Zlokovic, B.V., C.L. Martel, E. Matsubara, J.G. McComb, G. Zheng, R.T. McCluskey, B. Frangione, and J. Ghiso. 1996. Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid beta at the blood-brain and blood-cerebrospinal fluid barriers. *Proc.Natl.Acad.Sci.U.S.A.* 93:4229-4234.
31. Golabek, A.A., C. Soto, T. Vogel, and T. Wisniewski. 1996. The interaction between apolipoprotein E and Alzheimer's amyloid beta-peptide is dependent on beta-peptide conformation. *J.Biol.Chem.* 271:10602-10606.
32. Wu, Z., F.M. Hofman, and B.V. Zlokovic. 2003. A simple method for isolation and characterization of mouse brain microvascular endothelial cells. *J.Neurosci.Methods* 130:53-63.
33. Li, Y., W. Lu, M.P. Marzolo, and G. Bu. 2001. Differential functions of members of the low density lipoprotein receptor family suggested by their distinct endocytosis rates. *J.Biol.Chem.* 276:18000-18006.
34. Li, Y., P. van Kerkhof, M.P. Marzolo, G.J. Strous, and G. Bu. 2001. Identification of a major cyclic AMP-dependent protein kinase A phosphorylation site within the cytoplasmic tail of the low-density lipoprotein receptor-related protein: implication for receptor-mediated endocytosis. *Mol.Cell.Biol.* 21:1185-1195.

35. Zlokovic, B.V., S. Yamada, D. Holtzman, J. Ghiso, and B. Frangione. 2000. Clearance of amyloid beta-peptide from brain: transport or metabolism? *Nat.Med.* 6:718-719.
36. Cirrito, J.R., P.C. May, M.A. O'Dell, J.W. Taylor, M. Parsadanian, J.W. Cramer, J.E. Audia, J.S. Nissen, K.R. Bales, S.M. Paul, R.B. DeMattos, and D.M. Holtzman. 2003. In vivo assessment of brain interstitial fluid with microdialysis reveals plaque-associated changes in amyloid-beta metabolism and half-life. *J.Neurosci.* 23:8844-8853.
37. Davis, J., F. Xu, R. Deane, G. Romanov, M.L. Previti, K. Zeigler, B.V. Zlokovic, and W.E. Van Nostrand. 2004. Early-onset and robust cerebral microvascular accumulation of amyloid beta-protein in transgenic mice expressing low levels of a vasculotropic Dutch/Iowa mutant form of amyloid beta-protein precursor. *J.Biol.Chem.* 279:20296-20306.
38. DeMattos, R.B., M.A. O'Dell, M. Parsadanian, J.W. Taylor, J.A. Harmony, K.R. Bales, S.M. Paul, B.J. Aronow, and D.M. Holtzman. 2002. Clusterin promotes amyloid plaque formation and is critical for neuritic toxicity in a mouse model of Alzheimer's disease. *Proc.Natl.Acad.Sci.U.S.A.* 99:10843-10848.
39. Sata, M., A. Saiura, A. Kunisato, A. Tojo, S. Okada, T. Tokuhisa, H. Hirai, M. Makuuchi, Y. Hirata, and R. Nagai. 2002. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat.Med.* 8:403-409.
40. Cheng, T., D. Liu, J.H. Griffin, J.A. Fernandez, F. Castellino, E.D. Rosen, K. Fukudome, and B.V. Zlokovic. 2003. Activated protein C blocks p53-mediated apoptosis in ischemic human brain endothelium and is neuroprotective. *Nat.Med.* 9:338-342.
41. Guenette, S.Y., Y. Chang, B.T. Hyman, R.E. Tanzi, and G.W. Rebeck. 2002. Low-density lipoprotein receptor-related protein levels and endocytic function are reduced by overexpression of the FE65 adaptor protein, FE65L1. *J.Neurochem.* 82:755-762.
42. Guo, H., D. Liu, H. Gelbard, T. Cheng, R. Insalaco, J.A. Fernandez, J.H. Griffin, and B.V. Zlokovic. 2004. Activated protein C prevents neuronal apoptosis via protease activated receptors 1 and 3. *Neuron* 41:563-572.
43. Dahlgren, K.N., A.M. Manelli, W.B. Stine, Jr., L.K. Baker, G.A. Krafft, and M.J. LaDu. 2002. Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J.Biol.Chem.* 277:32046-32053.
44. Kayed, R., E. Head, J.L. Thompson, T.M. McIntire, S.C. Milton, C.W. Cotman, and C.G. Glabe. 2003. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 300:486-489.
45. Van Uden, E., M. Mallory, I.V Einbergs, M. Alford, E. Rockenstein, and E. Masliah. 2002. Increased extracellular amyloid deposition and neurodegeneration in human amyloid precursor protein transgenic mice deficient in receptor-associated protein. *J.Neurosci.* 22:9298-9304.
46. Levy, E., M.D. Carman, I.J. Fernandez-Madrid, M.D. Power, I. Lieberburg, S.G. Van Duinen, G.T. Bots, W. Luyendijk, and B. Frangione. 1990. Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. *Science* 248:1124-1126.
47. Grabowski, T.J., H.S. Cho, J.P. Vonsattel, G.W. Rebeck, and S.M. Greenberg. 2001. Novel amyloid precursor protein mutation in an Iowa family with dementia and severe cerebral amyloid angiopathy. *Ann.Neurol.* 49:697-705.
48. Kawarabayashi, T., L.H. Younkin, T.C. Saido, M. Shoji, K.H. Ashe, and S.G. Younkin. 2001. Age-dependent changes in brain, CSF, and plasma amyloid (beta) protein in the Tg2576 transgenic mouse model of Alzheimer's disease. *J.Neurosci.* 21:372-381.
49. Flood, D.G., A.G. Reaume, K.S. Dorfman, Y.G. Lin, D.M. Lang, S.P. Trusko, M.J. Savage, W.G. Annaert, B. De Strooper, R. Siman, and R.W. Scott. 2002. FAD mutant PS-1 gene-targeted mice: increased A beta 42 and A beta deposition without APP overproduction. *Neurobiol.Aging* 23:335-348.
50. Melman, L., H.J. Geuze, Y. Li, L.M. McCormick, P. van Kerkhof, G.J. Strous , A.L. Schwartz, and G. Bu. 2002. Proteasome regulates the delivery of LDL receptor-related protein into the degradation pathway. *Mol.Biol.Cell* 13:3325-3335.

51. Walsh, D.M., I. Klyubin, J.V. Fadeeva, W.K. Cullen, R. Anwyl, M.S. Wolfe, M.J. Rowan, and D.J. Selkoe. 2002. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416:535-539.
52. Gong, Y., L. Chang, K.L. Viola, P.N. Lacor, M.P. Lambert, C.E. Finch, G.A. Krafft, and W.L. Klein. 2003. Alzheimer's disease-affected brain: presence of oligomeric A beta ligands (ADDLs) suggests a molecular basis for reversible memory loss. *Proc.Natl.Acad.Sci.U.S.A.* 100:10417-10422.
53. Selkoe, D.J. 2001. Clearing the brain's amyloid cobwebs. *Neuron* 32:177-180.
54. Zlokovic, B.V. and B. Frangione. 2003. Transport-clearance hypothesis for Alzheimer's disease and potential therapeutic implications. *In* Aβ metabolism in Alzheimer's Disease. Georgetown, TX: Landes Bioscience, 114-122.
55. Ghiso, J. and B. Frangione. 2002. Amyloidosis and Alzheimer's disease. *Adv.Drug Deliv.Rev.* 54:1539-1551.
56. Mackic, J.B., M. Stins, J.G. McComb, M. Calero, J. Ghiso, K.S. Kim, S.D. Yan, D. Stern, A.M. Schmidt, B. Frangione, and B.V. Zlokovic. 1998. Human blood-brain barrier receptors for Alzheimer's amyloid-beta 1-40. Asymmetrical binding, endocytosis, and transcytosis at the apical side of brain microvascular endothelial cell monolayer. *J.Clin.Invest.* 102:734-743.
57. Willnow, T.E., A. Rohlmann, J. Horton, H. Otani, J.R. Braun, R.E. Hammer, and J. Herz. 1996. RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor-related endocytic receptors. *EMBO J.* 15:2632-2639.
58. DeMattos, R.B., K.R. Bales, D.J. Cummins, S.M. Paul, and D.M. Holtzman. 2002. Brain to plasma amyloid-beta efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. *Science* 295:2264-2267.
59. Matsuoka, Y., M. Saito, J. LaFrancois, M. Saito, K. Gaynor, V. Olm, L. Wang, E. Casey, Y. Lu, C. Shiratori, C. Lemere , and K. Duff. 2003. Novel therapeutic approach for the treatment of Alzheimer's disease by peripheral administration of agents with an affinity to beta-amyloid. *J.Neurosci.* 23:29-33.

chapter 5

Cellular uptake of C4b-binding protein is coordinated by heparan sulfate proteoglycans and LDL receptor-related protein

Patricia P.E.M. Spijkers[1], Anna M. Blom[2] and Peter J. Lenting[1]

[1] Laboratory for Thrombosis and Haemostasis, Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands, [2] Department of Clinical Chemistry, Lund University, The Wallenberg Laboratory, University Hospital Malmö, Sweden.

In preparation

Abstract

C4b-binding protein (C4BP) is a plasma protein involved in both complement and coagulation pathways. C4BP interacts with anticoagulant protein S via its β-chain, while the α-chain comprises binding sites for C4b, heparin and bacterial components. Recently, low-density lipoprotein receptor-related protein (LRP) was found to mediate the cellular uptake of C4BP *in vitro* and *in vivo* [Westein et al, 2002]. Moreover, this uptake involved the binding of LRP to the C4BP α-chains. In the present study, the interaction between C4BP and LRP is further characterized. By using an array of C4BP mutants with replacements of positively charged residues in the α-chain, we demonstrated that several cationic amino acids contribute to the interaction with LRP when assessed by surface plasmon resonance analysis. Furthermore, the triple mutant rC4BPα/R39Q-R64Q-R66Q is also less efficiently degraded by CHO cells expressing LRP. Since C4BP efficiently binds heparin, we explored the potential cooperative action of LRP with cell-surface exposed heparan sulfate proteoglycans (HSPGs) in the endocytosis of C4BP. Indeed, CHO cells lacking HSPGs bound and internalized less C4BP than wild-type CHO cells, which endogenously express LRP. Finally, we assessed the effect of protein S on the interaction between C4BP and LRP. Using two distinct approaches, we observed that protein S is unable to interfere with complex formation between LRP and C4BP. In conclusion, binding and uptake of C4BP is coordinated by both HSPGs and LRP. Furthermore, the formation of the trimolecular LRP/C4BP/protein S complex suggests that LRP indirectly contributes to the clearance of C4BP-bound protein S.

Introduction

C4b-binding protein (C4BP) is a component of both complement and coagulation pathways[1;2]. In the complement pathway, it enhances factor I-mediated degradation of C4b and C3b[3;4], and prevents the assembly of the C3-convertase complex by binding C4b[5]. C4BP is an approximately 570-kDa plasma protein with an average plasma concentration of 150 mg/L[6-8]. It is synthesized in the liver as a heterologous, multimeric protein: it contains 6 or 7 identical α-chains of 70 kDa (C4BPα) and 80% of the molecules also contains a unique β-chain of 45 kDa (C4BPαβ)[9;10]. All chains are interconnected via their C-terminal domains through disulfide bridges[11]. The α- and β-chains are composed of 8 or 3 so-called complement control protein (CCP)- or Sushi domains, respectively.

C4BPαβ plays a role in the coagulation pathway via its β-chain, which has been found to bind protein S[2;12]. Protein S acts as a cofactor for activated protein C in the degradation of coagulation factors Va and VIIIa[13;14]. 60% of plasma protein S is associated with C4BPαβ. This interaction inhibits the cofactor activity of protein S[15]. The α-chains harbor binding sites for C4b[11;16]. C4BP is also able to bind other ligands, such as serum amyloid P[17], factor VIII[18], components of pathogenic bacteria[19] and heparin[3;16]. The plasma concentration of C4BP is determined by the balance between synthesis and clearance. C4BPα is increased dramatically during an inflammatory process, whereas C4BPαβ displays stable levels[20]. Recently, we identified low-density lipoprotein (LDL) receptor-related protein (LRP) as a receptor that mediates the cellular clearance of C4BP *in vitro* and *in vivo*[21].

LRP is a member of the LDL receptor family that is expressed in a wide spectrum of cells, including hepatocytes, fibroblasts, neurons and monocytes[22]. LRP consists of a non-covalently linked heavy and light chain. The 85-kDa light chain comprises the intracellular and transmembrane domain. The intracellular domain harbors 2 NP*X*Y and 1 Y*XX*L motifs that are implicated in binding of adaptor proteins involved in endocytosis and cellular signaling[23;24]. The 515-kDa heavy chain contains four clusters of complement-type repeats. Clusters II and IV play a dominant role in ligand binding[25;26]. Ligands that are recognized by LRP are structurally and functionally unrelated[21]. Among them are apolipoproteins, lipases, coagulation factors and β2-integrins[27-30].

The molecular mechanism of cellular C4BP binding has not been completely investigated yet. For other heparin-binding LRP ligands, such as tissue factor pathway inhibitor, factor VIII, activated factor IX and thrombospondin[31-33], it has been described that their cellular binding is facilitated by heparan sulfate proteoglycans (HSPGs). HSPGs are abundantly expressed at the cell surface and consist of a core protein and heterogeneous heparan sulfate side chains[34]. After

sequestration from the plasma by HSPGs, ligands can be efficiently transferred to their specific receptor, such as LRP.

Heparin has been demonstrated to prevent the binding of C4BP to LRP[21]. The present study focused on the role of the heparin-binding sites of C4BP in its binding to the cell surface and LRP. Our data show that rC4BPα mutants with decreased heparin-affinity[35] were able to bind to immobilized LRP less efficiently. The R39Q-R64Q-R66Q triple mutant showed reduced clearance by mouse fibroblasts. The initial binding of rC4BP to CHO cells was predominantly dependent on the presence of HSPGs, resulting in a delayed LRP-mediated cellular clearance. Finally, the protein S/C4BPαβ complex was able to bind to LRP, suggesting that LRP indirectly contributes to the binding and probably clearance of protein S.

Experimental Procedures

Materials

The Biacore2000 biosensor system and reagents, including an amine-coupling kit and CM5 biosensor chips (research grade), were from Biacore AB (Uppsala, Sweden). All cell culture disposables were from Nunc (Roskilde, Denmark). Bovine Serum Albumin (BSA) fraction V was from Sigma (Zwijndrecht, The Netherlands).

Proteins

Recombinant C4BP, consisting of the α-chains but lacking the β-chain (rC4BPα), was produced using stably transfected baby hamster kidney cell lines, purified by immunoaffinity chromatography as reported previously[36], and stored in 125 mM NaCl, 0.005% (v/v) Tween 20, 25 mM Hepes (pH 7.4) at -20ºC until use. rC4BPα isoforms mutated in positively charged regions were previously described in ref 35. Plasma-derived C4BP containing both α- and β-chains (C4BPαβ) was purified as described[37]. rC4BPα was radio-labeled with $Na^{125}I$ (Amersham Biosciences) using IodoBeads (Pierce, Rockford, IL) according to the manufacturer's protocol. Free ^{125}I was removed by extensive dialysis against 125 mM NaCl, 25 mM Hepes pH 7.4. Purified full-length LRP was purchased from Biomac (Leipzig, Germany). Recombinant LRP cluster IV was purified from the cell culture supernatant using a GST-RAP sepharose column, as reported previously[26]. Protein S was isolated from plasma as described by Hackeng et al[38].

Cell culture

Mouse embryonic fibroblasts (MEF-1, ATCC CRL-2241) and their LRP-deficient counterparts PEA-13 cells[39] (ATCC CRL-2216), CHO-K1 (wild-type, ATCC CRL-

61), CHO LRP-/-[40] and xylosyltransferase-deficient CHO cells (resulting in the inability to produce cell surface heparan sulfate proteoglycans (HSPG), ATCC CRL-2242) were from the American Tissue Culture Collection. MEFs and PEAs were cultured in DMEM:F12 medium (Invitrogen, Breda, The Netherlands) and CHO cells in HAM's F12 medium (Cambrex, Verviers, Belgium). Both cell culture media were supplemented with 10% fetal bovine serum (Cambrex, Verviers, Belgium) and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Breda, The Netherlands). Cells were grown in a humidified incubator containing 5% CO_2 at 37ºC.

Surface plasmon resonance (SPR)

Binding studies were performed employing a Biacore2000 biosensor system, and SPR analysis was done essentially as described[21;32;41]. LRP was immobilized on a CM5 sensor chip at 7 fmol/mm^2 using the amine-coupling kit as instructed by the supplier. Routinely, a control channel was activated and blocked using the amine-coupling reagents in the absence of protein. rC4BPα was injected until saturation was achieved. Directly after binding, rC4BPα at a higher concentration was injected. Alternatively, C4BPαβ was perfused over the LRP chip until saturation, directly followed by an injection of protein S. For other experiments, C4BPαβ or C4BPα were immobilized on a CM5 sensor chip at 7.2 and 6.9 fmol/mm^2, respectively, as described for LRP. After saturation of immobilized C4BPαβ with protein S, cluster IV was allowed to bind to the complex. Binding to coated channels was corrected for binding to non-coated channels (< 5% of binding to coated channels). SPR analysis was performed in 125 mM NaCl, 1 mM $CaCl_2$, 0.005% (v/v) Tween 20, 25 mM Hepes (pH 7.4) at 25ºC at 10 μL/min. Regeneration of the surface of the LRP sensor chip was performed using 100 mM H_3PO_4.

Analysis of SPR data

The maximally obtained response was determined from each injection. Using GraphPad software, response units were plotted against the protein concentration. K_d values were determined employing one-site binding nonlinear regression.

Cellular binding and degradation

For cellular binding experiments, CHO cells seeded at least 48h before the start of the experiment on glass coverslips in a 24-wells plate and grown until 90-95% confluence in normal culture medium. Cells were extensively washed with HAM's F12 medium, supplemented with 1% BSA and 2 mM $CaCl_2$ (assay medium), followed by an incubation for 30 min at 37ºC to deplete serum compo-

nents. Cells were transferred to ice and then incubated with 250 μl assay medium containing 10-100 nM ^{125}I-labeled rC4BPα or ^{125}I-labeled C4BPαβ mixed with non-labeled rC4BPα or C4BPαβ to a 1:1 molar ratio for 2h. Subsequently, cells were washed 3 times to remove non-bound material and coverslips were transferred to a new plate. Finally, cells were lysed in 1 M NaOH and supernatant was counted in a γ-counter. In each experiment, also a control coverslip without cells was included to determine non-specific binding. Non-specific binding appeared to be less than 20% compared to the wells that contained cells.

For cellular degradation experiments, MEF and PEA, or CHO cells were directly seeded in 24-wells plates at least 48h before the experiment and grown until 90-95% confluence in normal culture medium. Cells were extensively washed and incubated in assay medium at 37ºC for 30 min. 50 nM ^{125}I-rC4BPα mixed with 50 nM non-labeled rC4BPα was added for 1h at 4ºC. Cells were washed three times with 500 μl of assay medium to remove non-bound material. Subsequently, incubation was allowed to proceed at 37ºC in a volume of 500 μl. At indicated time points, 30 μl samples were taken to determine the amount of degraded material and mixed with 10% trichloroacetic acid (TCA) for at least 30 min. Degraded material is defined as the radioactivity that is soluble in 10% TCA. In all experiments a control was included in which the amount of degradation was examined in the absence of cells.

Results

rC4BPα mutants display decreased binding to LRP

To investigate whether the heparin-binding region of the C4BPα chains contribute to the interaction with LRP, we used a number of previously described mutants (*Fig 1*). In these mutants, one or three positively charged residues have been replaced by glutamine, and each mutation is associated with suboptimal heparin-binding[35]. The interaction of the purified mutants with LRP was tested in equilibrium-binding assays using SPR analysis. Various concentrations of wt-rC4BPα or its mutants were applied to immobilized LRP until equilibrium was reached. For wt-rC4BPα, this approach resulted in a binding-isotherm that allowed the calculation of an apparent K_d, the value of which being 10.4 nM *(Fig 2)*. With regard to the mutants, all of them were less efficient than wt-rC4BPα, albeit to a different extent (*Table 1*). Most severely affected was the triple mutant rC4BPα/R39Q-R64Q-R66Q, which displayed 20.6-fold les affinity for LRP than wt-rC4BPα. Apparently, the LRP-binding site includes residues that are also involved in heparin binding.

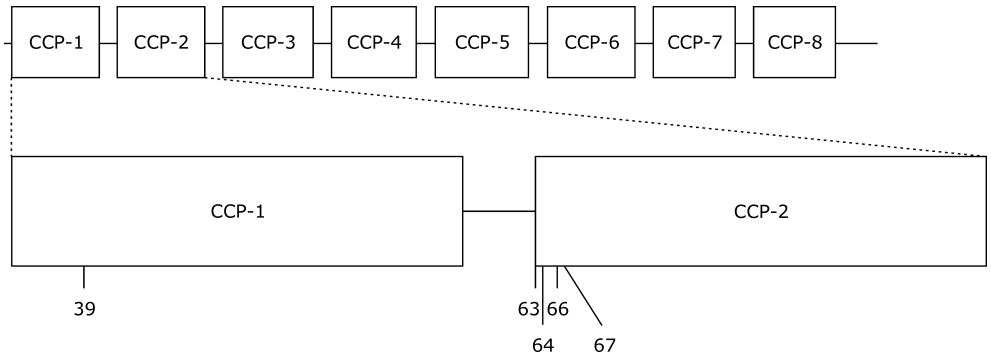


Figure 1. Schematic representation of the C4BP α-chain. The α-chain of C4BP contains 8 complement control protein domains (CCP). CCP-1 and –2 are enlarged. Indicated are positively charged amino acids that were mutated to glutamine residues. In the present study, the following mutants were investigated: R39Q, R63Q, R66Q, H67Q, R39Q-R64Q-R66Q.

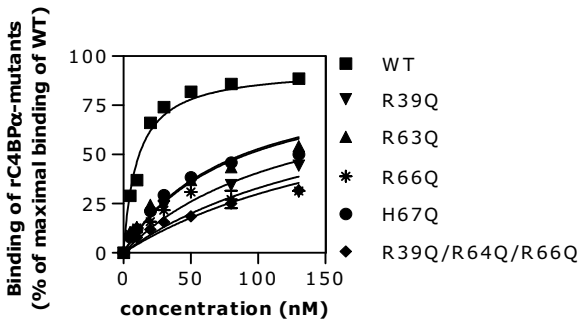


Figure 2. Binding of rC4BPα mutants to immobilized LRP. LRP was immobilized on a CM5 sensor chip at 7 fmol/mm^2. rC4BPα was injected until saturation was achieved. Directly after binding, rC4BPα at a higher concentration was injected. Data were fitted using a one-site regression model.

Table 1. Kinetic parameters for the binding of rC4BPα mutants to LRP.

rC4BPα mutant	$K_{d,app}$ (nM)	standard error
WT	10.4	0.7
R39Q	130.9	4.7
R63Q	78.2	3.5
R66Q	185.5	16.7
H67Q	80.9	3.5
R39Q-R64Q-R66Q	214.0	8.4

Reduced cellular uptake of rC4BPα/R39Q-R64Q-R66Q by LRP-expressing fibroblasts

In a second approach, we tested whether impaired binding of C4BP to LRP is indeed associated with a reduced cellular uptake of this protein. Therefore, experiments were performed using mouse fibroblasts that are genetically deficient for LRP (*ie* PEA-13 cells) or their counterparts that express LRP endogenously (*ie* MEF-1 cells). As expected, ^{125}I-labeled wt-rC4BPα was efficiently degraded by MEF-1 cells, whereas the amount of degraded wt-rC4BPα was reduced in the LRP-deficient PEA-13 cells (*Fig 3*). With regard to the triple mutant rC4BPα/R39Q-R64Q-R66Q, the amount of degraded protein in LRP-expressing MEF-1 cells was reduced to levels that paralleled that of wt-rC4BPα in LRP-deficient cells. Thus, abrogation of the combined heparin/LRP-binding site in C4BP results in impaired cellular uptake of the protein.

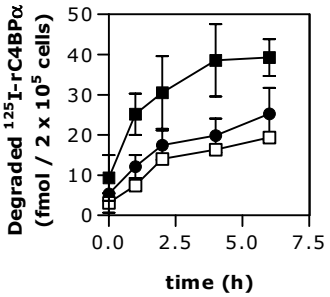


Figure 3. Cellular degradation of wt and mutant rC4BPα. LRP+ MEF-1 cells (squares) and LRP- PEA-13 cells (circles) were incubated with a 1:1 mixture of unlabeled and ^{125}I-labeled wt-rC4BPα (closed symbols) or rC4BPα R39Q-R64Q-R66Q (open symbols) (final concentration 100 nM) at 4ºC for 60min. After washing, cells were subsequently incubated at 37°C for indicated time points, and degradation of rC4BPα was determined as described under "Experimental Procedures". Data represent mean ± SEM (n=2).

HSPGs are required for optimal endocytosis of C4BP

The observation that the triple mutant rC4BPα/R39Q-R64Q-R66Q is taken up by the cell in a suboptimal manner, does not automatically allow the conclusion that the reduced uptake is selectively due to an aberrant interaction with LRP. For a number of LRP ligands such as factor VIII and thrombospondin, it has been described that LRP-dependent endocytosis is preceded by the sequestration of these ligands on HSPGs that are present at the cellular surface. It was of interest therefore to examine the contribution of HSPGs to the cellular uptake of C4BP. To this end, binding and degradation of wt-rC4BPα by normal CHO cells (which express LRP endogenously) and HSPG-deficient CHO cells were compared. Binding of ^{125}I-labeled wt-rC4BPα to normal CHO cells was saturable and dose-dependent, with half-maximal binding occurring at approximately 70 nM (*Fig 4A*). In addition, radioactive TCA-soluble products appeared in time in the medium, indicating that the radiolabeled wt-rC4BPα was degraded by these nor-

mal CHO cells (*Fig 4B*). In contrast, binding and subsequent degradation were severely reduced in CHO cells that lack HSPG at their cellular surface (*Fig 4A and B*). Taken together, these data indicate that HSPGs and LRP act in concert in the binding and internalization of C4BP.

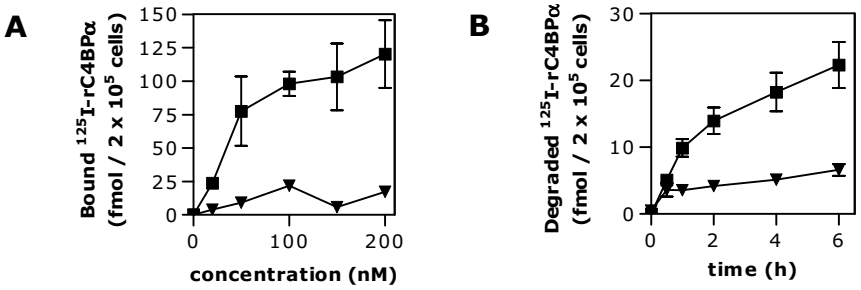


Figure 4. Cellular binding and degradation of ^{125}I-rC4BPα to CHO cells. *A*. CHO cells (WT (squares) and HSPG (triangles)) were grown on coverslips. After incubation in HAM's F12, 1% BSA and 2 mM $CaCl_2$ at 37ºC for 30 min, indicated concentrations of ^{125}I-rC4BPα and nonlabeled rC4BPα were added simultaneously in a 1:1 molar ratio on ice for 2h. Cells were washed extensively and coverslips were transferred to a new plate. Then, cells were lysed and supernatant was counted in a γ-counter. *B.* CHO cells were incubated with a 1:1 mixture of unlabeled and ^{125}I-labeled wt-rC4BPα (final concentration 100 nM) at 4ºC for 60min. After washing, cells were subsequently incubated at 37ºC for indicated time points, and degradation of rC4BPα was determined as described under "Experimental Procedures". Counts were corrected for non-specific binding by subtracting values from wells without cells. Data represent mean ± SEM (n=3).

Simultaneous binding of C4BPαβ to LRP and protein S

In plasma, the β-chain containing C4BP molecules (spanning approximately 80% of the C4BP population) circulate in complex with anticoagulant protein S. We examined therefore the possibility that not only C4BPαβ alone but also the C4BPαβ/protein S complex may interact with LRP. Two distinct experimental approaches employing SPR-analysis were used in this respect. First, immobilized LRP was saturated with C4BPαβ followed by application of protein S. As shown in figure 5A, C4BPαβ efficiently bound to immobilized LRP, resulting in a response of 261 RU that gradually declined to 214 RU just before application of protein S. These 214 RU correspond to approximately 0.4 fmol C4BPα/mm^2. Shortly after finalizing LRP injection, protein S was applied. Perfusion of protein S resulted in an increased response, which was calculated to be 34 RU, corresponding to 0.45 fmol protein S/mm^2. Control experiments demonstrated that protein S alone

does not associate with LRP[21], indicating that the observed response originates from binding of protein S to LRP-bound C4BPαβ. In an alternative approach, C4BP consisting of both the α- and β-chains or only of its α-chains was immobilized onto a CM5-sensorchip (7.2 fmol/mm^2 and 6.9 fmol/mm^2 for C4BPαβ and C4BPα, respectively). Protein S was subsequently perfused over both channels, resulting in the absence of protein S on the C4BPα-channel and binding of 500 RU (*ie* 6.6 fmol protein S/mm^2) onto the C4BPαβ-channel (*Fig 5B*). Subsequently, both channels were perfused with recombinant LRP cluster IV. As depicted in figure 5B, binding of near equal amounts of LRP cluster IV to the C4BPα- and C4BPαβ-channel was observed. These results are consistent with the view that C4BPαβ is able to interact simultaneously with LRP and protein S.

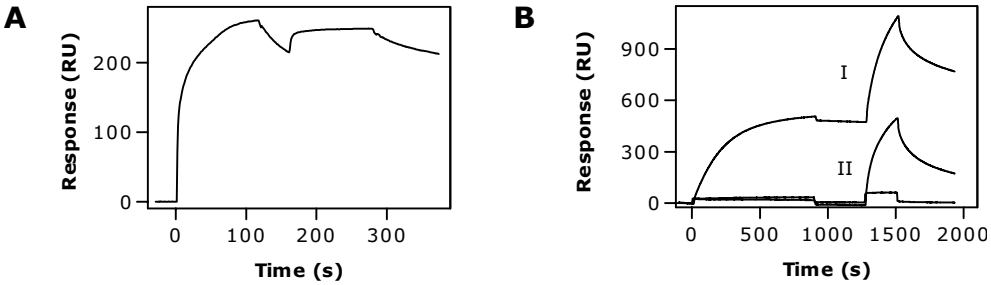


Figure 5. Binding of the C4BPαβ / protein S complex to LRP. *A.* Immobilized LRP (7.0 fmol/mm^2) was saturated with C4BPαβ followed by application of protein S. *B.* C4BP consisting of both the α- and β-chains (*line I*) or only of its α-chains (*line II*) were immobilized onto a CM5-sensorchip (7.2 fmol/mm^2 and 6.9 fmol/mm^2 for C4BPαβ and C4BPα, respectively). Protein S was subsequently perfused over both channels. Then, both channels were perfused with recombinant LRP cluster IV.

Discussion

C4BP is a multifunctional protein associated with various physiological processes, like complement regulation, coagulation and removal of apoptotic cells[14;42]. Recently, we described that C4BP is a ligand for the endocytic receptor LRP[21], and in the present study the interaction between both proteins was investigated in more detail. By using a number of previously described mutants (*Fig 1* and ref 35), we have now identified a number of positively charged amino acids located in the C4BP α-chains that are critical for optimal interaction between C4BP and LRP. Conversion of these residues (*ie* R39, R63, R64, R66, H67) into glutamine residues all resulted in reduced binding to LRP as assessed by SPR

analysis. In addition, the mutant with the triple mutation (rC4BPα/R39Q-R64Q-R66Q) did not only display reduced binding to LRP, but also was taken up by LRP-expressing cells at levels that parallel those in the absence of LRP (*Fig 2*). The same mutations also affect the interaction with other components, like factor I, C4b, heparin and bacterial components[19;35]. The triple mutant showed a strongly reduced cofactor function in the factor I-mediated C4b binding. In addition, all mutants demonstrated decreased affinity for heparin and streptococcal M proteins. Whether the interaction between C4BP and LRP is completely dependent on charged residues interactions seems unlike, because even in the triple mutant significant residual binding was observed. Furthermore, binding could not be completely inhibited in the presence of high salt concentrations (> 300 mM, data not shown). This suggests that also hydrophobic residues within the C4BP α-chains may contribute to the interaction with LRP. In this respect, C4BP is similar to activated factor IXa, which interaction with LRP also involves a number of charged and hydrophobic residues[43].

The presence of the heparin-binding site within the C4BP α-chains opens the possibility that C4BP may also associate to HSPGs that were present at the cellular surface. Indeed, a strongly reduced binding and uptake of C4BP was observed in cells that lack the expression of HSPGs at their cell surface. The notion that an LRP ligand binds also to cell surface HSPGs is not unique for C4BP, as several other LRP ligands such as thrombospondin, factor VIII and activated factor IX also have been described to interact with HSPGs[32;33;44]. The mechanism that underlies the cooperation between LRP and HSPGs is unclear. It is assumed that HSPGs first bind the bulk of ligands in order to increase their local concentration. In a second step, these ligands are then transported to LRP that facilitates internalization. Since LRP is known to participate in heterologous receptor complexes[30;45;46], it is tempting to speculate that part of the LRP and HSPG molecules combine into the same receptor complex. Alternatively, the possibility exists that both receptors meet during dynamic lateral movements at the cell surface. One other issue that is worth mentioning relates to the observations that HSPGs have the capacity to internalize proteins themselves[34]. Since there is considerable residual uptake of C4BP in LRP-deficient cells, it is conceivable that HSPGs are also directly involved in the internalization of C4BP.

We have previously shown that LRP contributes to the initial phase of C4BP clearance[21]. Since in the human situation (but not in mice) the C4BPαβ isoform circulates in complex with anticoagulant protein S[2;12], it seems reasonable to assume that C4BP may facilitate clearance of protein S via LRP. Such a facilitation requires formation of trimolecular complexes. Indeed, in two experimental approaches we demonstrated the possibility of the formation of the LRP/C4BP/protein S complex. In this regard it is important to note that protein S itself is unable to interact with LRP[21]. Supporting evidence for the view that

C4BP bridges protein S and LRP is obtained by *in vivo* survival experiments in mice, which showed that the half-life time of C4BP is roughly identical to that of C4BP-bound protein S (Denis and Lenting, unpublished observation).

C4BP interacts with several other proteins as well, such as amyloid P component[17], factor VIII[18] and bacterial proteins[19]. Therefore, C4BP may function as a chaperone for these proteins to mediate their interaction with LRP. Interestingly, LRP may be involved in the binding of bacteria via the interaction of bacterial surface proteins with C4BP, thereby serving as an entry receptor for pathogenic bacteria.

Taken together, C4BP binds to LRP via positively charged residues that are also involved in binding of C4b and heparin. We demonstrated that HSPGs are involved in the primary binding of C4BP to the cellular surface. Docking of C4BP is followed by an LRP-dependent internalization process. LRP is also able to indirectly bind protein S via C4BP that serves as a physical bridge, suggesting that LRP can also mediate the clearance of protein S, and probably, other C4BP-binding proteins.

Acknowledgements

This study was supported by a grant from the Dutch Organization of Scientific Research ZonMW (no. 902-26-236) to P.J.L.

References

1. Dahlback, B. 1991. Protein S and C4b-binding protein: components involved in the regulation of the protein C anticoagulant system. *Thromb.Haemost.* 66:49-61.
2. Dahlback, B. and J. Stenflo. 1981. High molecular weight complex in human plasma between vitamin K-dependent protein S and complement component C4b-binding protein. *Proc.Natl.Acad.Sci.U.S.A.* 78:2512-2516.
3. Fujita, T. and V. Nussenzweig. 1979. The role of C4-binding protein and beta 1H in proteolysis of C4b and C3b. *J.Exp.Med.* 150:267-276.
4. Blom, A.M., B.O. Villoutreix, and B. Dahlback. 2003. Mutations in alpha-chain of C4BP that selectively affect its factor I cofactor function. *J.Biol.Chem.* 278:43437-43442.
5. Gigli, I., T. Fujita, and V. Nussenzweig. 1979. Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. *Proc.Natl.Acad.Sci.U.S.A.* 76:6596-6600.
6. Scharfstein, J., A. Ferreira, I. Gigli, and V. Nussenzweig. 1978. Human C4-binding protein. I. Isolation and characterization. *J.Exp.Med.* 148:207-222.
7. Griffin, J.H., A. Gruber, and J.A. Fernandez. 1992. Reevaluation of total, free, and bound protein S and C4b-binding protein levels in plasma anticoagulated with citrate or hirudin. *Blood* 79:3203-3211.

8. Kusada-Funakoshi, M., J. Sasaki, Y. Takada, T. Soji, and K. Arakawa. 1991. Evidence that C4b-binding protein (proline-rich protein) is synthesized by hepatocytes. *Biochem.Med.Metab.Biol.* 45:350-354.
9. Sanchez-Corral, P., G.O. Criado, and S. Rodriguez de Cordoba. 1995. Isoforms of human C4b-binding protein. I. Molecular basis for the C4BP isoform pattern and its variations in human plasma. *J.Immunol.* 155:4030-4036.
10. Blom, A.M. 2002. Structural and functional studies of complement inhibitor C4b-binding protein. *Biochem.Soc.Trans.* 30:978-982.
11. Dahlback, B., C.A. Smith, and H.J. Muller-Eberhard. 1983. Visualization of human C4b-binding protein and its complexes with vitamin K-dependent protein S and complement protein C4b. *Proc.Natl.Acad.Sci.U.S.A.* 80:3461-3465.
12. Hillarp, A. and B. Dahlback. 1988. Novel subunit in C4b-binding protein required for protein S binding. *J.Biol.Chem.* 263:12759-12764.
13. DiScipio, R.G. and E.W. Davie. 1979. Characterization of protein S, a gamma-carboxyglutamic acid containing protein from bovine and human plasma. *Biochemistry* 18:899-904.
14. Rezende, S.M., R.E. Simmonds, and D.A. Lane. 2004. Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex. *Blood* 103:1192-1201.
15. Dahlback, B. 1986. Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. *J.Biol.Chem.* 261:12022-12027.
16. Hessing, M., C. van 't Veer, and B.N. Bouma. 1990. The binding site of human C4b-binding protein on complement C4 is localized in the alpha'-chain. *J.Immunol.* 144:2632-2637.
17. Schwalbe, R.A., B. Dahlback, and G.L. Nelsestuen. 1990. Independent association of serum amyloid P component, protein S, and complement C4b with complement C4b-binding protein and subsequent association of the complex with membranes. *J.Biol.Chem.* 265:21749-21757.
18. Koppelman, S.J., C. van 't Veer, J.J. Sixma, and B.N. Bouma. 1995. Synergistic inhibition of the intrinsic factor X activation by protein S and C4b-binding protein. *Blood* 86:2653-2660.
19. Blom, A.M., K. Berggard, J.H. Webb, G. Lindahl, B.O. Villoutreix, and B. Dahlback. 2000. Human C4b-binding protein has overlapping, but not identical, binding sites for C4b and streptococcal M proteins. *J.Immunol.* 164:5328-5336.
20. Garcia de Frutos, P., R.I. Alim, Y. Hardig, B. Zoller, and B. Dahlback. 1994. Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S. *Blood* 84:815-822.
21. Westein, E., C.V. Denis, B.N. Bouma, and P.J. Lenting. 2002. The alpha -chains of C4b-binding protein mediate complex formation with low density lipoprotein receptor-related protein. *J.Biol.Chem.* 277:2511-2516.
22. Moestrup, S.K., J. Gliemann, and G. Pallesen. 1992. Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res.* 269:375-382.
23. Li, Y., M.P. Marzolo, P. van Kerkhof, G.J. Strous, and G. Bu. 2000. The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. *J.Biol.Chem.* 275:17187-17194.
24. Gotthardt,M., M. Trommsdorff, M.F. Nevitt, J. Shelton, J.A. Richardson, W. Stockinger, J. Nimpf, and J. Herz. 2000. Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. *J.Biol.Chem.* 275:25616-25624.
25. Willnow, T.E., K. Orth, and J. Herz. 1994. Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *J.Biol.Chem.* 269:15827-15832.
26. Neels, J.G., B.M. van Den Berg, A. Lookene, G. Olivecrona, H. Pannekoek, and A.J. van Zonneveld. 1999. The second and fourth cluster of class A cysteine-rich repeats of the low density lipoprotein receptor-related protein share ligand-binding properties. *J.Biol.Chem.* 274:31305-31311.

27. Herz, J. and D.K. Strickland. 2001. LRP: a multifunctional scavenger and signaling receptor. *J.Clin.Invest.* 108:779-784.
28. Strickland, D.K., S.L. Gonias, and W.S. Argraves. 2002. Diverse roles for the LDL receptor family. *Trends Endocrinol.Metab.* 13:66-74.
29. Hussain, M.M., D.K. Strickland, and A. Bakillah. 1999. The mammalian low-density lipoprotein receptor family. *Annu.Rev.Nutr.* 19:141-72.:141-172.
30. Spijkers, P.P., P. da Costa Martins, E. Westein, C.G. Gahmberg, J.J. Zwaginga, and P.J. Lenting. 2005. LDL-receptor-related protein regulates beta2-integrin-mediated leukocyte adhesion. *Blood* 105:170-177.
31. Warshawsky, I., G.J. Broze, Jr., and A.L. Schwartz. 1994. The low density lipoprotein receptor-related protein mediates the cellular degradation of tissue factor pathway inhibitor. *Proc.Natl.Acad.Sci.U.S.A.* 91:6664-6668.
32. Neels, J.G., B.M. van den Berg, K. Mertens, H. ter Maat, H. Pannekoek, A.J. van Zonneveld, and P.J. Lenting. 2000. Activation of factor IX zymogen results in exposure of a binding site for low-density lipoprotein receptor-related protein. *Blood* 96:3459-3465.
33. Mikhailenko, I., M.Z. Kounnas, and D.K. Strickland. 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. *J.Biol.Chem.* 270:9543-9549.
34. Bernfield, M., M. Gotte, P.W. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, and M. Zako. 1999. Functions of cell surface heparan sulfate proteoglycans. *Annu.Rev.Biochem.* 68:729-777.
35. Blom, A.M., J. Webb, B.O. Villoutreix, and B. Dahlback. 1999. A cluster of positively charged amino acids in the C4BP alpha-chain is crucial for C4b binding and factor I cofactor function. *J.Biol.Chem.* 274:19237-19245.
36. van de Poel, R.H., J.C. Meijers, J. Rosing, G. Tans, and B.N. Bouma. 2000. C4b-binding protein protects coagulation factor Va from inactivation by activated protein C. *Biochemistry* 39:14543-14548.
37. Hessing, M., D. Kanters, T.M. Hackeng, and B.N. Bouma. 1990. Identification of different forms of human C4b-binding protein lacking beta-chain and protein S binding ability. *Thromb.Haemost.* 64:245-250.
38. Hackeng, T.M., M. Hessing, C. van 't Veer, F. Meijer-Huizinga, J.C. Meijers, P.G. de Groot, J.A. van Mourik, and B.N. Bouma. 1993. Protein S binding to human endothelial cells is required for expression of cofactor activity for activated protein C. *J.Biol.Chem.* 268:3993-4000.
39. Willnow, T.E. and J. Herz. 1994. Genetic deficiency in low density lipoprotein receptor-related protein confers cellular resistance to Pseudomonas exotoxin A. Evidence that this protein is required for uptake and degradation of multiple ligands. *J.Cell Sci.* 107:719-726.
40. FitzGerald, D.J., C.M. Fryling, A. Zdanovsky, C.B. Saelinger, M. Kounnas, J.A. Winkles, D. Strickland, and S. Leppla. 1995. Pseudomonas exotoxin-mediated selection yields cells with altered expression of low-density lipoprotein receptor-related protein. *J.Cell Biol.* 129:1533-1541.
41. Lenting,P.J., J.G.Neels, B.M.van Den Berg, P.P.Clijsters, D.W.Meijerman, H.Pannekoek, J.A.van Mourik, K.Mertens, and A.J.van Zonneveld. 1999. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *J.Biol.Chem.* 274:23734-23739.
42. Blom, A.M., B.O. Villoutreix, and B. Dahlback. 2004. Complement inhibitor C4b-binding protein-friend or foe in the innate immune system? *Mol.Immunol.* 40:1333-1346.
43. Rohlena, J., J.A. Kolkman, R.C. Boertjes, K. Mertens, and P.J. Lenting. 2003. Residues Phe342-Asn346 of activated coagulation factor IX contribute to the interaction with low density lipoprotein receptor-related protein. *J.Biol.Chem.* 278:9394-9401.
44. Sarafanov, A.G., N.M. Ananyeva, M. Shima, and E.L. Saenko. 2001. Cell surface heparan sulfate proteoglycans participate in factor VIII catabolism mediated by low density lipoprotein receptor-related protein. *J.Biol.Chem.* 276:11970-11979.
45. Loukinova, E., S. Ranganathan, S. Kuznetsov, N. Gorlatova, M.M. Migliorini, D. Loukinov, P.G. Ulery, I. Mikhailenko, D.A. Lawrence, and D.K. Strickland. 2002. Platelet-derived growth factor

(PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). Evidence for integrated co-receptor function between LRP and the PDGF receptor. *J.Biol.Chem.* 277:15499-15506.

46. Bacskai, B.J., M.Q. Xia, D.K. Strickland, G.W. Rebeck, and B.T. Hyman. 2000. The endocytic receptor protein LRP also mediates neuronal calcium signaling via N-methyl-D-aspartate receptors. *Proc.Natl.Acad.Sci.U.S.A.* 97:11551-11556.

chapter 6

General Discussion

Eureka!
(*Archimedes*)

Receptors have long been thought to interact with a single ligand, but current insights have recognized the existence of receptors that are able to bind multiple ligands[1;2]. One well-known group of these so-called multiligand receptors is the low-density lipoprotein (LDL)-receptor family. At present, 12 members are known, which show structural homology[3-5]. In addition to their multiligand nature, they also display multifunctionality and are expressed in a broad spectrum of cell types. The intriguing aspect in this regard is that despite their versatile nature, these receptors distinguish themselves from each other by distinct cell-expression patterns and cell-specific functions.

Novel Aspects of LDL Receptor-Related Protein (LRP) Biology

Based on its structural homology to the LDL receptor and its capacity to bind lipoproteins, LRP has initially been considered as a second receptor involved in lipoprotein metabolism[6;7]. Indeed, the LDL receptor and LRP act in concert in the clearance of LDL particles[8]. However, it soon became clear that LRP functions as a multiligand receptor, because more and more ligands were identified in time[9;10]. Most of these ligands are internalized upon binding to LRP and subsequently degraded in lysosomal compartments[3;11;12]. LRP not only targets ligands to lysosomes, but also participates in other transport pathways, like transcytosis[13], nuclear targeting[14] and phagocytosis[15;16]. A number of ligands have been found to modulate cellular properties through their interaction with LRP. In addition, LRP can initiate cellular signaling pathways[16-18], or can influence signaling by other receptors, thereby functioning as a co-receptor[19-21].

In the present thesis, various aspects of LRP function in relation to its cellular environment are described.

LRP and its regulation of β2-integrin function

Another example of multiligand receptors is the β2-integrin family. These integrins are exclusively expressed by leukocytes and have a role in mediating cell adhesion to different surfaces[22;23]. Their main counter receptors are the intercellular adhesion molecules (ICAMs) present at the endothelium[24], but they also interact with several other ligands, like fibrinogen[25], glycoprotein Ibα[26], factor X[27] and neutrophil inhibitory factor (NIF)[28].

We demonstrated that LRP is able to directly bind to β2-integrins at the cell surface of blood-derived polymorphonuclear cells (PMNs) and monocytes or the monocytic cell line U937 (chapter 2). This interaction is at least mediated by the extracellular domains of LRP and the α-subunits of β2-integrins. Evidence for this

conclusion includes 1) granulocytes, monocytes and U937 cells bind to immobilized cluster II and IV, which are part of the extracellular chain of LRP, 2) the LRP-antagonist receptor-associated protein (RAP) inhibits this interaction, and 3) adhesion of U937 cells to immobilized cluster II and IV is inhibited by antibodies against the I-domains of the α-subunits, which are located at the extracellular domain. Whether the intracellular domains also contribute to complex formation remains to be investigated.

Binding of U937 cells to cluster II and IV can be inhibited by antibodies directed against the I-domains of the α_L, α_M and α_X subunits. Thus, LRP is able to interact with each of these α-subunits at the cell surface. The importance of the I-domains in LRP-binding is in line with previous findings that show that other ligands interact with this region as well[29-31]. However, adhesion is also decreased when an antibody against the β-subunit is used. This may suggest that LRP is also able to bind to the β-subunit. Indeed, some ligands are known to directly interact with the β-subunit[32]. Alternatively, the anti-β2-subunit antibody may prevent binding of LRP to the α-subunit via sterical hindrance, or by disrupting the interaction between the α- and β-subunits. The β-subunit has been shown to be involved in the proper conformation and ligand-binding capacity of the α-subunit[33-37]. By making use of the purified β-subunit, one may be able to discriminate between these proposed mechanisms.

Proper functioning of β2-integrins requires their clustering upon immunological activation[38]. The redistribution of β2-integrins is a process that depends on the liberation of this receptor from the actin cytoskeleton[39]. This process is influenced by a number of intracellular components, such as calpain[40], activated phospholipase A2[41], the small GTPase RAP1[42] and its regulator RAPL[43]. Whether or not LRP is involved in this intracellular process is unclear. However, the cytoplasmic domains of both LRP and β2-integrins contain a number of phosphorylation sites that may affect interactions with adaptor proteins[17;44].

Redistribution leads to the translocation of β2-integrins to lipid rafts[45]. Our confocal microscopy and immunoprecipitation experiments clearly demonstrate that LRP is colocalized with β2-integrins in these clusters. Interestingly, clustering is completely abolished in U937 cells lacking LRP. This suggests that the presence of LRP is necessary for the clustering and, thus, functioning of the β2-integrins. Indeed, adhesion of LRP-negative U937 cells to endothelial cells is significantly reduced compared to LRP-positive U937 cells. This functional liaison between LRP and β2-integrins opens the possibility that LRP may also mediate (pathological) processes in which leukocytes are involved. For example, LRP may contribute to β2-integrin-dependent migration of leukocytes to the subendothelial space, and as such facilitate the development of atherosclerotic lesions. LRP may also control β2-integrin-dependent phagocytosis in macrophages. Future

studies should be designed that address the issue in which processes LRP plays a regulatory role.

LRP and its expression in lymphocytes

Since LRP plays an important role in monocyte and PMN adhesion, it was of interest to investigate the contribution of LRP to the function in other leukocytes as well. Whereas the expression and function of LRP in monocytes and dendritic cells have been well characterized [15;16;46-48], LRP expression patterns and functions in other leukocytes have not been fully understood yet.

In chapter 3, we described the presence of both LRP mRNA and protein in different lymphocyte subsets: T-lymphocytes, B-lymphocytes and natural killer (NK) cells. Interestingly, a different localization of LRP in T-lymphocytes compared to B-lymphocytes and NK cells is observed. Whereas LRP is present at the cell surface of NK cells and B-lymphocytes, only a small amount of T-lymphocytes have LRP at their surface. Thus, LRP appears to be located in intracellular storage compartments in T-lymphocytes.

We speculate that LRP may be recruited to the cell membrane upon activation in T-lymphocytes. Stimulation of T-lymphocytes by interleukin-2 (IL-2) and phytohaemagglutinin (PHA) indeed increases the number of cells positive for cell surface LRP from 2% to 12% of the T-lymphocytes after 48h. However, this increase is transient and not immediate, making it unlikely that LRP is redistributed from intracellular storage pools to the cell surface. In a second approach, we showed that fast upregulation of LRP takes place in a mixed-lymphocyte reaction (MLR) in 60-70% of the T-lymphocytes. This suggests that LRP is promptly translocated from storage pools to the cell surface in these cells. A fast translocation of LRP to the cell surface is also observed in insulin-treated adipocytes[49] and NGF-treated neurons[50]. It is important to note that in resting neurons and adipocytes LRP is also present at the cell surface, whereas in T-lymphocytes the total LRP pool is maintained within the cell.

It appeared that allogenic monocytes were able to induce this observed increase in LRP cell surface expression, whereas mixing T-lymphocytes from 2 donors did not increase cell surface expression. This may point to a role for LRP in conditions in which antigen presenting cells (APCs) trigger T-lymphocyte activation, like in graft-versus-host disease (GVHD)[51]. Many receptors that play a role in the interaction with APCs are present at the cell surface of T-lymphocytes[52-57]. Interestingly, one of these cell surface receptors, CTLA-4, is also recruited to the cell membrane in a mixed lymphocyte reaction (MLR)[55]. It is possible that CTLA-4 and LRP are stored in similar storage pools. Another receptor that is important for T-lymphocyte activation is $\alpha_L\beta 2$[57]. Since LRP and $\alpha_L\beta 2$ are functionally interlinked in monocytes, it deserves further study to de-

termine if such connection also exists in T-lymphocytes. If so, then LRP may serve as a novel target to interfere with immune disorders such as GVHD. It should be noted that such studies also should focus on the elucidation of the intracellular signaling pathways that force the translocation of LRP to the cell surface.

LRP and its function as brain endothelial clearance receptor for amyloid β (Aβ) peptides

LRP is a key component in the pathogenesis of Alzheimer's disease (AD). A genetic polymorphism in the *LRP* gene itself is associated with late-onset AD[58-61]. In addition, the LRP ligands amyloid precursor protein (APP), Apolipoprotein E (ApoE) and α_2-macroglobulin (α_2M) are also genetically linked to AD[10]. LRP mediates the endocytosis of APP in neurons, leading to increased Aβ production[62-64]. Although LRP can mediate the re-uptake of these Aβ peptides in neurons[65-67], this mechanism is unlikely to contribute to Aβ clearance *in vivo*, but rather leads to the formation of amyloid plaques[68]. Thus, an alternative pathway should exist that results in the removal of Aβ peptides from the brain to the circulation. In chapter 4, the contribution of brain endothelial cell LRP in the clearance of Aβ peptides is described.

Aβ peptides are known to bind to LRP indirectly via complex formation with ApoE or α_2M[65-67]. However, our study clearly shows that Aβ peptides also directly interact with LRP clusters. The affinity of Aβ40 for LRP is higher than of Aβ42, which contains 2 extra hydrophobic residues, and of the Aβ40 isoform harboring the Dutch/Iowa mutation (DIAβ40). This increased affinity correlates with the β-sheet content of Aβ-peptides: Aβ40 contains only 25% β-sheet, and Aβ42 and DIAβ40 75% and 80%, respectively. LRP mediates binding and internalization of Aβ peptides by brain endothelial cells. However, this uptake is not followed by degradation. This suggests that LRP may mediate the transcytosis of Aβ over the blood-brain barrier (BBB). *In vivo* experiments showed that Aβ40 is indeed more rapidly cleared from brain tissue than the low-affinity binders Aβ42 or DIAβ40. Remarkably, the presence of high levels of Aβ peptides decreases their efflux across the BBB, caused by an induction of LRP degradation in the proteasome pathway. The relevance of this observation *in vivo* has been demonstrated by studying the phenotype of RAP-null mice (which show severe LRP deficiency) crossed with APP-overexpressing mice[69]. In these mice Aβ peptides accumulate in the interstitial fluid of the brain. This implicates that Aβ peptides are inefficiently cleared across the BBB. Indeed, Aβ peptide clearance is decreased significantly compared to wild-type mice in mice expressing either the low-clearance mutant DIAβ40 or overexpressing APP. Interestingly, these latter mice show reduced LRP levels in brain, suggesting that an excess of Aβ availability

also downregulates LRP in brain endothelial cells *in vivo*. In AD patients carrying the DIAβ mutant also an elevated Aβ deposition is observed[70]. Studying LRP levels of brain endothelial cells from these patients reveals a reduction in LRP expression.

Receptor for advances glycation end products (RAGE) is another Aβ receptor present at brain endothelium. In contrast to LRP, it mediates continuous influx of Aβ from blood to the brain[71]. An imbalance between influx and efflux may increase Aβ accumulation in brain[72]. It has been demonstrated that Aβ-peptide-binding agents present in blood shift the transport balance towards the blood, thereby lowering the Aβ concentration in brain tissue. Therefore, sequestering of Aβ peptides via LRP may be a novel therapeutic target in the development of AD.

LRP and its role in C4b-binging protein (C4BP) clearance

Mediating internalization of soluble proteins is the classical function of LRP[3;11;12]. Recently, we identified C4BPα as a new ligand for LRP[73]. C4BP is a component of both complement system and coagulation and has been found to bind several proteins, such as C4b, amyloid P component, bacterial components and protein S[74-78]. Heparin has been described to bind to C4BP as well[79;80] and to inhibit the interaction between C4BP and LRP[73]. This would suggests that positively charged residues in C4BP are involved in the interaction with LRP. Analyzing C4BP mutants in which positive amino acids are mutated into glutamine resulted in the finding that a triple mutant C4BP showed a severe reduction in LRP binding (chapter 5). In addition, the triple mutant is less efficiently degraded in CHO cells containing LRP. The observed residual binding suggests that other interactions are also involved. Indeed, experiments using high salt concentrations reveal that these high concentrations are not able to completely inhibit C4BP/LRP binding, suggesting that hydrophobic interactions may also play a role. In this respect, the binding between activated factor IX and LRP has been shown to be partially dependent on hydrophobic interactions[81].

The importance of positively charged amino acids in LRP binding would suggest that heparan sulfate proteoglycans (HSPGs) also play a role in the binding of C4BP. CHO cells lacking functional HSPGs show strongly reduced binding of C4BP. Also for other LRP ligands it has been demonstrated that these HSPGs serves as a primary docking site[82-84], thereby increasing their local concentration. Subsequently, ligands can efficiently be transferred to LRP, which mediate their internalization.

The mutated positively charged amino acids in the triple mutant have also been demonstrated to be important in binding of other C4BP-binding proteins[76;85], suggesting that these proteins may interfere with the interaction between C4BP and LRP. Alternatively, because of C4BP's multivalency, a trimolecu-

lar complex can occur consisting of LRP, C4BP and the C4BP-binding protein S. Protein S binds to the β-chain of C4BP[77;86]. We demonstrated that LRP is still able to bind C4BP in complex with protein S. Since protein S does not bind to LRP itself[73], C4BP is proposed to link protein S and LRP. Thus, LRP may indirectly regulate the plasma concentration of these C4BP-binding proteins.

The Versatility of LRP

In chapters 2-5 we highlighted 4 distinct aspects of the multifunctional protein LRP. This receptor can exert many diverse functions in various cell types. Moreover, the function of LRP in one cell type may not be restricted to a single process. Thus, the question rises as to which regulatory mechanisms may specify LRP function. In the next paragraphs, we discuss to what extent the microenvironment plays a role in determining the specificity of LRP.

LRP's ligands considered

LRP is a receptor that is able to recognize multiple structurally unrelated ligands[9;10]. These ligands can prompt various cellular events, like endocytosis[3;11;12], cell adhesion and growth[48;87], and neuronal transmission[88;89]. The spatial localization of LRP ligands may be one mode in which the function of LRP is specified. Some ligands show a highly cell-restricted expression pattern, like β2-integrins that are only present on leukocytes[22]. In this regard, we showed that LRP directly binds to the α-subunit of β2-integrins (chapter 2). Interestingly, in CHO cells LRP mediates the uptake of soluble I-domain of α_M (data not shown), whereas it does not regulate surface expression of β2-integrins in monocytes (chapter 2). It cannot be excluded that delivery to the lysosomal degradation pathway is limited to soluble ligands (refs 12;90-92 and chapter 5) More likely, the endocytic process is cell-type-dependent in cells (see 'Cell-restricted functions of LRP').

The function of LRP may also be restricted by a temporarily availability of its ligands. One clear example hereof is coagulation factor IX. In its naïve state, factor IX is unable to ligate to LRP. However, factor XIa-mediated activation of this zymogen exposes a binding site for LRP[82]. Another profound case of temporarily present agents are proteins derived from bacteria and viruses during an inflammatory event, like HIV tat protein and pseudomonas exotoxin A[93-96]. To this end, LRP may serve as an entry receptor, thereby contributing to the hazardous properties of these compounds. At the same time, it is of interest to consider the presence of soluble LRP, which has the potential to act as a first defense barrier, preventing cellular penetration of these infective agents[97].

Third, the structure of a ligand may allow dimerization or multimerization of LRP. Backsai et al[88] proposed that LRP/α_2M-mediated NMDA-induced calcium influx may be due to LRP dimerization, since LRP-activating antibodies, but not their Fab-fragments caused elevated intracellular calcium levels. α_2M is a multivalent protein, which may be able to cluster LRP, and thereby inducing signaling. C4BPα can be internalized via LRP (ref 73 and chapter 5), but also shows other cellular effects. For instance, it stimulates B-lymphocyte proliferation via CD40[98]. Since LRP is also present at B-lymphocytes (chapter 3), it would be of interest to study the involvement of LRP in this process. Moreover, because of its multivalency, C4BP may be able to multimerize LRP to evoke cellular signaling, or it may cause the formation of heterologous receptor complexes that consist of C4BP-binding receptors.

Since LRP's ligands are structurally dissimilar, but have the potential to induce similar physiological processes, the structure of these proteins may not be a major determinant of LRP's function. Thus, a model in which the spatial and temporal occurrence of ligands may be preferable over their structure for influencing the role of LRP under specific circumstances.

Cell-restricted functions of LRP

Hepatocytes are identified as master endocytic cells that mediate the clearance of various plasma proteins in an LRP-mediated fashion[99-101]. Since hepatocytes are located directly adjacent to the blood stream, hepatic LRP can efficiently internalize its ligands, thereby regulating their plasma concentration. Although studies on the presence of LRP in endothelial cells are inconclusive, LRP has been described to be expressed by brain endothelium[102], another cell type that is constitutively exposed to blood. LRP is involved in rapid clearance of Aβ peptides from the brain in these cells (chapter 4). Whereas other cell types, like adipocytes[49;103;104] and fibroblasts (chapter 5), are also competent to internalize LRP ligands, other cells are mainly involved in distinct cellular events[10]. One striking example of cell-restricted function is illustrated in chapter 4. Although neurons and brain endothelium both express LRP[4;5;102;105], LRP-mediated transcytosis of Aβ peptides is predominantly limited to brain endothelium.

In neurons, LRP shows various unrelated functions. Among them are modulation of N-methyl-D-aspartate (NMDA) receptor-mediated calcium signaling[88], processing of APP[62] and stimulation of neurite outgrowth[106]. These functions may become apparent under distinct conditions of activation of neuronal cells. For example, modulation of NMDA receptor signaling may occur during synaptic transmission of an action potential[89], while under conditions of neuronal damage LRP may be particularly involved in neurite outgrowth[106;107].

LRP displays also multiple functions in monocytes. Activated monocytes adhere to the endothelium in an LRP-dependent mode (chapter 2). Upon subsequent differentiation into macrophages, LRP expression is increased dramatically[108]. In the macrophage, LRP contributes to phagocytosis[15;16] and to 12/15 lipoxygenase-mediated oxidation of LDL[46]. In contrast to neurons, the function of LRP in monocytes/macrophages is thus clearly spatially and temporarily determined. However, precise mechanisms that regulate LRP function are presently unknown. In both neurons and monocytes, it seems conceivable that cofactors that are (temporarily) active or present during these processes influence LRP function.

Receptor cross-talk

LRP is able to efficiently act in concert with other proteins present at the cell surface. HSPGs are a group of receptors abundantly present at the cell surface[109]. Heparin-binding LRP ligands (*e.g.* factor VIII and tissue-factor pathway inhibitor (TFPI)) are efficiently captured from the plasma to the plasma membrane, resulting in a high concentration pool at the cell surface. Ligands can then be directed to LRP for endocytosis or other cellular events[84;110;111]. Chapter 5 illustrates that endocytosis of binding of C4BP (a protein with heparin-binding sites[79;112]) is also a multistep process, which first involves binding to HSPGs and subsequently binding to and endocytosis by LRP. Whether LRP and HSPGs coexist in a heterologous complex or modulate each others function is unclear so far.

LRP is described to be involved in the modification of cellular signaling events by other receptors. For this function as coreceptor, LRP may indirectly interact with other cell surface receptors via intracellular adaptor proteins. For instance, LRP is able to cooperate with the platelet-derived growth factor (PDGF) receptor[19;20]. Stimulation of the PDGF receptor results in phosphorylation of the LRP cytoplasmic tail[19;20], enabling its conjugation with the adaptor protein Shc, which also binds to the PDGF receptor. Via this bridge LRP is able to integrate with the PDGF-induced signaling in order to minimize the cellular responses provoked by PDGF.

Another example in which LRP limits signaling raised by other receptors is that of the NMDA receptor. Long-term exposure of α_2M to LRP on neurons downregulates NMDA receptor expression and thereby diminishes calcium influx via the NMDA receptor[21]. LRP is involved in short-term stimulation of calcium influx as well[88]. To accomplish this receptor cross-talk, LRP and NMDA receptor are interlinked via the intracellular adaptor protein PSD-95[17;89].

Concerning leukocytes, we demonstrated that LRP regulates the clustering of β2-integrins (chapter 2). Both receptors may form a complex at the cell surface

via an interaction between their extracellular domains. To exert a regulatory function, LRP may interact with β2-integrins via their cytoplasmic tails as well. To this end, LRP may be directly involved in the redistribution of β2-integrins. The process of relocalization depends on the detachment of β2-integrins from the actin cytoskeleton[33;39;113;114]. How or whether LRP is involved in this process, is still unclear. However, LRP has been recently described to be required for actin reorganization during the engulfment of apoptotic cells[115]. Both receptors contain several putative phosphorylation sites that may affect interactions with adaptor proteins[16-18;44;116]. Therefore, these adaptor proteins may serve to link the intracellular domains of β2-integrins and LRP, thereby enabling LRP to modulate β2-integrin clustering, probably via actin remodeling.

Besides an indirect interaction via adaptor proteins, LRP is also able to directly interact with transmembrane receptors. LRP mediates the internalization of APP via a direct interaction[117]. It is noteworthy that neuronal LRP is only able to efficiently internalize APP, but not Aβ peptides[102]. Apparently, motifs in APP's intracellular tail are required for endocytosis by LRP, possibly via adaptor proteins. Interestingly, LRP's intracellular tail is connected with the intracellular domain of APP via the adaptor protein Fe65[118;119]. Whether or not this interaction is required for endocytosis or signaling has still to be investigated. Chapter 4 reports that brain endothelial cells are capable to internalize Aβ. However, it is still unclear whether motifs in Aβ peptides or the endocytosis machinery of these cells is responsible for this process.

Taken together, the microenvironment of LRP, and in particular the presence of other membrane receptors, may define LRP functions in a specific cell-type.

Subcellular localization-dependent function of LRP

Initial studies on LRP showed that LRP is localized in clathrin-coated pits at the cell surface for its function as endocytic receptor. LRP interacts with adaptor protein-2 in the clathrin complex. Motifs in the intracellular tail are specifically involved in endocytosis. Whereas for the LDL receptor the NP*X*Y motif is required for efficient endocytosis[120], the main endocytosis motifs for LRP are a Y*XX*L and a di-leucine motif[121]. In chapter 5 we described that LRP is required for the endocytosis of C4BP. It seems conceivable that clathrin-coated pits are also involved in C4BP endocytosis.

Apart from the clathrin-coated pits, LRP has also been detected in lipid rafts[19]. It is tempting to speculate that LRP is recruited to lipid rafts in its function as signaling receptor[122]. LRP functions as a modulator of PDGF receptor signaling[20]. To do so, LRP and PDGF receptor are localized in caveolae[19]. In addition, LRP is described to be present in rafts in complex with β2-integrins in activated monocytes (chapter 2). We found that LRP does not downregulate cell surface expres-

sion of β2-integrins, suggesting that LRP is not involved in endocytosis in this interaction in rafts in these cells. However, LRP localized in lipid rafts can also mediate internalization of ligands, as described for APP, of which the proper internalization and processing is dependent on its localization in these cell membrane domains[123]. Probably caveolae are involved, since this lipid raft subtype is known to play an important role in non-clathrin-mediated endocytosis[124].

Localization of LRP at the cell surface may be directed by the interaction between cytoplasmic tail and intracellular adaptor proteins. This is illustrated by Marzolo et al[125] who demonstrated that the NP*X*Y motif of LRP is responsible for its basolateral sorting in MDCK cells. In other polarized cells, like neurons, LRP localization appears to be restricted to only one plasma membrane domain[105;126]. In brain endothelial cells, LRP is supposed to be present at their basolateral membrane and to mediate clearance of Aβ peptides (chapter 4 and ref 102), whereas RAGE is involved in internalization of Aβ peptides at the apical membrane[71].

Thus, subcellular localization of LRP appears to be an important factor in the regulation of its function. The presence of coreceptors and intracellular adaptor proteins, and LRP's association with the cytoskeleton may be key factors in localizing LRP in cell membrane domains. Due to this spatial dissection, LRP is exposed to a selective set of ligands, which may specify LRP's function. In addition, function can be determined by the phosphorylation state of LRP's intracellular tail. Serine/threonine phosphorylation leads to an increase in endocytosis rate[127;128]. In contrast, cellular signaling events may be dependent on tyrosine phosphorylation[16-18;129]. It would be interesting to study which nature of phosphorylation is preferred at a specific site at the cell surface.

Structural requirements of LRP in relation to its function

We demonstrated that leukocytes equally adhere to immobilized cluster II and IV. In this respect, leukocytes are similar to most other LRP ligands, which show similar affinity for isolated recombinant clusters II and IV[130]. One of these ligands is the tPA/PAI-1 complex. Interestingly, studies using minireceptors only containing cluster II and IV revealed that the tPA/PAI-1 complex prefers binding to cluster II. Possibly, the covalent attachment to the transmembrane domain may limit ligand access to the minireceptor containing cluster IV[131]. The ligand binding repeats in a cluster show similarities, but display differential ligand recognition. Primary conformation and spatial arrangements between the individual repeats are thought to ensure ligand specificity[1]. Further molecular dissection of the clusters showed that ligands prefer binding to certain successive repeats, which may overlap with repeats that bind other ligands[132]. Future studies may provide evidence whether ligand binding to a specific cluster or repeat

evokes a particular LRP-mediated cellular function. In this aspect, it is of interest to mention that RAP admittedly is able to bind to LRP with high affinity[133] and can be internalized via LRP, but many studies demonstrated that RAP is unable to stimulate cellular signaling pathways. Thus, structural information within RAP may determine its effect on LRP. Alternatively, RAP may change the conformation of LRP, thereby making it impossible to transduce a signal. Regarding LRP conformation, it has been generally postulated that one single RAP molecule can interact with cluster II, III and IV of LRP simultaneously. It is conceivable that other ligands may induce a distinct conformational change in LRP, thereby transmitting a particular signal to LRP's intracellular tail and allowing binding of specific adaptor molecules.

A tool to study apparently distinct functions of the clusters are LRP minireceptors that contain only one cluster (mainly II and IV). Though, the difference between the distance between cluster II and the cell membrane in full-length and truncated LRP may be a disadvantage of this method. Probably, the spatial occurrence of cluster II in full-length LRP may specify the function of LRP raised by binding of a ligand to this cluster compared to binding to cluster IV[131;134;135].

Regulation of LRP expression

In most cell types LRP is constitutively expressed at the plasma membrane, where its presence is required to exert its function. Under certain conditions LRP's cell surface expression is regulated by either *de novo* synthesis or translocation from intracellular storage pools.

Although exact mechanisms on how transcription is regulated are unknown[136], NGF, vascular endothelial growth factor, transforming growth factor (TGF)-β1 and TGF-β2 are described to induce LRP mRNA expression[137;138]. A rapid induction of LRP protein expression at the cell surface can be accomplished by translocation of intracellularly stored LRP, as has been demonstrated for insulin- or NGF-stimulated cells[49;137]. This is further exemplified in chapter 3, which describes that LRP protein is rapidly recruited to the cell membrane in T-lymphocytes. These cells are the only cells described that maintain the total LRP pool intracellularly, in contrast to other cell types which show both cell surface and intracellular localization of LRP. The underlying mechanisms on how T-lymphocytes regulate this localization are unclear. In these examples signaling induced by another receptor may regulate LRP expression. In chapter 4 we described that the LRP ligand Aβ peptide directly downregulates the expression of LRP by targeting it to the proteasomal degradation pathway.

These studies indicate that the function and activity of LRP may be temporally controlled by regulating its cell surface expression.

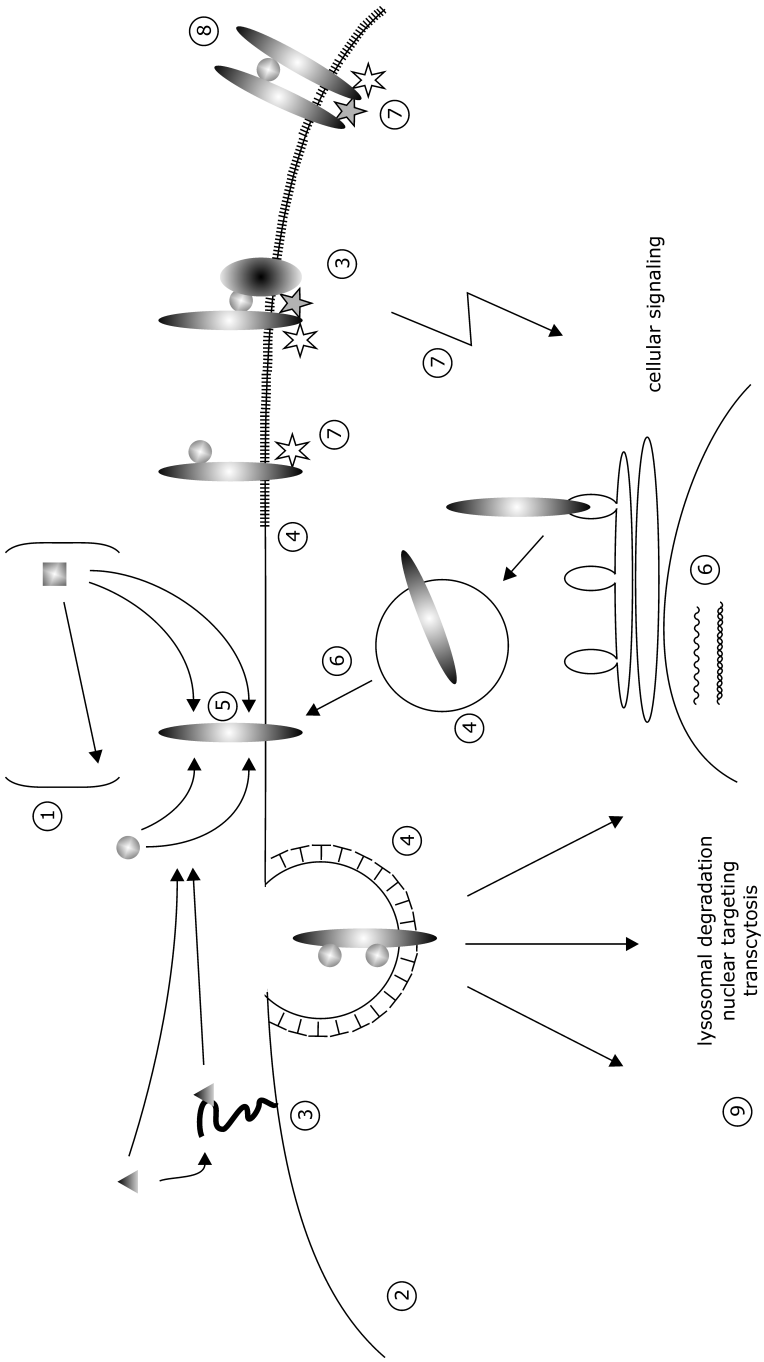


Figure 1. Regulation of LRP function. The versatility of LRP may be determined by its microenvironment. 1, occurrence and structure of ligands; 2, cellular expression patterns; 3, receptor cross-talk; 4, subcellular localization; 5, structure of LRP; 6, regulation of mRNA and protein expression; 7, intracellular adaptor proteins and cellular signaling; 8, receptor dimerization; 9, intracellular transport pathways.

Summary and Future Prospects

In summary, the multifunctionality and the broad cellular expression of LRP prompted us to address the question whether its microenvironment play a pivotal role in directing cell-restricted functions. Information present in ligands and LRP structure may determine cellular events induced by receptor-ligand interaction. In addition, accessory proteins located either at the cell surface (coreceptors) or intracellularly (adaptor proteins) contribute to specifying functions of LRP at different steps of cellular responses (*Fig 1*).

Future research may be focused to consider details in ligand-receptor interactions in respect to components present in the environment, and how these interactions may influence LRP's functions in respect to intracellular transport pathways and cellular signaling. A proteomic approach may aid to identify LRP-binding proteins under specific (patho-)physiological conditions.

References

1. Krieger, M. and J. Herz. 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu.Rev.Biochem.* 63:601-637.
2. Krieger, M. and D.M. Stern. 2001. Series introduction: multiligand receptors and human disease. *J.Clin.Invest.* 108:645-647.
3. Li, Y., J. Cam, and G. Bu. 2001. Low-density lipoprotein receptor family: endocytosis and signal transduction. *Mol.Neurobiol.* 23:53-67.
4. Herz, J. 2001. The LDL receptor gene family: (un)expected signal transducers in the brain. *Neuron* 29:571-581.
5. Herz, J. and H.H. Bock. 2002. Lipoprotein receptors in the nervous system. *Annu.Rev.Biochem.* 71:405-434.
6. Willnow, T.E., Z. Sheng, S. Ishibashi, and J. Herz. 1994. Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor antagonist. *Science* 264:1471-1474.
7. Rohlmann, A., M. Gotthardt, R.E. Hammer, and J. Herz. 1998. Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *J.Clin.Invest.* 101 :689-695.
8. van Vlijmen, B.J., A. Rohlmann, S.T. Page, A.Bensadoun, I.S. Bos, T.J. van Berkel, L.M. Havekes, and J. Herz. 1999. An extrahepatic receptor-associated protein-sensitive mechanism is involved in the metabolism of triglyceride-rich lipoproteins. *J.Biol.Chem.* 274:35219-35226.
9. Strickland, D.K., S.L. Gonias, and W.S. Argraves. 2002. Diverse roles for the LDL receptor family. *Trends Endocrinol.Metab.* 13:66-74.
10. Herz, J. and D.K. Strickland. 2001. LRP: a multifunctional scavenger and signaling receptor. *J.Clin.Invest.* 108:779-784.
11. Mukherjee, S., R.N. Ghosh, and F.R. Maxfield. 1997. Endocytosis. *Physiol.Rev.* 77:759-803.
12. Hussain, M.M., D.K. Strickland, and A. Bakillah. 1999. The mammalian low-density lipoprotein receptor family. *Annu.Rev.Nutr.* 19:141-172.
13. Demeule, M., J. Poirier, J. Jodoin, Y. Bertrand, R.R. Desrosiers, C. Dagenais, T. Nguyen, J. Lanthier, R. Gabathuler, M. Kennard, W.A. Jefferies, D. Karkan, S. Tsai, L. Fenart, R. Cecchelli,

and R. Beliveau. 2002. High transcytosis of melanotransferrin (P97) across the blood-brain barrier. *J.Neurochem.* 83:924-933.

14. Muramatsu, H., K. Zou, N. Sakaguchi, S. Ikematsu, S. Sakuma, and T. Muramatsu . 2000. LDL receptor-related protein as a component of the midkine receptor. *Biochem.Biophys.Res.Commun.* 270:936-941.
15. Patel, M., J. Morrow, F.R. Maxfield, D.K. Strickland, S. Greenberg, and I. Tabas. 2003. The cytoplasmic domain of the low density lipoprotein (LDL) receptor-related protein, but not that of the LDL receptor, triggers phagocytosis. *J.Biol.Chem.* 278:44799-44807.
16. Su, H.P., K. Nakada-Tsukui, A.C. Tosello-Trampont, Y. Li, G. Bu, P.M. Henson, and K.S. Ravichandran. 2002. Interaction of CED-6/GULP, an adapter protein involved in engulfment of apoptotic cells with CED-1 and CD91/low density lipoprotein receptor-related protein (LRP). *J.Biol.Chem.* 277:11772-11779.
17. Gotthardt, M., M. Trommsdorff, M.F. Nevitt, J. Shelton, J.A. Richardson, W. Stockinger, J. Nimpf, and J. Herz. 2000. Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. *J.Biol.Chem.* 275:25616-25624.
18. Barnes, H., B. Larsen, M. Tyers, and P. van der Geer. 2001. Tyrosine-phosphorylated low density lipoprotein receptor-related protein 1 (Lrp1) associates with the adaptor protein SHC in SRC-transformed cells. *J.Biol.Chem.* 276:19119-19125.
19. Boucher, P., P. Liu, M. Gotthardt, T. Hiesberger, R.G. Anderson, and J. Herz. 2002. Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low Density lipoprotein receptor-related protein in caveolae. *J.Biol.Chem.* 277:15507-15513.
20. Loukinova, E., S. Ranganathan, S. Kuznetsov, N. Gorlatova, M.M. Migliorini, D. Loukinov, P.G. Ulery, I. Mikhailenko, D.A. Lawrence, and D.K. Strickland. 2002. Platelet-derived growth factor (PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). Evidence for integrated co-receptor function between LRP and the PDGF receptor. *J.Biol.Chem.* 277:15499-15506.
21. Qiu, Z., D.K. Strickland, B.T. Hyman, and G.W. Rebeck. 2002. alpha 2-Macroglobulin exposure reduces calcium responses to N-methyl-D-aspartate via low density lipoprotein receptor-related protein in cultured hippocampal neurons. *J.Biol.Chem.* 277:14458-14466.
22. Harris, E.S., T.M. McIntyre, S.M. Prescott, and G.A. Zimmerman. 2000. The leukocyte integrins. *J.Biol.Chem.* 275:23409-23412.
23. McEver, R.P. 2001. Adhesive interactions of leukocytes, platelets, and the vessel wall during hemostasis and inflammation. *Thromb.Haemost.* 86:746-756.
24. Gahmberg, C.G. 1997. Leukocyte adhesion: CD11/CD18 integrins and intercellular adhesion molecules. *Curr.Opin.Cell Biol.* 9:643-650.
25. Wright, S.D., J.I. Weitz, A.J. Huang, S.M. Levin, S.C. Silverstein, and J.D. Loike. 1988. Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. *Proc.Natl.Acad.Sci.U.S.A.* 85:7734-7738.
26. Simon, D.I., Z. Chen, H. Xu, C.Q. Li, J. Dong, L.V. McIntire, C.M. Ballantyne, L. Zhang, M.I. Furman, M.C. Berndt, and J.A. Lopez. 2000. Platelet glycoprotein ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J.Exp.Med.* 192:193-204.
27. Altieri, D.C. and T.S. Edgington. 1988. The saturable high affinity association of factor X to ADP-stimulated monocytes defines a novel function of the Mac-1 receptor. *J.Biol.Chem.* 263:7007-7015.
28. Moyle, M., D.L. Foster, D.E. McGrath, S.M. Brown, Y. Laroche, J. De Meutter, P. Stanssens, C.A. Bogowitz, V.A. Fried, J.A. Ely, H.R. Soyle, and G.P. Vlasuk. 1994. A hookworm glycoprotein that inhibits neutrophil function is a ligand of the integrin CD11b/CD18. *J.Biol.Chem.* 269:10008-10015.
29. Diamond, M.S., J. Garcia-Aguilar, J.K. Bickford, A.L. Corbi, and T.A. Springer. 1993. The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. *J.Cell Biol.* 120:1031-1043.

30. Shimaoka, M., J. Takagi, and T.A. Springer. 2002. Conformational regulation of integrin structure and function. *Annu.Rev.Biophys.Biomol.Struct.* 31:485-516.
31. Dickeson, S.K. and S.A. Santoro. 1998. Ligand recognition by the I domain-containing integrins. *Cell Mol.Life Sci.* 54:556-566.
32. Solovjov, D.A., E. Pluskota, and E.F. Plow. 2005. Distinct roles for the alpha and beta subunits in the functions of integrin alpha Mbeta 2. *J.Biol.Chem.* 280: 1336-1345.
33. Liu, S., D.A. Calderwood, and M.H. Ginsberg. 2000. Integrin cytoplasmic domain-binding proteins. *J.Cell Sci.* 113:3563-3571.
34. Ehirchiou, D., Y.M. Xiong, Y. Li, S. Brew, and L. Zhang. 2005. Dual function for a unique site within the beta 2I-domain of integrin alpha Mbeta 2. *J.Biol.Chem.* 280: 8324-8331.
35. Bajt, M.L., T. Goodman, and S.L. McGuire. 1995. Beta 2 (CD18) mutations abolish ligand recognition by I domain integrins LFA-1 (alpha L beta 2, CD11a/CD18) and MAC-1 (alpha M beta 2, CD11b/CD18). *J.Biol.Chem.* 270:94-98.
36. Goodman, T.G. and M.L. Bajt. 1996. Identifying the putative metal ion-dependent adhesion site in the beta2 (CD18) subunit required for alphaLbeta2 and alphaMbeta2 ligand interactions. *J.Biol.Chem.* 271:23729-23736.
37. Xiong, Y.M., T.A. Haas, and L. Zhang. 2002. Identification of functional segments within the beta2I-domain of integrin alphaMbeta2. *J.Biol.Chem.* 277:46639-46644.
38. Constantin, G., M. Majeed, C. Giagulli, L. Piccio, J.Y. Kim, E.C. Butcher, and C. Laudanna. 2000. Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* 13:759-769.
39. Van Kooyk, Y. and C.G. Figdor. 2000. Avidity regulation of integrins: the driving force in leukocyte adhesion. *Curr.Opin.Cell Biol.* 12:542-547.
40. Stewart, M.P., A. McDowall, and N. Hogg. 1998. LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a Ca2+-dependent protease, calpain. *J.Cell Biol.* 140:699-707.
41. Myou, S., X. Zhu, E. Boetticher, Y. Qin, S. Myo, A. Meliton, A. Lambertino, N.M. Munoz, K.J. Hamann, and A.R. Leff. 2002. Regulation of adhesion of AML14.3D10 cells by surface clustering of beta2-integrin caused by ERK-independent activation of cPLA2. *Immunology* 107:77-85.
42. de Bruyn, K.M., S. Rangarajan, K.A. Reedquist, C.G. Figdor, and J.L. Bos. 2002. The small GTPase Rap1 is required for Mn(2+)- and antibody-induced LFA-1- and VLA-4-mediated cell adhesion. *J.Biol.Chem.* 277:29468-29476.
43. Katagiri, K., A. Maeda, M. Shimonaka, and T. Kinashi. 2003. RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nat.Immunol.* 4:741-748.
44. Fagerholm, S., N. Morrice, C.G. Gahmberg, and P. Cohen. 2002. Phosphorylation of the cytoplasmic domain of the integrin CD18 chain by protein kinase C isoforms in leukocytes. *J.Biol.Chem.* 277:1728-1738.
45. Krauss, K. and P. Altevogt. 1999. Integrin leukocyte function-associated antigen-1-mediated cell binding can be activated by clustering of membrane rafts. *J.Biol.Chem.* 274:36921-36927.
46. Xu, W., Y. Takahashi, T. Sakashita, T. Iwasaki, H. Hattori, and T. Yoshimoto. 2001. Low density lipoprotein receptor-related protein is required for macrophage-mediated oxidation of low density lipoprotein by 12/15-lipoxygenase. *J.Biol.Chem.* 276:36454-36459.
47. Hart, J.P., M.D. Gunn, and S.V. Pizzo. 2004. A CD91-positive subset of CD11c+ blood dendritic cells: characterization of the APC that functions to enhance adaptive immune responses against CD91-targeted antigens. *J.Immunol.* 172:70-78.
48. Spijkers, P.P., P. da Costa Martins, E. Westein, C.G. Gahmberg, J.J. Zwaginga, and P.J. Lenting. 2005. LDL-receptor-related protein regulates beta2-integrin-mediated leukocyte adhesion. *Blood* 105:170-177.
49. Descamps, O., D. Bilheimer, and J. Herz. 1993. Insulin stimulates receptor-mediated uptake of apoE-enriched lipoproteins and activated alpha 2-macroglobulin in adipocytes. *J.Biol.Chem.* 268:974-981.

50. Bu, G., Y. Sun, A.L. Schwartz, and D.M. Holtzman. 1998. Nerve growth factor induces rapid increases in functional cell surface low density lipoprotein receptor-related protein. *J.Biol.Chem.* 273:13359-13365.
51. Ferrara, J.L. and H.J. Deeg. 1991. Graft-versus-host disease. *N.Engl.J.Med.* 324:667-674.
52. Sharpe, A.H. and G.J. Freeman. 2002. The B7-CD28 superfamily. *Nat.Rev.Immunol.* 2:116-126.
53. Riley, J.L. and C.H. June. 2005. The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation. *Blood* 105:13-21.
54. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe. 2005. The B7 Family Revisited. *Annu.Rev.Immunol.* 23:515-548.
55. Cam, J.A., C.V. Zerbinatti, Y. Li, and G. Bu. 2005. Rapid endocytosis of the LDL receptor-related protein modulates cell surface distribution and processing of the beta amyloid precursors protein. *J.Biol.Chem.* 280:15464-15470.
56. Springer, T.A. 1990. Adhesion receptors of the immune system. *Nature* 346:425-434.
57. Pribila, J.T., A.C. Quale, K.L. Mueller, and Y. Shimizu. 2004. Integrins and T cell-mediated immunity. *Annu.Rev.Immunol.* 22:157-180.
58. Kang, D.E., T. Saitoh, X. Chen, Y. Xia, E. Masliah, L.A. Hansen, R.G. Thomas, L.J. Thal, and R. Katzman. 1997. Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), an apolipoprotein E receptor, with late-onset Alzheimer's disease. *Neurology* 49:56-61.
59. Hollenbach, E., S. Ackermann, B.T. Hyman, and G.W. Rebeck. 1998. Confirmation of an association between a polymorphism in exon 3 of the low-density lipoprotein receptor-related protein gene and Alzheimer's disease. *Neurology* 50:1905-1907.
60. Lambert, J.C., F. Wavrant-De Vrieze, P. Amouyel, and M.C. Chartier-Harlin. 1998. Association at LRP gene locus with sporadic late-onset Alzheimer's disease. *Lancet* 351:1787-1788.
61. Wavrant-DeVrieze, F., J.C. Lambert, L. Stas, R. Crook, D. Cottel, F. Pasquier, B. Frigard, M. Lambrechts, E. Thiry, P. Amouyel, J.P. Tur, M.C. Chartier-Harlin, J. Hardy, and F. van Leuven. 1999. Association between coding variability in the LRP gene and the risk of late-onset Alzheimer's disease. *Hum.Genet.* 104:432-434.
62. Kounnas, M.Z., R.D. Moir, G.W. Rebeck, A.I. Bush, W.S. Argraves, R.E. Tanzi, B.T. Hyman, and D.K. Strickland. 1995. LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell* 82:331-340.
63. Pietrzik, C.U., T. Busse, D.E. Merriam, S. Weggen, and E.H. Koo. 2002. The cytoplasmic domain of the LDL receptor-related protein regulates multiple steps in APP processing. *EMBO J.* 21:5691-5700.
64. Ulery, P.G., J. Beers, I. Mikhailenko, R.E. Tanzi, G.W. Rebeck, B.T. Hyman, and D.K. Strickland. 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. *J.Biol.Chem.* 275:7410-7415.
65. Qiu, Z., D.K. Strickland, B.T. Hyman, and G.W. Rebeck. 1999. Alpha2-macroglobulin enhances the clearance of endogenous soluble beta-amyloid peptide via low-density lipoprotein receptor-related protein in cortical neurons. *J.Neurochem.* 73:1393-1398.
66. Kang, D.E., C.U. Pietrzik, L. Baum, N. Chevallier, D.E. Merriam, M.Z. Kounnas, S.L. Wagner, J.C. Troncoso, C.H. Kawas, R. Katzman, and E.H. Koo. 2000. Modulation of amyloid β-protein clearance and Alzheimer's disease susceptibility by the LDL receptor-related protein pathway. *J.Clin.Invest.* 106:1159-1166.
67. Narita, M., D.M. Holtzman, A.L. Schwartz, and G. Bu. 1997. Alpha2-macroglobulin complexes with and mediates the endocytosis of beta-amyloid peptide via cell surface low-density lipoprotein receptor-related protein. *J.Neurochem.* 69:1904-1911.
68. Zerbinatti, C.V., D.F. Wozniak, J. Cirrito, J.A. Cam, H. Osaka, K.R. Bales, M. Zhuo, S.M. Paul, D.M. Holtzman, and G. Bu. 2004. Increased soluble amyloid-beta peptide and memory deficits in amyloid model mice overexpressing the low-density lipoprotein receptor-related protein. *Proc.Natl.Acad.Sci.U.S.A.* 101:1075-1080.

69. Van Uden, E., M. Mallory, I. Veinbergs, M. Alford, E. Rockenstein, and E. Masliah. 2002. Increased extracellular amyloid deposition and neurodegeneration in human amyloid precursor protein transgenic mice deficient in receptor-associated protein. *J.Neurosci.* 22:9298-9304.
70. Vinters, H.V. and E.S. Farag. 2003. Amyloidosis of cerebral arteries. *Adv.Neurol.* 92:105-112.
71. Deane, R., Y.S. Du, R.K. Submamaryan, B. LaRue, S. Jovanovic, E. Hogg, D. Welch, L. Manness, C. Lin, J. Yu, H. Zhu, J. Ghiso, B. Frangione, A. Stern, A.M. Schmidt, D.L. Armstrong, B. Arnold, B. Liliensiek, P. Nawroth, F. Hofman, M. Kindy, D. Stern, and B. Zlokovic. 2003. RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain. *Nat.Med.* 9:907-913.
72. Zlokovic, B.V. 2004. Clearing amyloid through the blood-brain barrier. *J.Neurochem.* 89:807-811.
73. Westein, E., C.V. Denis, B.N. Bouma, and P.J. Lenting. 2002. The alpha -chains of C4b-binding protein mediate complex formation with low density lipoprotein receptor-related protein. *J.Biol.Chem.* 277:2511-2516.
74. Fujita, T., I. Gigli, and V. Nussenzweig. 1978. Human C4-binding protein. II. Role in proteolysis of C4b by C3b-inactivator . *J.Exp.Med.* 148:1044-1051.
75. Schwalbe, R.A., B. Dahlback, and G.L. Nelsestuen. 1990. Independent association of serum amyloid P component, protein S, and complement C4b with complement C4b-binding protein and subsequent association of the complex with membranes. *J.Biol.Chem.* 265:21749-21757.
76. Blom, A.M., K. Berggard, J.H. Webb, G. Lindahl, B.O. Villoutreix, and B. Dahlback. 2000. Human C4b-binding protein has overlapping, but not identical, binding sites for C4b and streptococcal M proteins. *J.Immunol.* 164:5328-5336.
77. Dahlback, B. and J. Stenflo. 1981. High molecular weight complex in human plasma between vitamin K-dependent protein S and complement component C4b-binding protein. *Proc.Natl.Acad.Sci.U.S.A.* 78:2512-2516.
78. Hillarp, A. and B. Dahlback. 1988. Novel subunit in C4b-binding protein required for protein S binding. *J.Biol.Chem.* 263:12759-12764.
79. Fujita, T. and V. Nussenzweig. 1979. The role of C4-binding protein and beta 1H in proteolysis of C4b and C3b. *J.Exp.Med.* 150:267-276.
80. Hessing, M., C. van 't Veer, and B.N. Bouma. 1990. The binding site of human C4b-binding protein on complement C4 is localized in the alpha'-chain. *J.Immunol.* 144:2632-2637.
81. Rohlena, J., J.A. Kolkman, R.C. Boertjes, K. Mertens, and P.J. Lenting. 2003. Residues Phe342-Asn346 of activated coagulation factor IX contribute to the interaction with low density lipoprotein receptor-related protein. *J.Biol.Chem.* 278:9394-9401.
82. Neels, J.G., B.M. van den Berg, K. Mertens, H. ter Maat, H. Pannekoek, A.J. van Zonneveld, and P.J. Lenting. 2000. Activation of factor IX zymogen results in exposure of a binding site for low-density lipoprotein receptor-related protein. *Blood* 96:3459-3465.
83. Mikhailenko, I., M.Z. Kounnas, and D.K. Strickland. 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. *J.Biol.Chem.* 270:9543-9549.
84. Sarafanov, A.G., N.M. Ananyeva, M. Shima, and E.L. Saenko. 2001. Cell surface heparan sulfate proteoglycans participate in factor VIII catabolism mediated by low density lipoprotein receptor-related protein. *J.Biol.Chem.* 276:11970-11979.
85. Blom, A.M., J. Webb, B.O. Villoutreix, and B. Dahlback. 1999. A cluster of positively charged amino acids in the C4BP alpha-chain is crucial for C4b binding and factor I cofactor function. *J.Biol.Chem.* 274:19237-19245.
86. Dahlback, B. 1991. Protein S and C4b-binding protein: components involved in the regulation of the protein C anticoagulant system. *Thromb.Haemost.* 66:49-61.
87. Swertfeger, D.K., G. Bu, and D.Y. Hui. 2002. Low density lipoprotein receptor-related protein mediates apolipoprotein E inhibition of smooth muscle cell migration. *J.Biol.Chem.* 277:4141-4146.

88. Bacskai, B.J., M.Q. Xia, D.K. Strickland, G.W. Rebeck, and B.T. Hyman. 2000. The endocytic receptor protein LRP also mediates neuronal calcium signaling via N-methyl-D-aspartate receptors. *Proc.Natl.Acad.Sci.U.S.A.* 97:11551-11556.
89. May, P., A. Rohlmann, H.H. Bock, K. Zurhove, J.D. Marth, E.D. Schomburg, J.L. Noebels, U. Beffert, J.D. Sweatt, E.J. Weeber, and J. Herz. 2004. Neuronal LRP1 functionally associates with postsynaptic proteins and is required for normal motor function in mice. *Mol.Cell Biol.* 24:8872-8883.
90. Gliemann, J., A. Nykjaer, C.M. Petersen, K.E. Jorgensen, M. Nielsen, P.A. Andreasen, E.I. Christensen, A. Lookene, G. Olivecrona, and S.K. Moestrup. 1994. The multiligand alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein (alpha 2MR/LRP). Binding and endocytosis of fluid phase and membrane-associated ligands. *Ann.N.Y.Acad.Sci.* 737:20-38.
91. Strickland, D.K. and S. Ranganathan. 2003. Diverse role of LDL receptor-related protein in the clearance of proteases and in signaling. *J.Thromb.Haemost.* 1:1663-1670.
92. Strickland, D.K., M.Z. Kounnas, and W.S. Argraves. 1995. LDL receptor-related protein: a multiligand receptor for lipoprotein and proteinase catabolism. *FASEB J.* 9:890-898.
93. Kounnas, M.Z., R.E. Morris, M.R. Thompson, D.J. FitzGerald, D.K. Strickland, and C.B. Saelinger. 1992. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J.Biol.Chem.* 267:12420-12423.
94. Hofer, F., M. Gruenberger, H. Kowalski, H. Machat, M. Huettinger, E. Kuechler, and D. Blass. 1994. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. *Proc.Natl.Acad.Sci.U.S.A.* 91:1839-1842.
95. Shakibaei, M. and U. Frevert. 1996. Dual interaction of the malaria circumsporozoite protein with the low density lipoprotein receptor-related protein (LRP) and heparan sulfate proteoglycans. *J.Exp.Med.* 184:1699-1711.
96. Liu, Y., M. Jones, C.M. Hingtgen, G. Bu, N. Laribee, R.E. Tanzi, R.D. Moir, A. Nath, and J.J. He. 2000. Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. *Nat.Med.* 6:1380-1387.
97. Quinn, K.A., V.J. Pye, Y.P. Dai, C.N. Chesterman, and D.A. Owensby. 1999. Characterization of the soluble form of the low density lipoprotein receptor-related protein (LRP). *Exp.Cell Res.* 251:433-441.
98. Brodeur, S.R., F. Angelini, L.B. Bacharier, A.M. Blom, E. Mizoguchi, H. Fujiwara, A. Plebani, L.D. Notarangelo, B. Dahlback, E. Tsitsikov, and R.S. Geha. 2003. C4b-binding protein (C4BP) activates B cells through the CD40 receptor. *Immunity* 18:837-848.
99. Kowal, R.C., J. Herz, J.L. Goldstein, V. Esser, and M.S. Brown. 1989. Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins. *Proc.Natl.Acad.Sci.U.S.A.* 86:5810-5814.
100. Verges, M., A. Bensadoun, J. Herz, J.D. Belcher, and R.J. Havel. 2004. Endocytosis of hepatic lipase and lipoprotein lipase into rat liver hepatocytes in vivo is mediated by the low density lipoprotein receptor-related protein. *J.Biol.Chem.* 279:9030-9036.
101. Yu, K.C., W. Chen, and A.D. Cooper. 2001. LDL receptor-related protein mediates cell-surface clustering and hepatic sequestration of chylomicron remnants in LDLR-deficient mice. *J.Clin.Invest.* 107:1387-1394.
102. Shibata, M., S. Yamada, S.R. Kumar, M. Calero, J. Bading, B. Frangione, D.M. Holtzman, C.A. Miller, D.K. Strickland, J. Ghiso, and B.V. Zlokovic. 2000. Clearance of Alzheimer's amyloid-ss(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. *J.Clin.Invest.* 106:1489-1499.
103. Obunike, J.C., P. Sivaram, L. Paka, M.G. Low, and I.J. Goldberg. 1996. Lipoprotein lipase degradation by adipocytes: receptor-associated protein (RAP)-sensitive and proteoglycan-mediated pathways. *J.Lipid Res.* 37:2439-2449.
104. Vassiliou, G., F. Benoist, P. Lau, G.N. Kavaslar, and R. McPherson. 2001. The low density lipoprotein receptor-related protein contributes to selective uptake of high density lipoprotein cho-

lesteryl esters by SW872 liposarcoma cells and primary human adipocytes. *J.Biol.Chem.* 276:48823-48830.

105. Moestrup, S.K., J. Gliemann, and G. Pallesen. 1992. Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res.* 269:375-382.
106. Holtzman, D.M., R.E. Pitas, J. Kilbridge, B. Nathan, R.W. Mahley, G. Bu, and A.L. Schwartz. 1995. Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line. *Proc.Natl.Acad.Sci.U.S.A.* 92:9480-9484.
107. Nathan, B.P., Y. Jiang, G.K. Wong, F. Shen, G.J. Brewer, and R.G. Struble. 2002. Apolipoprotein E4 inhibits, and apolipoprotein E3 promotes neurite outgrowth in cultured adult mouse cortical neurons through the low-density lipoprotein receptor-related protein. *Brain Res.* 928:96-105.
108. Watanabe, Y., T. Inaba, H. Shimano, T. Gotoda, K. Yamamoto, H. Mokuno, H. Sato, Y. Yazaki, and N. Yamada. 1994. Induction of LDL receptor-related protein during the differentiation of monocyte-macrophages. Possible involvement in the atherosclerotic process. *Arterioscler.Thromb.* 14:1000-1006.
109. Bernfield, M., M. Gotte, P.W. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, and M. Zako. 1999. Functions of cell surface heparan sulfate proteoglycans. *Annu.Rev.Biochem.* 68:729-777.
110. Ji, Z.S., W.J. Brecht, R.D. Miranda, M.M. Hussain, T.L. Innerarity, and R.W. Mahley. 1993. Role of heparan sulfate proteoglycans in the binding and uptake of apolipoprotein E-enriched remnant lipoproteins by cultured cells. *J.Biol.Chem.* 268:10160-10167.
111. Ji, Z.S., H.L. Dichek, R.D. Miranda, and R.W. Mahley. 1997. Heparan sulfate proteoglycans participate in hepatic lipase and apolipoprotein E-mediated binding and uptake of plasma lipoproteins, including high density lipoproteins. *J.Biol.Chem.* 272:31285-31292.
112. Hessing, M., D. Kanters, T.M. Hackeng, and B.N. Bouma. 1990. Identification of different forms of human C4b-binding protein lacking beta-chain and protein S binding ability. *Thromb.Haemost.* 64:245-250.
113. Calderwood, D.A., S.J. Shattil, and M.H. Ginsberg. 2000. Integrins and actin filaments: reciprocal regulation of cell adhesion and signaling. *J.Biol.Chem.* 275:22607-22610.
114. Sampath, R., P.J. Gallagher, and F.M. Pavalko. 1998. Cytoskeletal interactions with the leukocyte integrin beta2 cytoplasmic tail. Activation-dependent regulation of associations with talin and alpha-actinin. *J.Biol.Chem.* 273:33588-33594.
115. Kinchen, J.M., J. Cabello, D. Klingele, K. Wong, R. Feichtinger, H. Schnabel , R. Schnabel, and M.O. Hengartner . 2005. Two pathways converge at CED-10 to mediate actin rearrangement and corpse removal in C. elegans. *Nature* 434:93-99.
116. Dib, K. 2000. BETA 2 integrin signaling in leukocytes. *Front.Biosci.* 5:D438-51.
117. Knauer, M.F., R.A. Orlando, and C.G. Glabe. 1996. Cell surface APP751 forms complexes with protease nexin 2 ligands and is internalized via the low density lipoprotein receptor-related protein (LRP). *Brain Res.* 740:6-14.
118. Trommsdorff, M., J.P. Borg, B. Margolis, and J. Herz. 1998. Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J.Biol.Chem.* 273:33556-33560.
119. Kinoshita, A., C.M. Whelan, C.J. Smith, I. Mikhailenko, G.W. Rebeck, D.K. Strickland, and B.T. Hyman. 2001. Demonstration by fluorescence resonance energy transfer of two sites of interaction between the low-density lipoprotein receptor-related protein and the amyloid precursor protein: role of the intracellular adapter protein Fe65. *J.Neurosci.* 21:8354-8361.
120. Chen, W.J., J.L. Goldstein, and M.S. Brown. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J.Biol.Chem.* 265:3116-3123.
121. Li, Y., M.P. Marzolo, P. van Kerkhof, G.J. Strous, and G. Bu. 2000. The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. *J.Biol.Chem.* 275:17187-17194.

122. Fielding, C.J. and P.E. Fielding. 2004. Membrane cholesterol and the regulation of signal transduction. *Biochem.Soc.Trans.* 32:65-69.
123. Ikezu, T., B.D. Trapp, K.S. Song, A. Schlegel, M.P. Lisanti, and T. Okamoto. 1998. Caveolae, plasma membrane microdomains for alpha-secretase-mediated processing of the amyloid precursor protein. *J.Biol.Chem.* 273:10485-10495.
124. Anderson, R.G. 1998. The caveolae membrane system. *Annu.Rev.Biochem.* 67:199-225.
125. Marzolo, M.P., M.I. Yuseff, C. Retamal, M. Donoso, F. Ezquer, P. Farfan, Y. Li, and G. Bu. 2003. Differential distribution of low-density lipoprotein-receptor-related protein (LRP) and megalin in polarized epithelial cells is determined by their cytoplasmic domains. *Traffic* 4:273-288.
126. Brown, M.D., G.A. Banker, I.M. Hussaini, S.L. Gonias, and S.R. Vandenberg. 1997. Low density lipoprotein receptor-related protein is expressed early and becomes restricted to a somatodendritic domain during neuronal differentiation in culture. *Brain Res.* 747:313-317.
127. Li, Y., P. van Kerkhof, M.P. Marzolo, G.J. Strous, and G. Bu. 2001. Identification of a major cyclic AMP-dependent protein kinase A phosphorylation site within the cytoplasmic tail of the low-density lipoprotein receptor-related protein: implication for receptor-mediated endocytosis. *Mol.Cell.Biol.* 21:1185-1195.
128. Ranganathan, S., C.X. Liu, M.M. Migliorini, C.A. Von Arnim, I.D. Peltan, I. Mikhailenko, B.T. Hyman, and D.K. Strickland. 2004. Serine and threonine phosphorylation of the low density lipoprotein receptor-related protein by protein kinase Calpha regulates endocytosis and association with adaptor molecules. *J.Biol.Chem.* 279:40536-40544.
129. Barnes, H., E.J. Ackermann, and P. van der Geer. 2003. v-Src induces Shc binding to tyrosine 63 in the cytoplasmic domain of the LDL receptor-related protein 1. *Oncogene* 22:3589-3597.
130. Neels, J.G., I.R. Horn, B.M. van den Berg, H. Pannekoek, and A.J.van Zonneveld. 1998. Ligand-receptor interaction of the low density lipoprotein receptor-related protein, a multiligand endocytic receptor. *Fibrinol.Proteol.* 12:219-240.
131. Willnow, T.E., K. Orth, and J. Herz. 1994. Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *J.Biol.Chem.* 269:15827-15832.
132. Meijer, A.B., J. Rohlena, C. van der Zwaan, R.C. Boertjes, P.J. Lenting, and K. Mertens. 2005. Complement-type repeats involved in the interaction of low density lipoprotein receptor-related protein with factor VIII light chain, activated factor IX, and α_2-macroglobulin. *Manuscript in preparation*.
133. Bu, G. 1998. Receptor-associated protein: a specialized chaperone and antagonist for members of the LDL receptor gene family. *Curr.Opin.Lipidol.* 9:149-155.
134. Neels, J.G., B.M. van den Berg, A. Lookene, G. Olivecrona, H. annekoek, and A.J.van Zonneveld. 1999. The second and fourth cluster of class A cysteine-rich repeats of the low density lipoprotein receptor-related protein share ligand-binding properties. *J.Biol.Chem.* 274:31305-31311.
135. Obermoeller-McCormick, L.M., Y. Li, H. Osaka, D.J. FitzGerald, A.L. Schwartz, and G. Bu. 2001. Dissection of receptor folding and ligand-binding property with functional minireceptors of LDL receptor-related protein. *J.Cell Sci.* 114:899-908.
136. Kutt, H., J. Herz, and K.K. Stanley. 1989. Structure of the low-density lipoprotein receptor-related protein (LRP) promoter. *Biochim.Biophys.Acta* 1009:229-236.
137. Kibbey, R.G., J. Rizo, L.M. Gierasch, and R.G. Anderson. 1998. The LDL receptor clustering motif interacts with the clathrin terminal domain in a reverse turn conformation. *J.Cell Biol.* 142:59-67.
138. Hollborn, M., G. Birkenmeier, A. Saalbach, I. Iandiev, A. Reichenbach, P. Wiedemann, and L. Kohen. 2004. Expression of LRP1 in retinal pigment epithelial cells and its regulation by growth factors. *Invest.Ophthalmol.Vis.Sci.* 45:2033-2038.

Samenvatting in het Nederlands

De meest fundamentele ideeën van de wetenschap zijn
in wezen eenvoudig en kunnen in de regel worden uitgedrukt
in een taal die voor iedereen begrijpelijk is
(*Albert Einstein*)

Inleiding

Cellen communiceren met hun omgeving via bepaalde eiwitten, zogenaamde receptoren, die zich bevinden op het oppervlak van de cel (= celmembraan). Vaak geldt dat receptoren alleen één soort eiwit kunnen herkennen aan het oppervlak van de cel. Een eiwit dat bindt aan zo'n receptor wordt ligand genoemd. Echter, er zijn ook receptoren bekend die meerdere soorten liganden kunnen binden. Dit is de groep van de zogenaamde 'multiligand receptoren'.

Een groep van aan elkaar verwante receptoren is de low-density lipoprotein (LDL) receptor familie. De familie bestaat uit 12 leden die qua structuur veel op elkaar lijken. Een tweede overeenkomst is dat ze allemaal in staat zijn om meerdere soorten liganden te binden. De LDL receptoren zijn dus multiligand receptoren. De receptoren hebben ook verschillen ten opzichte van elkaar. Zo zijn enkele leden groter dan de rest. Bovendien komen ze niet allemaal in dezelfde celtypen voor en binden ze ook niet allemaal dezelfde liganden.

Het onderzoek zoals beschreven in het proefschrift is gericht op één receptor uit de familie, namelijk LDL receptor-related protein (LRP). Zoals de naam al doet vermoeden, lijkt deze receptor veel op de LDL receptor. Grote verschillen zijn dat LRP ruim 4x zo groot is en veel meer soorten liganden kan binden. Voorbeelden van liganden zijn stollingsfactoren, lipoproteïnen en groeifactoren. De liganden vertonen soms, maar meestal geen structurele overeenkomsten.

Het belang van LRP voor het leven is gebleken toen er transgene muizen werden gemaakt waarin LRP afwezig is. De muizen sterven vroeg in de embryonale ontwikkeling. De functie van LRP is net als zijn liganden erg divers. Voor de diverse functies is de staart van LRP die zich aan de binnenkant van de cel bevindt, erg belangrijk. Hieraan kunnen andere eiwitten binden die de functie van LRP kunnen bepalen. Een bekende functie van LRP is de opname van liganden uit het plasma of van het celoppervlak via het gedeelte van LRP dat uit de cel steekt. Dit proces heet endocytose en leidt uiteindelijk tot de afbraak van de liganden in zogenaamde lysosomen ('mini-maagjes' van de cel). Daarnaast kan LRP ervoor zorgen dat er een signaal in de cel ontstaat dat uiteindelijk bepaalde effecten teweegbrengt, zoals celgroei- en ontwikkeling. Een bijzondere vorm van signalering is dat LRP de functie van andere receptoren op het celoppervlak kan beïnvloeden.

LRP komt voor in verschillende cellen en weefsels in ons lichaam, zoals lever-, hersen- en vetcellen. Niet alle bovengenoemde functies komen in alle cellen voor. De combinatie van cellen en liganden kan de functie van LRP onder die bijzondere omstandigheden bepalen.

Dit proefschrift beschrijft een aantal nieuwe aspecten van LRP. Er is gekeken naar de functie van LRP in verschillende cellen. Een korte samenvatting van de gevonden resultaten is hieronder beschreven.

LRP en de regulatie van de functie van β2-integrinen

LRP is dus een receptor die veel liganden kan binden. Er zijn ook andere groepen liganden bekend die dezelfde eigenschap hebben. Een voorbeeld zijn de β2-integrinen. Dit zijn receptoren die op het oppervlak van witte bloedcellen voorkomen. Deze cellen zijn betrokken bij onze afweer. β2-integrinen zijn onder andere betrokken bij de binding van de witte bloedcellen aan de vaatwand indien de cellen geactiveerd worden door andere onderdelen van het afweersysteem. Hierdoor kunnen de cellen door de vaatwand heen migreren en zo naar de plaats gaan waar ze nodig zijn om bijvoorbeeld de oorzaak van de infectie te bestrijden.

β2-integrinen zijn een complex van 2 eiwitten. Ze bestaan uit een α-deel en een β2-deel. Er zijn verschillende α-delen bekend: α_L, α_D, α_X en α_D. In alle α-delen is een bepaald gebied aanwezig dat betrokken is in de binding aan andere liganden, het zogenaamde I-domein.

In hoofdstuk 2 hebben we beschreven dat de ligand-bindende delen van LRP, de zogenaamde clusters II en IV, en het I-domein van het α-deel van de β2-integrinen direct aan elkaar konden binden. Ook waren LRP en β2-integrinen elkaars liganden op het oppervlak van een groep witte bloedcellen, de monocyten. Het is gebleken dat LRP en β2-integrinen aan elkaar bonden in zowel ongestimuleerde als gestimuleerde monocyten.

Het was al bekend dat als monocyten gestimuleerd worden, β2-integrinen groepjes aan het celoppervlak vormen. Dit is noodzakelijk om efficiënte binding van de monocyten aan de vaatwand te krijgen. Wij hebben gevonden dat in deze groepjes ook LRP aanwezig was. De functie van LRP in deze situatie hebben we bekeken door monocyten te gebruiken waarin LRP niet aanwezig was. Zulke monocyten bleken dan niet meer in staat om de groepjes β2-integrinen te vormen. Dit had als gevolg dat deze monocyten ook niet meer zo efficiënt aan de vaatwand konden binden.

Uit deze resultaten blijkt dus dat LRP de functie van β2-integrinen kan beïnvloeden en dat LRP noodzakelijk is voor het goed functioneren van β2-integrinen. In dit geval is LRP een zogenaamde co-receptor voor β2-integrinen. Vervolgonderzoek moet uitwijzen hoe LRP precies de β2-integrinen beïnvloedt.

LRP en zijn expressie in lymfocyten

LRP komt in veel celtypen voor en in sommige celtypen heeft LRP een unieke functie. Omdat de aanwezigheid van LRP van vitaal belang is, is het noodzakelijk dat de expressie van LRP in celtypen goed wordt onderzocht en dat de (specifieke) functie van LRP in deze cellen opgehelderd wordt. In hoofdstuk 2 hebben we beschreven dat LRP belangrijk is voor de functie van monocyten. LRP komt ook voor in de witte-bloedceltypen granulocyten en dendritische cellen. Het is nog niet duidelijk of LRP ook aanwezig is in T-cellen. Er is nog niets bekend over de expressie in B-cellen en natural killer cellen (NK cellen). Elke celsoort heeft een unieke receptor op zijn celoppervlak waaraan deze cel te herkennen is. Deze heten CD-markers. T-cellen zijn te herkennen aan CD3. Er zijn 2 soorten T-cellen: helper T-cellen (CD4) en cytotoxische T-cellen (CD8). B-cellen hebben CD19 en NK cellen CD16 en CD56.

In hoofdstuk 3 wordt onderzoek naar de aanwezigheid van LRP beschreven in deze celtypen. Eerst werd gekeken naar mRNA. mRNA wordt afgelezen van DNA en mRNA wordt weer afgelezen om uiteindelijk eiwit te vormen. Zowel LRP mRNA als LRP eiwit was aanwezig in de witte-bloedceltypen T-cellen, B-cellen en NK cellen. Toen we keken naar de plaats waar LRP zit in de cellen, dan zagen we iets opmerkelijks. In B-cellen en NK cellen zat LRP op het celoppervlak. Echter, in T-cellen was LRP voornamelijk aan de binnenkant van de cel aanwezig.

Omdat LRP in het algemeen zijn functie aan het oppervlak van de cel heeft, hebben we gekeken of LRP vervoerd kan worden van de binnenkant naar het oppervlakte van de cel. Van andere celtypen is bekend dat dit kan gebeuren door cellen te stimuleren. We hebben gekeken of LRP ook naar buiten komt als T-cellen gestimuleerd worden. T-cellen kunnen op verschillende manieren gestimuleerd worden. Als eerste kan dat door het eiwit interleukine-2, dat is betrokken bij de celgroei die plaats vindt na stimulatie. Na stimulatie met interleukine-2 kwam er na 2 dagen meer LRP op het celoppervlak in 12% van de cellen. Als LRP al in de cel 'klaar ligt', dan zijn 2 dagen vrij lang voordat het op het oppervlak komt. Een andere manier van stimuleren is zoals ook gebeurt bij de afstoot van organen na transplantatie. De T-cellen van de donor kunnen dan cellen aanvallen van de ontvanger. Dit hebben we nagebootst door witte bloedcellen van één persoon samen te voegen met witte bloedcellen van een tweede persoon. Dan zagen we dat bij ongeveer 70% van de T-cellen LRP verschoof van binnen de cel naar de oppervlak in een periode van minder dan een half uur. Toen we verschillende witte bloedcellen zuiverden, zagen we dat als een T-cel van persoon 1 met een monocyt van persoon 2 mixten, dat dan LRP op het oppervalk kwam, maar niet als de gezuiverde T-cellen gemixt werden.

Uit deze resultaten kunnen we nog niet bepalen wat de rol van LRP is. Daarom moet er meer onderzoek gedaan worden om de rol van LRP in de afstoot van donorcellen te bepalen.

LRP en de rol in de ziekte van Alzheimer

De ziekte van Alzheimer kenmerkt zich door afbraak van delen van de hersenen en de hierop volgende uitval van bepaalde hersenfuncties. De oorzaak is dat eiwitten die niet meer de goede structuren hebben worden afgezet en in de hersenen en daar dan de cellen beschadigen.

Op het oppervlak van hersencellen zit een eiwit, genaamd APP, dat door LRP opgenomen kan worden. In de cel wordt het dan geknipt en weer uitgescheiden. Deze uitgescheiden vorm (amyloid β) kan dan schadelijk zijn als het gaat klonteren en zogenaamde plaques gaat vormen. Ter bescherming kan het weer worden opgenomen door de omliggende cellen. Ook bij dit proces is LRP betrokken: het zorgt voor opname van amyloid β door de vaatwandcellen in de hersenen.

In hoofdstuk 4 wordt beschreven hoe LRP de opname van amyloid β op vaatwandcellen reguleert. Het bleek dat de slechte vorm van amyloid β (Aβ42) slechter bond aan LRP op de vaatwandcellen dan de goede vorm (Aβ40). Daardoor kon er meer slecht Aβ42 in de hersenen komen, wat dan leidde tot een grotere kans op de ziekte van Alzheimer. Amyloid β dat via LRP werd opgenomen, werd aan de andere kant van de vaatwandcellen uitgescheiden en kwam zo het bloed in. Zo kon amyloid β worden afgevoerd en was het niet schadelijk. Het was ook gebleken in muisexperimenten dat een hoge concentratie van amyloid β de concentratie van LRP op het oppervlak van de vaatwandcellen verminderde, doordat LRP in de cel werd afgebroken. Hierdoor kon er minder amyloid β worden afgevoerd en ontstonden er eerder schadelijke plaques. Patiënten met de ziekte van Alzheimer hadden ook minder LRP in de vaatwandcellen van hun hersenen. Amyloid kon dan niet via LRP afgevoerd worden en daardoor ontstond er een hogere concentratie amyloid β dat de plaques in de hersenen veroorzaakte.

LRP heeft dus in potentie de capaciteit om de ziekte van Alzheimer te voorkomen. In vervolgexperimenten kan gekeken worden hoe LRP amyloid β kan opruimen in patiënten, bijvoorbeeld door de opname van amyloid β te bevorderen of de afbraak van LRP tegen te gaan.

LRP en de klaring van C4BP

Een van de best gekarakteriseerde functies van LRP is zijn rol in de endocytose van liganden. Als een ligand bindt aan LRP aan het celoppervlak, dan wordt zowel LRP als het ligand opgenomen door de cel. Het complex komt dan in zogenaamde endosomen. De zuurgraad hierin is lager dan buiten de cel, wat resulteert in het uiteenvallen van het complex. LRP kan dan herbruikt worden en gaat dan weer naar het celoppervlak. Het ligand wordt getransporteerd naar lysosomen en wordt daar afgebroken. Op deze manier kan de concentratie van een eiwit in bloed geregeld worden.

Recent is op ons laboratorium gevonden dat C4b-binding protein (C4BP) een ligand voor LRP is. In het bloed heeft het verschillende functies. Het bindt de stollingsfactor, proteïne S, dat door deze binding zijn functie niet meer kan uitvoeren. Het kan ook het eiwit C4b binden dat betrokken is in de afweer tegen bacteriën. LRP zorgt er uiteindelijk voor dat C4BP wordt afgebroken in de cel.

In dit proefschrift hebben we nader gekeken naar deze opname van C4BP door de cel (hoofdstuk 5). Voordat het aan LRP bindt, werd het eerst uit het plasma gevangen door heparan sulfaat proteoglycanen (HSPGs), eiwitten die in grote hoeveelheden op het oppervlak van cellen aanwezig zijn. We hebben een aantal mutanten van C4BP gebruikt om te kijken welke delen van C4BP nu precies aan LRP bonden. Die mutaties zijn gemaakt in het deel van C4BP dat ook aan heparine bindt. Heparine is een soort suiker dat de bloedstolling remt. Een vorm van heparine is ook aanwezig in de HSPGs. Onze resultaten geven aan dat die delen van C4BP die ook aan heparine/HSPGs bonden ook aan LRP bonden. Omdat C4BP ook aan proteïne S bindt, hebben we gekeken of het C4BP/proteïne S complex ook nog aan LRP kon binden. Het is gebleken dat het complex inderdaad nog aan LRP kon binden.

Conclusie

In dit proefschrift hebben we 4 verschillende aspecten van LRP beschreven in 4 verschillende celtypen. In elke celsoort lijkt LRP een specifieke functie te hebben. De vraag die nu opkomt, is hoe het mogelijk is dat een en dezelfde receptor totaal verschillende functies heeft in diverse celtypen. Vervolgonderzoek moet uitwijzen hoe deze functies gereguleerd worden door te zoeken naar andere eiwitten in, op of rondom de cel die de specifieke functies van LRP kunnen beïnvloeden.

Dankwoord

Ik moet oppassen dat dit hoofdstuk niet het langste van het boekje wordt. Heel wat mensen heb ik namelijk een plaatsje moeten beloven in het meest gewilde hoofdstuk van dit boekje. Dat betekent dus dat zo'n proefschrift nooit in je eentje tot stand zou zijn gekomen.

Peter, bedankt voor de mogelijkheid dat ik op dit project kon werken. Jij als eiwitmens, ik meer celmens. Ik moest gaan kantklossen en op reis (dit laatste heb ik soms iets te letterlijk opgevat…), en uiteindelijk heb ik met alle bagage mijn doel bereikt! Je was altijd erg kritisch, meestal niet onterecht. En natuurlijk ook enthousiast als we op het punt stonden belangrijke resultaten te krijgen. En tja, die confocaal plaatjes kosten nu eenmaal veel tijd.

Flip, Jan-Willem, Harry, Martijn, Ton, bedankt voor jullie kritische blik op mijn resultaten tijdens de werkbesprekingen.

Lenting-groepje, bedankt voor alle interesse gedurende deze 4 jaar. Ondanks dat we op veel verschillende onderwerpen werken, was er altijd goed overleg. Erik, bedankt voor je hulp bij de Biacore (moest je maar ergens anders gaan zitten). Sarah, bedankt voor je interesse in alles en voor alle moeite voor de laatste C4BP experimenten (Dit stukje mag toch wel in het Nederlands?). Ronan, finally your experiments will work. You only have to wait for the ultimate blunder. Good luck with all your future experiments! But when will you start talking Dutch? Carina, het was altijd een gezellig uitstapje naar Lab I. Nog niet genoeg van VWF? Even blijven doorzetten nog! Alles komt uiteindelijk op zijn pootjes terecht. En natuurlijk ook bedankt voor de gezelligheid buiten het lab. Caroline, we worked together for only a couple of weeks, but anyway: good luck with the sacred protein LRP! Lucy, bedankt voor al je hulp bij de laatste loodjes, ook al werkt niet alles zoals je zou willen (welkom op de research!).

Lab II (van voor en na de grote exodus). In willekeurige volgorde: Maarten (Ja ik ben eigenwijs, en ja ik kom uit Den Bosch), Cees ("nee, geen Foo Fighters"), Rolluf ("nee, geen Radiohead!"), Menno "Flummel"

(je bent van me af!), Bettina, Barend, Mieke, Eszter, Remo, Laura, Caroline, Dafna, (kale)Bas ((ex)buurman, bedankt dat je het zo lang vol hebt kunnen houden), Winnie (ik kon alle verhalen over kinderen nog wel aan hoor), Jelle, Lucy, Marieke, Alex). Een hoop lol, een hoop rotzooi, een hoop discussies (poldermodel?), maar ondanks alles was er toch nog te werken (dat is toch de oorspronkelijke functie van G03.601?). Bedankt voor al het gezeur dat jullie gewild en ongewild wilden/moesten aanhoren! En ook sorry voor mijn zangkwaliteiten (Bløf!). En vooral: zet jullie CD-verzameling weer op. En wie moet nu trouwens alle falconbuizen gaan halen?

Lab I. Even weer die falconbuizen dus gaan halen en ik bleef soms weer veel te lang hangen op het 'theekransje' (Janine (overloper naar lab III), Chantal (gedeeltelijke overloper) en Carina).

Lab III, voorbeeldlab, hoe houden jullie het zo schoon! Marjolijn, al jaren vriendin, later ook collega's en nu moest ik zelfs van je rafts leren isoleren. Suzanne, bedankt voor je gezelschap tijdens de vrijdagochtendritjes. Ik was toch nooit zo vervelend? Beloof je om nooit meer te stressen? Irlando, bedankt voor alle mental coaching sessies.

Alex, Lex voor vrienden, bedankt voor al je hulp als stagiaire. Tussen alle bakkies door heb je toch veel bijgedragen aan dit proefschrift. Had je ooit gedacht dat het zo zou uitpakken? En je kon als een van de weinigen met mijn chaotisch gedrag omgaan! Hoofdstuk 3 is van ons! Succes met je tweede stage en het AIO-zijn later (ze hebben je gewaarschuwd).

Joukje, bedankt voor alle administratieve dingen. Hoe vaak heb ik trouwens gezegd dat een pakketje echt het laatste pakketje naar Amerika was?

Alle oud-labgenootjes, bedankt voor al jullie hulp. Bianca, bedankt voor het wegwijs maken op het lab.

Alle bewoners van andere labs: bedankt voor alle chemicaliën waarvoor ik bij jullie kwam shoppen. Hoeklab, daar kwam ik weer met mijn FACS problemen. Ik ben blij dat jullie nu pas verhuisd zijn, zodat ik niet zo ver hoefde te lopen voor de centrifuge. Jordan VI, bedankt voor de samenwerking op het CKL en voor het beantwoorden van al mijn leukovragen. Tuna, Saskia, Anton, Henk, Tineke en Elles, bedankt voor jullie hulp bij het lymfo-verhaal. Tja, wij als haemostaselab weten nu eenmaal niets van immunologie.

Niels, hier sta je dan in het dankwoord. Ik heb je het vaak genoeg moeten beloven! Hé, bedankt voor alles. Bedankt voor al je geduld (en jij hebt mij ook geleerd wat geduld is), theoretisch advies en gezelligheid. O nog iets: niet al te arrogant worden, hè?

Bedankt alle anderen in het ziekenhuis die ik vergeten ben. Niet boos worden hè?

Tanja-Maria, Eveliina, Leena, Maria, Liisa, Yvonne and Carl (and others!). Thank you for your hospitality during my stay in Helsinki. I really appreciate all technical advice, but, also advice for how to visit Helsinki in a few days! I love your city. Liisa, I hope you enjoyed your stay here in Utrecht. Thank you for your attempts to answer my e-mails about things you've never heard about.

Paula, I'm sorry I'm not so fast in perfusion experiments as you are. Many, many times I told you how much I'm sorry to ask you to perform these experiments. Here's my last sorry: Sorry! But anyway, it resulted in a really nice paper! Jaap-Jan, je kunt sneller denken dan dat wij kunnen bijhouden. Die halfuurtjes op het CLB waren daarom altijd erg vruchtbaar! Bedankt voor het brainstormen.

Ik heb eigenlijk altijd gezegd dat vrienden niet echt bijgedragen hebben aan dit boekje. Niemand snapt toch wat ik nou die 4 jaar heb gedaan. Maar ik kan het natuurlijk niet maken om jullie te vergeten! Al die etentjes en dagjes uit zijn natuurlijk net zo belangrijk als de hele

dag pipetteren op het lab. Marjolijn, Marjolein (leuk, dezelfde namen, maar jullie wisten altijd precies over wie we het hadden! En leuk dat jullie mijn paranimfen willen zijn), Linda + alle aanhang: waarom wonen we nu zo ver van elkaar? Niet nog verder weggaan, hè? De vakantie in Frankrijk als een van de hoogtepunten. De eigenlijk veeeeeeeeeel te weinig etentjes in het jaar (ja, we gaan nu écht vaker afspreken), waar het werk op een of andere manier toch altijd een gespreksonderwerp was. Marjolijn, jij bleef in ieder geval dichtbij wonen en natuurlijk woonde je ook op het hematlab. Marjolein: leuk dat je weer in de buurt komt wonen. We moeten meer afspreken dan! Linda: niet rond de 20e bevallen hè? Bianca, Daniëlle en Judith: vanaf nu beloof ik dat ik jullie vaker mail en sms en bel! Richard, Schotse Hooglander, jij bent volgens mij de enige leek die ooit het lab heeft bezocht. En je hebt onverwachts toch nog iets bijgedragen aan het proefschrift (weet je nog wat? ;-)). Carina, Michiel, Casper, Esther, Astrid, Bart, Linda, wandelmaatjes, bedankt voor jullie gezelschap tijdens de weinige sociale contacten in de laatste maanden. Michiel, zie je: ook tijdens het MSN-en lukt het me om dit proefschrift te schrijven! Bedankt voor je praatjes.

Bien, "druk" met afstuderen, maar toch nog tijd om met me naar Bløf te (moeten) gaan. De voorkant is echt mooi geworden! Succes verder.

Pap en mam, tja hier heb ik het dan al die jaar voor gedaan. Sorry dat ik nooit kon uitleggen wat ik nu gedaan heb. Nou, speciaal voor jullie de 'Samenvatting in het Nederlands'. Mam, tja, ik kwam niet zo vaak meer langs, maar je viel me vaak genoeg lastig op MSN om contact te hebben. Pap, het is toch niet echt 'iets met weefsels'. En standaard 8 uur werken ("als je om 8 uur begint, mag je toch om half 5 naar huis"), dat zit er dus ook niet in. Maar toch bedankt hoor!

Groetjes,

Patricia

Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 6 september 1978 te 's-Hertogenbosch. In 1996 behaalde zij het gymnasium diploma aan het Jeroen Bosch College te 's-Hertogenbosch. In hetzelfde jaar werd begonnen met de studie Medische Biologie aan de Universiteit Utrecht, waarvan in 1997 het propaedeuse diploma werd behaald (*cum laude*). Van dezelfde studie werd in 2001 het doctoraal diploma behaald met als hoofdvak Celbiologie (Prof. Dr. G. Strous, Dr. W. Stoorvogel en Dr. E.M. van Dam, Universitair Medisch Centrum Utrecht) en als bijvak Pediatrische Endocrinologie (Dr. S.C. van Buul-Offers en Dr. J.G. Koster, Universitair Medisch Centrum Utrecht). Van april 2001 tot juni 2005 was de schrijfster werkzaam als assistent in opleiding bij de vakgroep Haematologie van het Universitair Medisch Centrum Utrecht onder begeleiding van Prof. Dr. Ph.G. de Groot en Dr. P.J. Lenting. De resultaten van het onderzoek zijn beschreven in dit proefschrift, waarvan de verdediging op 20 september 2005 plaats zal vinden.