# The Biological Role of the Receptor-like Protein Tyrosine Phosphatase LAR

**- a mouse model to study loss-of-function of LAR tyrosine phosphatase activity -**

**Roel Q.J. Schaapveld**

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Cover. A whole-mount staining of the first inguinal mammary gland of a virgin female mouse

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## ABBREVATIONS

| | |
|---|---|
| CA | Carbonic Anhydrase |
| CAM | Cell Adhesion Molecule |
| CD45-AP | CD45 Associated Protein |
| CNS | Central Nervous System |
| CSF-1 | Colony Stimulating Factor 1 |
| DCC | Deleted in Colorectal Cancer |
| ECM | ExtraCellular Matrix |
| EGF | Epidermal Growth Factor |
| ES cell | Embryonic Stem cell |
| FA(s) | Focal Adhesion(s) |
| FAK | Focal Adhesion Kinase |
| Fn-III | Fibronectin type III |
| GEF | Guanine nucleotide Exchange Factor |
| GPI | GlycosylPhosphatidylInositol |
| GRB-2 | Growth factor Receptor Binding protein-2 |
| ICAM-1/ 3 | Intercellular Cell Adhesion Molecule-1/-3 |
| IDDM | Insulin-Dependent Diabetes Mellitus |
| Ig | Immunoglobulin |
| IR | Insulin Receptor |
| IRS-1/ 2 | Insulin Receptor Substrate-1/-2 |
| IRTK | Insulin Receptor Tyrosine Kinase |
| kDa | kilo Dalton |
| LAR | Leukocyte common Antigen Related molecule |

| | |
|---|---|
| LCA | Leukocyte Common Antigen (CD45) |
| LFA-1 | Lymphocyte Function-associated Antigen-1 |
| LIP-1 | LAR Interacting Protein-1 |
| LPAP | Lymphocyte Phosphatase-Associated Phosphoprotein |
| LRP | Leukocyte common antigen Related Protein |
| | |
| MAM | Meprin/A5/$\mu$ homology domain |
| MAP | Mitogen Activated Protein |
| me | mini-exon |
| | |
| N-CAM | Neural Cell Adhesion Molecule |
| Ng-CAM | Neuron-Glial Cell Adhesion Molecule |
| NGF | Nerve Growth Factor |
| | |
| PDGF | Platelet-Derived Growth Factor |
| PDZ | acronym for PSD-95, DlgA, and ZO-1 |
| PH | Pleckstrin Homology |
| PKC | Protein Kinase C |
| PMA | Phorbol 12-Myristate 13-Acetate |
| PSK | Protein Serine Kinase |
| PTK(s) | Protein Tyrosine Kinase(s) |
| PTPase(s) | Protein Tyrosine Phosphatase(s) |
| | |
| RPTK(s) | Receptor like Protein Tyrosine Kinase(s) |
| RPTPase(s) | Receptor like Protein Tyrosine Phosphatase(s) |
| | |
| SH2/SH3 | Src Homology-domain 2/Src Homology-domain 3 |
| Sos | Son of sevenless |
| | |
| TCR | T Cell Receptor |
| TNF | Tumor Necrosis Factor |
| TPA | 12-Tetradecanoate 13-Acetate |

# Chapter I

## General Introduction

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Receptor-like protein tyrosine phosphatases: Alike and yet so different
*Molecular Biology Reports*, in press (1997)

## GENERAL INTRODUCTION

### Protein tyrosine phosphatases

Protein tyrosine phosphatases (PTPases, protein-tyrosine-phosphate phosphohydrolase, EC 3 1 3 48) are a family of proteins of which the first one, PTP1B, was discovered in 1988 by Tonks and coworkers (Tonks *et al* , 1988a) PTPases obtained their name from the enzymatic role they perform inside cells, namely the removal of phosphate groups from phosphotyrosine residues of specific target proteins The phosphorylation of these tyrosine residues, catalyzed by the protein tyrosine kinases (PTKs), is known to regulate important cellular processes like metabolism, gene expression, division and differentiation, development, transport, and locomotion (Barford *et al* , 1995) Since PTPases act on the same kind of biochemical switches as the PTKs, they are thought to play an equally important biological role (Sun and Tonks, 1994, Figure 1) In support with this contention, Klarlund, already in 1985, showed that addition of the PTPase inhibitor vanadate to cells in culture leads to increased amounts of phosphotyrosine-containing proteins and cellular transformation Thus, a delicate balance between PTK and PTPase action is essential for normal functioning of cells

Next to the PTPases, two other protein families are capable as well to dephosphorylate tyrosine-phosphorylated proteins the structurally distinct low molecular weight PTPases and the structurally related dual specificity phosphatases which catalyze the dephosphorylation of phospho-tyrosine, threonine and -serine residues (Barford *et al* , 1995) To date, the protein family of PTPases comprises some 50 members (Barford *et al* , 1995) which display a bewildering variety of amino acid motifs, fused to the catalytic PTPase domains (Brady Kalnay and Tonks, 1995) The PTPase family can be divided in two main classes based on their subcellular localization, namely (i) intracellular, cytosolic or nuclear, PTPases that contain only one PTPase domain, and (ii) receptor like, transmembrane PTPases that have one or two tandemly repeated catalytic domains

### The catalytic PTPase domain

All PTPase family members possess at least one ~230-280 amino acid catalytic domain with a mean sequence identity of ~35% among all pairs of sequences (Barford *et al* ,

1995; Streuli, 1996). Each domain contains a highly conserved active-site region with the consensus motif [I/V]HCXAGXXR[S/T]G (where X is any amino acid). Site-directed mutagenesis studies showed that the cysteine residue within this motif is absolutely essential for activity (Guan and Dixon, 1991; Pot and Dixon, 1992). Trapping experiments showed that the cysteine forms a thiol-intermediate during the catalytic turnover in which the cleavage of the phosphoenzyme intermediate is the rate-limiting step (Cho *et al.*, 1992a, 1992b; Guan and Dixon, 1991; Pot and Dixon, 1992). The hydroxyl group of the conserved threonine residue in the signature motif is important in facilitating the breakdown of this intermediate (Zhang *et al.*, 1995). The invariant cysteine is adjacent to another conserved residue, histidine, which is significantly involved in catalysis, but not essential for PTPase activity Its role appears to be predominantly structural, in defining the positioning of the cysteine residue and the conformation of the phosphate binding loop (Barford *et al.*, 1994) The arginine residue at position +6 is also essential for catalytic activity (Streuli *et al.*, 1990) by having an important role in substrate binding and transition state stabilization (Zhang *et al.*, 1994a).

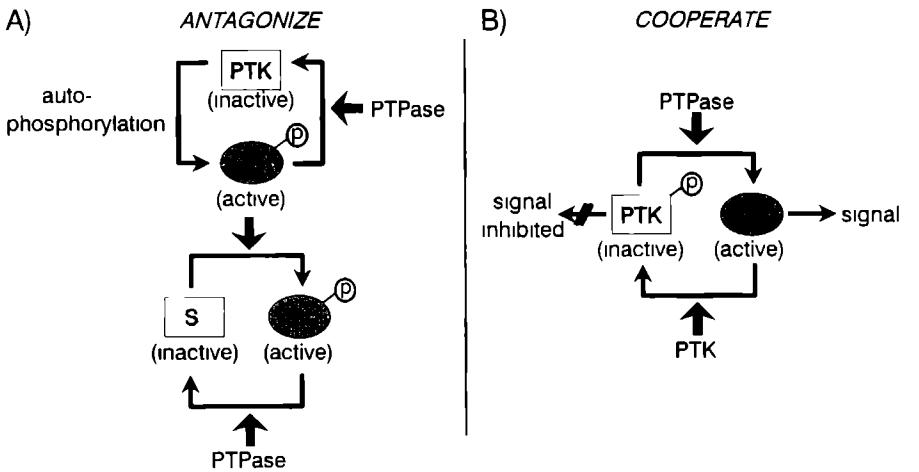



**Figure 1.** The concerted action of PTPases and PTKs in cell signalling.

(**A**) PTPases may act as antagonists of PTKs by dephosphorylating the PTKs' autophosphorylation sites, thereby inactivating the kinases itself, or by dephosphorylating their target substrates (S) (**B**) PTPases may act in cooperation with PTKs in the case of the Src-like PTKs, by dephosphorylating the C-terminal inhibitory phosphorylation site, thereby activating the kinase and promoting the signalling cascade (adapted from Sun and Tonks, 1994)

All amino acids in between the arginine and histidine residues form the base of the catalytic site cleft (Barford *et al* , 1994) The PTPases' specificity for phosphotyrosine containing residues probably results from the depth of this active-site cleft the shorter phosphoserine and phosphothreonine side chains would not reach the phosphate-binding site (Barford *et al* , 1994) Obviously, this structure has been well conserved as the three dimensional structure of PTP1B (Barford *et al* 1994) and the *Yersinia* dual-specificity PTPase (Stuckey *et al* , 1994), which share only twenty percent sequence homology, revealed an almost similar conformation of the phosphate-binding site Interestingly, the structure of the active-site cleft bears much more resemblance to Src homology 2 (SH2) domains, which also recognize phosphorylated tyrosines (Jia *et al* , 1995) than to the catalytic domains of the serine/threonine phosphatases or of the alkaline or acid phosphatases (Mauro and Dixon, 1994) The sequence context in which the phosphorylated tyrosine residue(s) must be recognized may be a crucial determinant in this property In contrast to the SH2 domains which contain binding sites C-terminal to the phosphorylated tyrosine (reviewed in Jia *et al* , 1995), PTPases display a subtle preference for multiple acidic side chains within five amino acids N-terminal to the phosphotyrosine residue (Zhang *et al* , 1993, 1994b)

**Regulation of PTPase activity**

Strikingly, intrinsic PTPase enzymatic activity is generally two to three orders of magnitude greater than that of the PTKs (Hunter, 1995) This can account for the low level of tyrosine-phosphorylated proteins, only 0 01-0 05% of all phosphorylated proteins and for the transient nature of tyrosine phosphorylation of cellular proteins (Hunter and Sefton, 1980, Hunter, 1995) Also, considering the large family of potentially redundant PTPases, the enzymatic activity must be highly regulated and restricted to prevent non specific dephosphorylation of proteins and to make PTK signalling effective (Hunter, 1995, Mauro and Dixon, 1994, Tonks *et al* , 1992) This can be accomplished in various ways (1) by modulating the steady state levels of the enzyme, (2) by post-translational modification (e g serine/threonine and/or tyrosine phosphorylation, proteolytic cleavage, etc ), (3) by interaction with as yet not identified activator/inhibitor molecules, and (4) by discrete subcellular localization in further restricting the substrate specificity (Hunter, 1995, Lammers *et al* , 1993, Mauro and Dixon, 1994, Tonks *et al* , 1992) The latter regulatory principle is quite evident for the non receptor, intracellular PTPases

**Intracellular PTPases**

The intracellular PTPases show a great diversity of protein modules outside the conserved catalytic domain These modules serve as 'zip codes' to target the PTPase to a particular subcellular localization, thereby defining and restricting its substrate specificity (Mauro and Dixon, 1994, Tonks *et al* , 1992) Examples of these flanking modules are (i) a short hydrophobic segment for targeting to the cytoplasmic face of intracellular membranes, (ii) a nuclear localization signal, (iii) SH2 domains, which target the PTPases to phosphotyrosine-containing proteins, (iv) a motif with homology to the actin-binding proteins band 4 1, ezrin, and talin, that directs the protein to the interfaces between the plasma membrane and the cytoskeleton, (v) domains related to retinal binding proteins, and (vi) PDZ-motifs as present in many proteins involved in submembranous signalling at specialized cell cell junctions (Barford *et al* , 1995, Hendriks, 1995, Kennedy, 1995, Mauro and Dixon, 1994, Ponting and Phillips, 1995) In addition, several PTPases contain sequences rich in proline, glutamic acid, serine, and threonine residues, so-called PEST sequences, which may serve as signals for proteolytic degradation and are characteristic of proteins with a short intracellular half life (for review see Rechsteiner and Rogers, 1996)

Despite extensive data on their structure and subcellular localization, the signalling role of the cytosolic PTPases is not understood completely Recent findings gave more insight in the essential role they may play as terminators or as positive regulators of multiple signalling pathways (reviewed by Streuli, 1996)

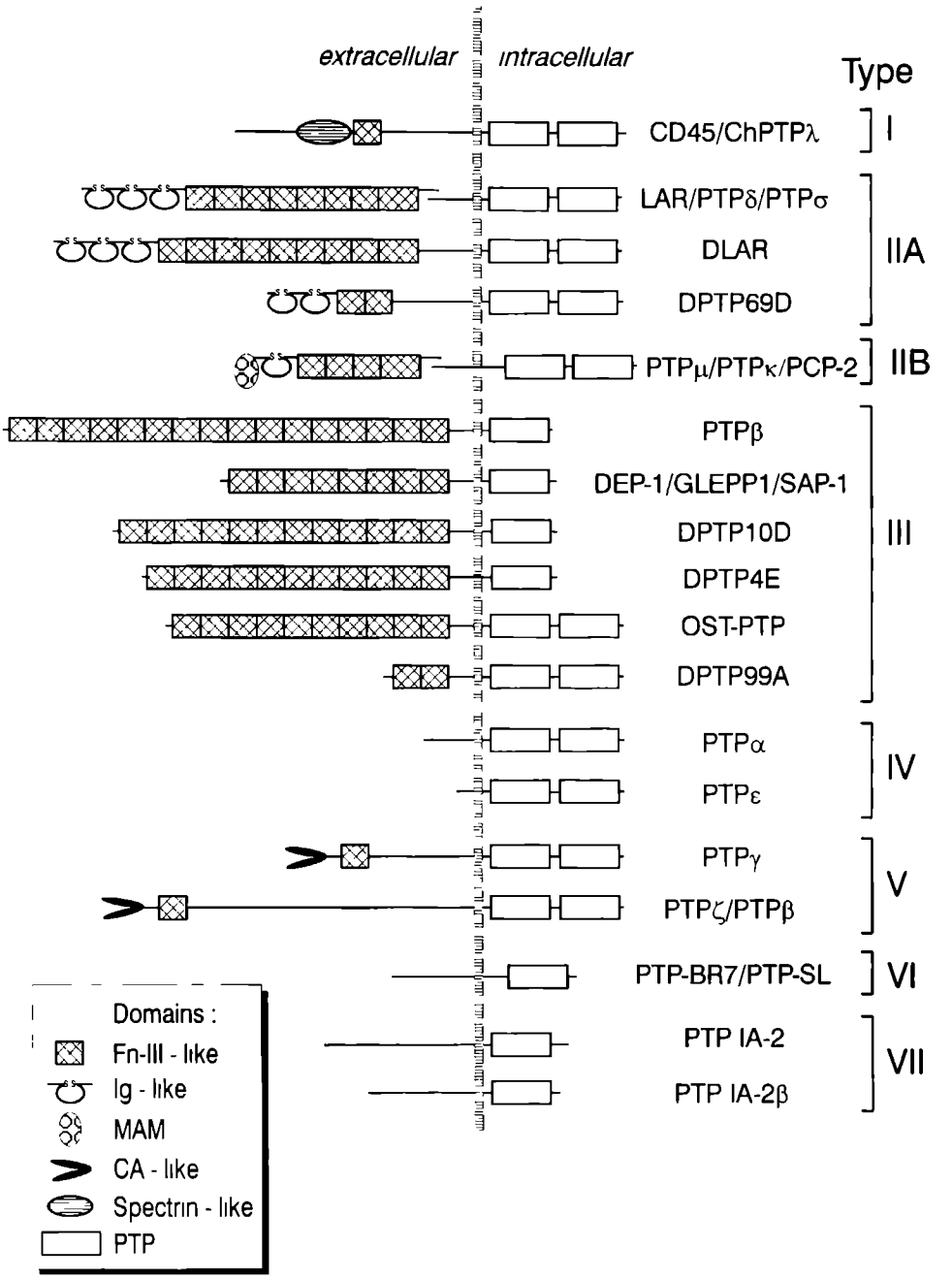
**Receptor-like protein tyrosine phosphatases**

A database search with the peptide sequence of the intracellular PTPase PTP1B led to the identification of the first transmembrane, receptor-like PTPase (RPTPase), the leukocyte common antigen CD45 (Charbonneau *et al* , 1988) which was cloned already three years earlier (Thomas *et al* , 1985) In 1988 it was then quickly shown that CD45 indeed could catalyze the dephosphorylation of phosphotyrosine-containing proteins (Tonks *et al* , 1988b), designating CD45 as the prototype of the RPTPase subfamily (Figure 2) All members possess an intracellular part containing one or two homologous PTPase domains, a single membrane-spanning region, and variable extracellular segments with potential ligand binding capacity Therefore, they have the potential to trigger signalling events at the cell membrane through ligand mediated dephosphorylation of tyrosine-

phosphorylated proteins (Tonks *et al.*, 1992) In this context, the binding of a ligand to the extracellular segment may modulate the cytoplasmic PTPase activity (Mauro and Dixon, 1994), but experimental proof for this mechanism is lacking thus far

The RPTPases can be classified in seven subtypes based on their extracellular motifs (Figure 2). The diversity of this subfamily is further increased by the existence of multiple isoforms resulting from alternative splicing and post-translational modifications (for review see Mourey & Dixon, 1994). In the following sections a detailed description on the distinct structural features and structure-function relationships of members of each of these subclasses will be given. The order of appearance of the different types largely follows the order as shown in Figure 2. An exception is made for the type II (A and B) and type III RPTPases, which are the cell adhesion molecule-like RPTPases and will be discussed in more detail at the end of this chapter.

**Figure 2.** The family of receptor-like protein tyrosine phosphatases
The figure shows the distinct subtypes of RPTPases based on their extracellular motifs. The classification is according to Brady-Kalnay and Tonks (1995) except for ChPTPλ, which we regard as a CD45-like RPTPase Only the largest isoform of each member is shown The identified structural features are indicated in the open box Fn-III, fibronectin type III-like repeat, Ig, immunoglobulin-like domain; MAM, domain with homology to meprin, the A5 glycoprotein, and RPTPμ; CA, carbonic anhydrase-like domain, PTP, tyrosine phosphatase domain References are indicated in the text.


extracellular
intracellular
Type
CD45/ChPTPλ
I
LAR/PTPδ/PTPσ
DLAR
DPTP69D
IIA
PTPμ/PTPκ/PCP-2
IIB
PTPβ
DEP-1/GLEPP1/SAP-1
DPTP10D
DPTP4E
OST-PTP
DPTP99A
III
PTPα
PTPε
IV
PTPγ
PTPζ/PTPβ
V
PTP-BR7/PTP-SL
VI
PTP IA-2
PTP IA-2β
VII
Domains :
Fn-III - like
Ig - like
MAM
CA - like
Spectrin - like
PTP

**The RPTPase prototype CD45**

The type I RPTPase CD45 (also known as LCA, T200 in T cells, and B220 in B cells, the mouse homolog is denoted Ly 5) is expressed on the surface of all haematopoietic cells and their precursors except mature erythrocytes and platelets (for review see Chan *et al* , 1994, Okumura and Thomas, 1995, Yakura, 1994) Its extracellular domain is highly glycosylated, is rich in cysteine residues, and shows alternative splicing of exons 4, 5, and 6, and in rat and mouse also of exon 7 The expression of the different isoforms, ranging in molecular mass from 180 to 235 kDa, is cell type specific and depends on the activation state of lymphoid cells (Kishihara *et al* , 1993)

The generation of different CD45 knock-out mice revealed that CD45 is not required for the proper development of B lymphocytes, but instead has a role in the regulation of certain subpopulations of B cells, i e in the negative and positive selection of B lymphocytes (Byth *et al* , 1996, Cyster *et al* , 1996, Kishihara *et al* , 1993) Furthermore, an important regulatory role for CD45 in the signal transduction via the T cell receptor (TCR) at multiple stages during T cell development has been demonstrated (Byth *et al* , 1996, Klaus *et al* , 1996) The catalytic activity of the first, membrane proximal PTPase domain is necessary and sufficient to rescue the TCR signalling in CD45-negative cells (Desai *et al* , 1993, 1994), but requires membrane anchoring close to the membrane-associated substrates (Niklinska *et al* , 1994)

It remains a point of controversy whether both PTPase domains of CD45 are enzymatically active (Streuli *et al* , 1990, Tan *et al* , 1993) because the *in vitro* data can be explained in various ways Inactivation of the first, membrane proximal domain, for example, might suppress the activity of the second domain, whereas partial or complete deletion of the structural integrity of the first domain may enable the second domain to become active (Pallen, 1993, Tan *et al* , 1993) Vice versa, the complete second domain may be necessary for the PTPase activity of the first domain (Johnson *et al* , 1992, Ng *et al* , 1995) This dispute on the enzymatic activity does not alter the fact that CD45 is the only RPTPase for which substrates have been identified (for review see Chan *et al* , 1994, Okumura and Thomas, 1995) CD45 is able to activate the Src family kinases $p56^{lck}$ and $p59^{lyn}$, important in antigen-induced receptor signalling, by specifically dephosphorylating the negative regulatory tyrosine phosphorylation site in their C termini In this mechanism, another cytoplasmic tyrosine kinase, $p50^{csk}$, is able to phosphorylate both the C-terminal tyrosine in $p56^{lck}$ and a site in the second PTPase domain of CD45 This activates CD45 and provides a binding-site for the SH2 domain of $p56^{lck}$ (Autero *et*

*al* , 1994) Others argue that the mechanism is more complex Burns *et al* (1994) found a hyperphosphorylation of the C-terminus and increased enzymatic activity for $p56^{lck}$ tyrosine kinase in CD45-negative T cells Ostergaard and Trowbridge (1990) showed that co-clustering of CD45 by CD4 decreases the CD4-associated $p56^{lck}$ autophosphorylation and enzymatic activity This would suggest that the interaction with CD45 inhibits rather than enhances $p56^{lck}$ activity Furthermore, Ng *et al* (1996) claimed that $p56^{lck}$ is able to interact with CD45 in the absence of any detectable tyrosine phosphorylation of CD45, suggesting a nonconvential SH2 domain interaction However, this interaction might not be direct *in vivo* In T cells, $p56^{lck}$ always co-precipitates with CD45 in the presence of a newly identified 30 32 kDa lymphocyte phosphatase-associated phosphoprotein, called LPAP or CD45AP (Koretzky *et al* , 1993, Ross *et al* , 1994, Schraven *et al* , 1991, 1992, 1994, Takeda *et al* , 1992, 1994) In contrast, LPAP/CD45AP can be co-precipitated with CD45 in the absence of $p56^{lck}$ (Koretzky *et al* , 1993) and the transmembrane domains of CD45 and LPAP are essential for this interaction (Bruyns *et al* , 1996, Cahir McFarland and Thomas, 1995, Kitamura *et al* , 1995) It has been suggested that LPAP is protected from degradation through its interaction with CD45 (Bruyns *et al* , 1996) but it is still not known what the function of LPAP is and also its requirement for the association of CD45 and $p56^{lck}$ is not understood Since tyrosine phosphorylation of LPAP is not detectable in the presence of enzymatically active CD45, LPAP might also be a substrate of CD45 in lymphocytes (Schraven *et al* , 1994) and important for the initiation of T cell signalling The identification of the CD3 $\zeta$ chain as another substrate of CD45 suggests in addition a role for CD45 in termination of the TCR signalling for which a dephosphorylated $\zeta$ chain is essential (Furukawa *et al* , 1994)

Phosphorylation on serine residues in CD45 may modulate the activity of the enzyme itself but the effects of this modification are still being disputed (Ostergaard and Trowbridge, 1991, Stover and Walsh, 1994, Tonks *et al* , 1990, Trowbridge *et al* , 1992, Valentine *et al* , 1991, Yamada *et al* , 1990) Another regulatory mechanism for CD45 enzyme activity might be the association to cytoskeletal proteins Binding to fodrin (non erythroid spectrin) or the structurally related cytoskeletal protein, spectrin, increases PTPase activity (Lokeshwar and Bourguignon, 1992) The fodrin binding site of CD45 was mapped to the N-terminal region of the second PTPase domain (Iida *et al* , 1994) Different isoforms of CD45 also specifically associate with an as yet unidentified widely expressed 116 kDa tyrosine-phosphorylated glycoprotein (Arendt and Ostergaard, 1995)

The efficacy of T and B cell activation through antigen recognition and the regulation

of intracellular signals generated via other surface receptors might be determined by CD45 receptor-ligand interactions Unfortunately, the identity of the ligand(s) of CD45 remains unclear although two candidates have been put forward Binding of CD22, a B cell surface protein of the Ig superfamily, blocks TCR-generated effects, suggesting that interaction with the extracellular domain may modulate CD45's PTPase activity (Aruffo *et al* , 1992, Sgroi *et al* , 1995, Stamenkovic *et al* , 1991) The other candidate-ligand is heparan sulfate on stromal cells suggesting a role for CD45 in adhesion of haematopoietic precursors to the bone marrow stroma (Coombe *et al* , 1994) The recently identified fibronectin type III (Fn-III)-like repeat in the extracellular domain (Bork and Doolittle, 1993) may mediate CD45's role in cell adhesion Moreover, also carbohydrate groups may be important in cell-cell interactions since the extracellular domain is heavily glycosylated and the CD45 isoforms differ in *O*-glycosylation Antibodies against the extracellular domain can influence the cell binding avidity Depending on the type of anti-CD45 monoclonal antibody used, some antibodies induce the adhesion between peripheral T cells and monocytes via lymphocyte function-associated antigen (LFA)-1, while others inhibit the LFA-1/intercellular adhesion molecule (ICAM)-1 or LFA-1/ICAM-3 dependent adhesion (reviewed in Okumura and Thomas, 1995) In addition, homotypic adhesion via LFA-1 of activated T cells, but not resting T cells, is induced by the engagement of CD45 (Spertini *et al* , 1994)

Whatever the mechanisms involved in CD45 binding, it is becoming increasingly clear that CD45 is part of a larger molecular assembly which may have a cell-type dependent composition Clearly, completion of the inventory of cell physiological ligand(s) of CD45 is needed in order to understand how CD45 activity is regulated and to define its role in cell-cell interaction processes

The second member of this subclass, ChPTPλ, was cloned from chicken and, quite surprisingly, was found to contain a spectrin-like repeat in its extracellular domain (Fang *et al* , 1994a) This repeat, present in many actin binding proteins, such as α- and β-spectrins, α-fodrin, α-actinin, and dystrophin, was subsequently also detected in CD45 Next to the spectrin-like repeat, the extracellular domain of ChPTPλ contains a Ser/Thr/Pro-rich region with many potential *O*-glycosylation sites, and a Fn-III-like repeat Also, the alternative splicing that affects the N-terminal part and the 70% similarity in the intracellular domains suggest that ChPTPλ may be regarded as the chicken homologue of CD45 Although it has been shown that ChPTPλ can

dephosphorylate pp60$^{c\ src}$ at the C terminal tyrosine residue at position 527 *in vitro* (Fang *et al* , 1994b), more studies will be necessary to verify or disclaim further overlap in the physiological roles of ChPTPλ and CD45 *in vivo*

**RPTPases with short extracellular extrusions**

The type IV RPTPases RPTPα (the mouse homolog is known as LRP) and RPTPε have two tandem cytoplasmic PTPase domains and short, highly glycosylated extracellular segments of only 123 or 27 residues, respectively (Jirik *et al* , 1990, Kaplan *et al* , 1990, Krueger *et al* , 1990, Matthews *et al* , 1990, Moriyama *et al* , 1992, Ohagi *et al* , 1990, Sap *et al* , 1990) In the case of RPTPα both cytoplasmic domains are enzymatically functional *in vitro*, but show distinct enzymatic activities and substrate specificities (Wang and Pallen, 1991, Pallen 1993) Interestingly, an enzymatically inactive alternatively spliced isoform that has an insertion within the first PTPase domain has been identified (Matthews *et al* , 1990, Wong *et al* , 1993), suggesting that activity of the first domain is required to uncover the cryptic intrinsic activity of the second domain giving rise to a fully active form of RPTPα (Pallen, 1993) RPTPα isoforms also arise from differences in glycosylation (Daum *et al* , 1991, 1994) Pulse chase experiments showed that a 100 kDa protein is the precursor of a 130 kDa protein which contains both *N* and *O* linked carbohydrates This translational modification might contribute to specificity in ligand binding Ligands, however, have not been identified sofar Both the 100 and 130 kDa isoenzymes are simultaneously expressed on the same cell and may form dimers, as has also been suggested for CD45 (Daum *et al* , 1994, Desai *et al* , 1993)

Recently, some progress has been made in elucidating the cellular role(s) and substrate(s) of RPTPα which is prominently expressed in brain and kidney but also present in a variety of other tissues (Kaneko *et al* , 1995, Matthews *et al* , 1990, Sap *et al* , 1990) Evidence is accumulating that RPTPα induces or acts in the signalling leading to neuronal differentiation possibly via dephosphorylating the regulatory tyrosine residue at position 527 in the C-terminus of pp60$^{c\ src}$ tyrosine kinase (den Hertog *et al* , 1993, Tsuneizumi *et al* , 1994, van Inzen *et al* , 1996) Overexpression of RPTPα in rat embryo fibroblasts also leads to persistent activation of pp60$^{c\ src}$ concomitant with cellular transformation, possibly via activation and translocation to the nucleus of mitogen activated protein (MAP) kinase and subsequent activation of the transcription factor c-Jun (Zheng *et al* , 1992, 1994) This makes RPTPα the only known PTPase to behave as an

oncogene in experimental systems, and also *in vivo* a role in the neoplastic transformation of colonic epithelial cells has been suggested (Tabiti *et al* , 1995, Zheng *at al* , 1992) Next to its possible function as a positive regulator of cell proliferation, RPTPα, and RPTPε as well, may have a role as a negative regulator of insulin receptor tyrosine kinase (IRTK) activity in baby hamster kidney cells overexpressing the insulin receptor (IR) (Møller *et al* , 1995)

Clearly, not only the cellular environment in proliferating and differentiating cells but also proper regulation of RPTPα activity is of critical importance for its biological role As already mentioned for CD45, phosphorylation on serine residues may be a mechanism for regulation of RPTPα PTPase activity Treatment of RPTPα transfected cells with the phorbol ester phorbol 12 myristate 13 acetate (PMA) resulted in enhanced PTPase activity This is parallelled by an increase in serine phosphorylation, probably mediated via activation of protein kinase C (PKC) (den Hertog *et al* , 1995, Tracy *et al* , 1995) As the position of the phosphorylated serine residues is close to the dimer interface they may have a role in RPTPα oligomerization (Bilwes *et al* , 1996) A model in which dimerization and consequent active site blockage prevents binding of phosphotyrosine containing substrates and subsequently downregulates RPTPα activity was proposed on the basis of the crystal structure of the membrane proximal PTPase domain (Bilwes *et al* , 1996)

RPTPα may also be regulated by tyrosine phosphorylation It has been shown that RPTPα is constitutively phosphorylated on tyrosine residue 789 which serves as a binding site for the adaptor growth factor receptor binding (GRB-2) protein (den Hertog *et al* , 1994, Su *et al* , 1994) This may provide a link to the *Ras* signal transduction pathway although the Ras guanine nucleotide exchange factor Son of Sevenless (Sos) was not detectable in this complex Recently, den Hertog and Hunter (1996) showed that not only the SH2 domain but also the C-terminal SH3 domain of GRB-2 is, most likely indirectly, involved in the binding to RPTPα This second RPTPα binding region is localized close to the catalytic site cleft of the N-terminal PTPase domain This suggests that binding of the GRB-2 C-terminal SH3 domain may block access to the catalytic site, and thereby inhibit RPTPα PTPase activity As a second consequence, the N-terminal SH3 domain of GRB-2 might not be able to bind to Sos due to steric hindrance (den Hertog and Hunter, 1996)

For the second type IV RPTPase, RPTPε, two major mRNAs were found that show

distinct tissue specificity the larger isoform being expressed in brain, heart, testes, and lung, and the slightly smaller mRNA in lymph nodes, thymus, spleen, and lung (Elson and Leder, 1995a) The larger mRNA specifies a molecule which undergoes tissue-specific *N* glycosylation of the extracellular domain (Elson and Leder, 1995a) The smaller isoform lacks the signal peptide, the extracellular domain and the transmembrane segment, making it the first known cytoplasmic PTPase with two PTPase domains (Elson and Leder, 1995b, Nakamura *et al* , 1996) The main regulation of expression of the distinct isoforms takes place at the level of transcription Presumably there is also regulation at the translational level because RPTPε mRNA expression is not invariably followed by RPTPε protein expression The cytoplasmic isoform is regarded as a delayed early response gene the transcription of which is regulated through a pathway that requires PKC For example, expression is upregulated in HL-60 cells upon addition of the phorbol ester 12 tetradecanoate 13 acetate (TPA) (Elson and Leder, 1995b)

The larger, transmembrane RPTPε isoform may play a role in v-Ha-*ras*- and c *neu*-mediated transformation of mammary epithelium The expression level of this isoform is low in normal mammary glands but is highly increased in mammary tumors initiated by v Ha *ras* or c *neu*, and not in mammary tumors initiated by c-*myc* or *int 2* (Elson and Leder, 1995a) It remains to be established whether RPTPε indeed plays an active role in the actual transformation mechanism of the mammary epithelial cells Another possible role for RPTPε may be in osteoclast formation as Schmidt *et al* (1996) found expression of RPTPε in osteoclasts and regulation of its PTPase activity by biphosphonates, potent inhibitors of bone resorption

**RPTPases with an extracellular carbonic anhydrase-like motif**

The RPTPases RPTPζ/β (Krueger *et al* , 1990) and RPTPγ (Kaplan *et al* , 1990, Barnea *et al* , 1993) have been classified as type V RPTPases Together with *Drosophila* DPTP99A, they are the only RPTPases, that have an aspartic acid residue instead of the conserved cysteine in their second PTPase domain (Hariharan *et al* , 1991, Kaplan *et al* , 1990, Krueger *et al* , 1990, Tian *et al* , 1991, Yang *et al* , 1991) Presumably this renders the second domain inactive In addition, both RPTPζ/β and RPTPγ possess a unique insert of 15 amino acids in the second PTPase domain that harbours a potential tyrosine phosphorylation site (Barnea *et al* , 1993, Kaplan *et al* , 1990) that may function in regulating their PTPase activity Extracellularly, they possess a single amino-terminal

motif with homology to carbonic anhydrase (CA), a Fn-III-like repeat, and a long cysteine-free spacer region (Barnea *et al* , 1993, Krueger and Saito, 1992, Levy *et al* , 1993) CAs catalyze the reversible hydration of a $CO_2$ molecule to a bicarbonate ion, and their physiological functions include $CO_2$ transport by blood, pH regulation in the kidney, and $CO_2$ exchange in mitochondria and chloroplasts (reviewed in Tashian, 1989) All CAs are zinc metalloenzymes but in the CA-like domains of RPTP$\zeta/\beta$ and RPTP$\gamma$ two conserved histidine residues are lacking This prevents binding of zinc and renders the CA-like domain catalytically inactive In contrast, many other amino acid residues are conserved, including two cysteine residues that are thought to form a disulfide bridge The CA-like domain may therefore have a three-dimensional structure quite similar to CAs and provide an ideal interface for the binding of small soluble ligands (Krueger and Saito, 1992)

RPTP$\zeta/\beta$ exists in three isoforms, one secreted and two membrane-bound, that differ by the absence of 860 amino acid residues from the spacer region (Levy *et al* 1993, Maurel *et al* , 1994) All three isoforms are exclusively expressed in the central and peripheral nervous system and restricted to cells of neuroectodermal origin Changes in expression of the different isoforms appear to be correlated with glial cell differentiation, suggesting a role in morphogenesis of the nervous system (Canoll *et al* , 1993, 1996, Levy *et al* , 1993, Sakurai *et al* , 1996) In line with this, the CA like domain of RPTP$\zeta/\beta$ was shown to bind to contactin, a glycosylphosphatidylinositol (GPI) anchored neuronal cell adhesion molecule of the immunoglobulin superfamily (also known as F11 in man, the mouse homolog is denoted F3) (Peles *et al* , 1995) Interestingly, contactin is also expressed as a soluble protein due to cleavage from the membrane (Brummendorf and Rathjen, 1993) enabling a unique mode of bidirectional signalling That is, contactin can function as the neuronal receptor for RPTP$\zeta/\beta$ and vice versa, the soluble form of contactin can act as the functional ligand of the transmembrane RPTP$\zeta/\beta$ isoforms (Peles *et al* , 1995)

An additional mechanism by which RPTP$\zeta/\beta$ may modulate neuronal migration was revealed by the identification of the extracellular domain of RPTP$\zeta/\beta$ as being the chondroitin sulphate proteoglycan called 6B4 proteoglycan or phosphacan (Barnea *et al* , 1994b Maeda *et al* , 1994, Maurel *et al* , 1994, Shitara *et al* , 1994) Both the large RPTP$\zeta/\beta$ transmembrane isoform and the soluble isoform, but not the short transmembrane isoform (denoted as dvRPTP$\zeta/\beta$), are expressed as chondroitin sulfate proteoglycans (Barnea *et al* , 1994b, Sakurai *et al* , 1996) that interact heterophilically

with the extracellular matrix (ECM) protein tenascin and the adhesion molecules neural cell adhesion molecule (N-CAM), neuron-glial cell adhesion molecule (Ng-CAM/L1), and TAG-1/axonin-1 (Barnea *et al* , 1994a Milev *et al* , 1994, 1996) It was found that these interactions, in contrast to the RPTP$\zeta/\beta$-contactin interaction mentioned above, inhibit cell adhesion and neurite outgrowth of chick brain neurons and are mediated by asparagine-linked oligosaccharides present in the CA like and Fn-III like domains (Milev *et al* , 1994, 1995, Peles *et al* , 1995) It is difficult to explain the different selective effects of phosphacan on neurite outgrowth and glial cell adhesion that have been reported (Maeda *et al* , 1995, 1996, Sakurai *et al* , 1996) Curently, the most attractive hypothesis is that phosphacan is likely to act as a competitor for ligand binding to the membrane bound forms of RPTP$\zeta/\beta$ and all three isoforms may interfere with the interactions of contactin with its natural ligand tenascin, or N-CAM and Ng-CAM/L1 Taking into account that TAG-1/axonin-1 is also a ligand of N-CAM and able to interact with tenascin, the existence of higly complex interactive processes for formation of neuronal networks is evident (Milev *et al* , 1996), but this may not come as a surprise

The biological significance of the other type V RPTPase, RPTP$\gamma$, is only poorly understood For quite some time RPTP$\gamma$ was considered as a candidate tumor suppressor due to the location of its gene (PTPRG) on human chromosome 3p14 2 (in first instance on 3p21), in a region found to be frequently deleted in certain types of renal and lung carcinoma (Barnea *et al* , 1993, LaForgia *et al* , 1991, 1993) Yet, although a homozygous deletion within the CA-like domain has been detected in a mouse tumor cell line (Wary *et al* , 1993), direct evidence that RPTP$\gamma$ is the product of a tumor suppressor gene is lacking sofar Moreover, a search for mutations in the PTPRG gene from tumors and tumor-derived cell lines failed to identify any abnormalities in all known exons (Druck *et al* , 1995, Tsukamoto *et al* , 1992) As with many other RPTPase genes, the observed distinctly sized mRNAs may represent normal isoforms of RPTP$\gamma$ which are generated by alternative splicing (Kastury *et al* , 1996, Tsukamoto *et al* , 1992) For instance, the most predominant transcript lacks a 87-bp juxtamembrane exon (Kastury *et al* , 1996), but the significance of the splicing out of this small exon is again elusive

**RPTPases with only one cytoplasmic PTPase domain**

PTP BR7 (also known as PC12-PTP) and PTP-SL together make up a new subfamily of

type VI RPTPases with only one cytoplasmic PTPase domain (Hendriks *et al* , 1995, Ogata *et al* , 1995, Sharma and Lombroso, 1995) These proteins display the highest homology to cytosolic PTPases, namely HePTP/LC-PTP and STEP Their extracellular domain does not show any structural homology to known molecules PTP-SL represents an N-terminal truncated form of PTP-BR7, resulting from the use of alternative promoters (A van den Maagdenberg, D Bachner, and W Hendriks, unpublished results) In mouse embryonic tissue only the ~4 1 kb transcript encoding PTP-BR7 is expressed whereas in the adult mouse brain both the ~4 1 kb PTP-BR7 transcript and a smaller, ~3 2 kb mRNA encoding PTP-SL are present

Unfortunately, there are not many clues to the functional roles of these type of RPTPases RNA *in situ* hybridization revealed that the expression is restricted to neuronal cells within the spinal ganglia and to developing and mature Purkinje cells within the cerebellum (A van den Maagdenberg, D Bachner, and W Hendriks, unpublished results, Ogata *et al* , 1995) This might point to a role in cerebellar development Also, upon treatment of PC12 cells with nerve growth factor (NGF) the expression of the PTP BR7 transcript is increased with a maximal induction after 8 hours, suggesting a possible involvement of PTP BR7 in the early stages of neuronal growth and differentiation (Sharma and Lombroso, 1995) Still, it remains to be shown that the increase in PTP-BR7 mRNA level is followed by an increase in protein product and/or enzymatic activity If so, PTP-BR7 might represent one of the three biochemically identified PTPase activities that are induced in NGF treated PC12 cells (Aparicio *et al* , 1992)

**RPTPases as autoantigens in insulin-dependent diabetes mellitus**

The type VII RPTPase-like proteins IA 2 (also known as PTPLP, ICA512, the mouse homolog is denoted as PTP35) (Kambayashi *et al* , 1995, Lan *et al* , 1994, Lu *et al* , 1994, Magistrelli *et al* , 1995) and IA-2$\beta$ (also known as PTP-NP, the rat homolog is denoted as Phogrin) (Chiang and Flanagan, 1996, Lu *et al* , 1996, Wasmeier and Hutton, 1996) are both RPTPase-like molecules with an unusual cysteine-rich region at their N-terminus in the extracellular domain and only a single PTPase domain intracellularly Interestingly, in this PTPase domain the conserved alanine in the signature motif has been substituted for an aspartate residue and it is still a question whether IA-2 or IA-2$\beta$ are enzymatically active For IA-2 it has been proposed it acts as a competitive inhibitor by protecting phosphotyrosine residues from the activity of genuine PTPases (Kambayashi *et*

*al* , 1995) The intracellular part of IA-2 is about 74% identical to the corresponding domain of IA-2$\beta$ but the extracellular domain differs substantially (only 26% identity) (Lu *et al* , 1996) Clearly, IA-2$\beta$ and IA-2 are encoded by different genes but whether they reside on different chromosomes or are arranged in tandem at the same locus still has to be investigated

IA-2, encoded by a 4 kb mRNA, is mainly expressed in brain and pancreatic islets (Hendriks *et al* , 1995, Kambayashi *et al* , 1995, Lan *et al* , 1994, Lu *et al* , 1994, Magistrelli *et al* , 1995) It was originally isolated from an insulinoma subtraction library (Lan *et al* , 1994) but it is still elusive whether its expression has any relation to unrestricted growth of these cells Magistrelli and coworkers (1995) found that the mRNA and protein expression of the mouse homolog of IA-2 is regulated during cell growth and stimulated by mitogens in mouse fibroblasts Mouse IA-2$\beta$ may have a function in neural development as an alternative spliced mRNA lacking the extracelllular region is expressed in early neural cell lineages At later stages of neurogenesis and in the adult brain the expression of the full-length mRNA is observed (Chiang and Flanagan, 1996)

IA-2 and IA-2$\beta$ have now been identified as the precursors of the 40 kDA and 37 kDA insulin-dependent diabetes mellitus (IDDM)-specific major autoantigens, respectively (Bonifacio *et al* , 1995, Lan *et al* , 1996, Lu *et al* , 1996, Magistrelli *et al* , 1995, Passini *et al* , 1995, Payton *et al* , 1995, Rabin *et al* , 1994) Autoantibodies recognize both common and distinct determinants on IA-2 and IA 2$\beta$ (Lu *et al* , 1996) Recently, IA-2 was identified as an intrinsic membrane protein of secretory granules which undergoes proteolytic cleavage at its luminal domain and that recycles to the Golgi complex to be sorted into newly formed secretory granules making IA-2 the first RPTPase residing in an intracellular compartment (Solimena *et al* , 1996) Also IA-2$\beta$ is localized to the secretory granule membranes in pancreatic islets and the precursors of pancreatic endocrine cells (Chiang and Flanagan, 1996, Lu *et al* , 1996, Wasmeier and Hutton, 1996) These findings suggest that a subset of the autoantibodies in IDDM is directed against proteins associated with secretory vesicles (Solimena *et al* , 1996) Moreover, the human IA-2 gene maps to chromosome region 2q35-q36 1 (van den Maagdenberg *et al* , 1996) which is within the area where the *IDDM7* or *IDDM13* susceptibility gene loci reside (Morahan *et al* , 1996), implying a direct role for IA-2 in autoimmune diabetes

**Cell adhesion molecule-like RPTPases**

Cell-cell and cell matrix interactions play an important role during development, for example in cell migration, tissue morphogenesis, and axonal growth in the nervous system (Doherty and Walsh, 1994, Parons, 1996) It has now been well established that protein tyrosine phosphorylation is a key event in these interactions Hence, RPTPases are considered good candidates to function as direct signal transducers upon cell surface recognition in cell-cell and cell-matrix interactions (Brady-Kalnay and Tonks, 1995)

The RPTPase that is subject of this thesis, the Leukocyte common Antigen-Related molecule LAR, belongs to a particular subset of RPTPases with a presumed role in these kind of interactions The extracellular part of these so-called cell-adhesion molecule (CAM)-like RPTPases contain multiple Fn-III-like repeats alone or in combination with immunoglubulin (Ig)-like domains They share these features with ECM proteins like fibronectin and tenascin, cell surface receptors such as the N-CAMs, the growth hormone-prolactin receptor family, and certain RPTKs like Axl and the Eph-subfamily These molecules are all involved in cell-cell or cell-matrix interactions The remaining part of this introduction will be dedicated to these CAM-like RPTPases that are classified as type II (A and B) and type III RPTPases (see Figure 1)

**The type III RPTPases: a function in contact inhibition?**

The type III RPTPases bear in their extracellular segment exclusively multiple Fn III-like repeats but can have either one or two cytosolic PTPase domains RPTP$\beta$, and its homolog in *Drosophila*, DPTP10D (discussed below) were the first RPTPases identified with only one cytoplasmic PTPase domain They bear sixteen or twelve Fn-III-like repeats in their extracellular segment, respectively (Krueger *et al* , 1990, Tian *et al* , 1991, Yang *et al* , 1991) This group has now been extended with the cloning of DEP 1 (also known as PTP$\eta$, the mouse homolog is denoted Byp) (Honda *et al* , 1994, Kuramochi *et al* , 1996, Ostman *et al* , 1994), GLEPP1 (also known as PTP U2, the mouse homolog is denoted as PTP$\phi$) (Pixley *et al* , 1995, Seimiya *et al* , 1995, Thomas *et al* , 1994, Wiggins *et al* , 1995), and SAP-1 (Matozaki *et al* , 1994), all with eight Fn-III-like repeats extracellularly, and DPTP4E in *Drosophila* which contains eleven Fn-III-like repeats in its extracellular domain (Oon *et al* , 1993) Also DPTP99A in *Drososphila* and OST-PTP which contain two cytoplasmic PTPase domains and two and ten Fn-III-like repeats extracellularly, respectively (Hariharan *et al* , 1991, Mauro *et al* , 1994), belong

to this class of RPTPases

The type III RPTPases with only one catalytic PTPase domain contain specific amino acids within the PTPase domain, which are not present in either cytosolic PTPases or other RPTPases with tandem PTPase domains (Thomas *et al* , 1994, Wiggins *et al* , 1995) Their mRNAs seem to come in many flavours, mainly due to alternative splicing Uniquely among RPTPases, for PTPϕ two membrane spanning molecules and a cytosolic isoform with identical single PTPase domains have been found (Pixley *et al* , 1995) Alternative splicing might also be responsible for the existence of Δ3'-RPTPβ, an RPTPβ mRNA truncated near its catalytic site (de Vries *et al* , 1991), PTP-oc, an osteoclast-specific 5'-truncated splice variant of GLEPP1 (Wu *et al* , 1996), and OB PTP, a rat olfactory bulb-specific GLEPP1 isoform with only seven Fn-III-like repeats in its extracellular domain (Yahagi *et al* , 1996)

There has been much speculation on the physiological role and regulation of the type III RPTPases For example, the intracellular domains of DEP-1, RPTPβ, and GLEPP1 contain multiple tyrosine residues suggesting that tyrosine phosphorylation may regulate phosphatase activity Moreover, the C termini of these RPTPases contain a conserved YEN(L/V) amino acid motif which is a potential binding site for the SH2 domain of the Abl tyrosine kinase SH3-binding protein 3BP2, implying a role for 3BP2 in the signalling downstream of these RPTPases (Kuramochi *et al* , 1996, Mayer *et al* , 1992, Songyang *et al* , 1994)

Compelling evidence for a specific function of these RPTPases was provided initially by Ostman *et al* (1994) They described that the expression of DEP-1 is dramatically increased in cell cultures at high density suggesting a role in contact inhibition of growth Earlier, Pallen and Tong (1991) had already found that PTPase activity is increased in cells harvested at high density Due to this increase, the levels of ligand-induced tyrosine phosphorylation of, for example, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors decrease in cells at high density Thus, this mechanism serves to prevent cellular transformation (Sorby and Ostman, 1996) Indeed, density-dependent growth inhibition can be overcome by treating cells with the a-specific PTPase inhibitor vanadate (reviewed in Brady-Kalnay and Tonks, 1994b) Conversely, upon stimulation of PTPase activity by O phospho-L-tyrosine, the growth of renal and breast carcinoma cell lines can be inhibited (Mishra and Hamburger, 1993) There is now accumulating evidence suggesting that individual type III RPTPases (e g DEP-1, RPTPβ, and OST PTP) might be candidates for mediating growth regulation by direct involvement

in density-dependent growth arrest (Gaits *et al* , 1994, 1995, Mauro *et al* , 1994, 1996, Southey *et al* , 1995) Still, for most members of this subclass this has to be experimentally verified

**The *Drosophila* RPTPases: essential in axon pathfinding**

Next to the two non-receptor PTPases DPTP61F (McLaughlin and Dixon, 1993) and *corkscrew* (Perkins *et al* , 1992), a family of five RPTPases has been identified in *Drosophila* (for review see Zinn, 1993) Of these five, the type III RPTPases DPTP4E and DPTP10D are the only ones that contain a single catalytic PTPase domain and eleven or twelve Fn III-like repeats in their extracellular segments, respectively In contrast, DPTP99A contains two tandemly repeated PTPase domains but only two Fn-III-like repeats in its extracellular part Finally, DLAR and DPTP69D (formerly DPTP) also contain two cytoplasmic PTPase domains, but extracellularly a combination of N terminal three Ig like and membrane-proximal nine Fn-III like repeats, or two Ig-like plus two Fn-III-like repeats, making them type IIA RPTPases (Desai *et al* , 1994, Hariharan *et al* , 1991, Oon *et al* , 1993, Streuli *et al* , 1989, Tian *et al* , 1991, Yang *et al* , 1991)

A transmembrane glycoprotein, gp150, has been identified that selectively interacts with the active site in the catalytic domain of DPTP10D (Tian and Zinn, 1994) This substrate-enzyme interaction is unusual in that it is stable and does not require phosphorylation of the tyrosine residues in the short gp150 cytoplasmic domain DPTP10D may therefore regulate the phosphorylation of gp150 and thereby control the interactions with, as yet unknown, downstream effectors (Tian and Zinn, 1994)

DPTP4E is expressed throughout many cell types in the fly whereas DLAR, DPTP10D, DPTP69D, and DPTP99A are much more selectively expressed on axons of the embryonic central nervous system (CNS) (for review see Chien, 1996, Desai *et al* , 1994) Considering the structural similarity and the significant overlap in their expression, they may have redundant functions in the developing CNS (Desai *et al* , 1994, Tian *et al* , 1991, Yang *et al* , 1991) Indeed, flies lacking DPTP99A show no alterations in the embryonic CNS (Hamilton *et al* , 1995) In mutant embryos lacking DPTP69D, however, motor neuron growth cones stop growing before they reach their muscle targets or they bypass their muscle targets (Desai *et al* , 1996) This phenotype was more severe in double mutants lacking DPTP69D and DPTP99A (Desai *et al* , 1996) In flies lacking DLAR, motor axons fail to enter their appropiate targets and display bypass phenotypes

due to wrong navigation at specific choice points (Krueger *et al* , 1996) This phenotype was not 100% penetrant Interestingly, this restricted nature of the DLAR phenotype contrasts with the widespread neural expression of DLAR Taken together, the specific mutant phenotypes demonstrate specific roles for these three RPTPases in steering (DLAR) and defasciculation (DPTP69D, DPTP99A), important processes in motor axon guidance (Desai *et al* , 1996, Krueger *et al* , 1996)

**Type IIB RPTPases mediate cell-cell aggregation via homophilic interactions**

The closely related type IIB RPTPases RPTP$\mu$ (Gebbink *et al* , 1991) and RPTP$\kappa$ (Jiang *et al* , 1993), and the recently cloned PCP 2 (Wang *et al* , 1996) harbor intracellularly the classical tandemly repeated PTPase domains, separated from the transmembrane segment by an unusually large juxtamembrane region The juxtamembrane region of RPTP$\mu$ bears a potential binding site for 14-3-3 proteins, as is also found in RPTP$\varepsilon$ (Muslin *et al* , 1996) The multifunctional family of phosphoserine binding 14-3-3 proteins play an important role in the formation of protein-protein complexes, and thus RPTP$\mu$'s location and function may be regulated by serine phosphorylation In addition to an Ig like and four Fn-III like repeats, the extracellular part contains a MAM domain, which is a $\sim$160 amino acids region including four cysteine residues that is also present in the unrelated membrane bound metalloendopeptidase meprin and the *Xenopus* neuronal cell surface A5 glycoprotein (Beckman and Bork, 1993) Both RPTP$\mu$ and $\kappa$ can undergo postranslational cleavage at a site in the fourth Fn-III-like repeat by a member of the subtilisin family of endoproteases, most likely PC5 (Campan *et al* , 1996) This generates two subunits which remain associated in a noncovalent manner (Brady-Kalnay and Tonks, 1994a, Gebbink *et al* , 1995, Jiang *et al* , 1993) The N terminal subunit which comprises almost the entire ectodomain can be shed from the surface of exponentially growing cells (Gebbink *et al* , 1995)

RPTP$\mu$ and RPTP$\kappa$ can mediate cell cell aggregation via homophilic, but not heterophilic, interactions (Brady Kalnay *et al* , 1993, Gebbink *et al* , 1993, Sap *et al* , 1994, Zondag *et al* , 1995) For these events to occur, the catalytic PTPase domains, proteolytic cleavage, or calcium are not required (Brady Kalnay *et al* , 1993, Gebbink *et al* , 1993, Sap *et al* , 1994) Using chimeric proteins it was found that the MAM domain might confer the homophilic binding specificity of RPTP$\mu$ (Zondag *et al* , 1995) However, another study pointed out that the Ig like domain is both necessary and

sufficient for the homophilic interaction of RPTPμ (Brady-Kalnay and Tonks, 1994a) Perhaps, in analogy to the CAM-like RPTK Axl/Ark/tyro7/UFO (Bellosta *et al* , 1995, Stitt *et al* , 1995, Varnum *et al* , 1995), RPTPμ might undergo a heterophilic interaction, i e binding a soluble ligand, at low cell density, and homophilic binding at high cell density (Brady-Kalnay and Tonks, 1995)

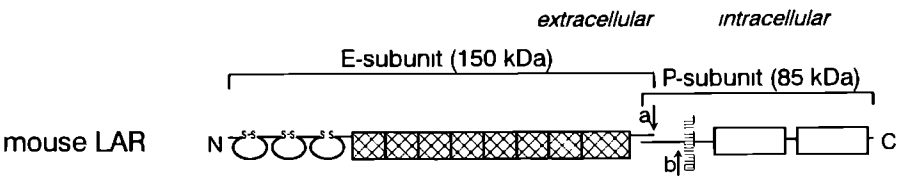
Immunofluorescense studies revealed that the cell surface expression of RPTPμ and RPTPκ is restricted to regions of cell-cell contact and increases with increasing cell density (Brady-Kalnay *et al* , 1995, Fuchs *et al* , 1996, Gebbink *et al* , 1995) This clustering at contact regions is accompanied by increased proteolytic cleavage of the protein (Campan *et al* , 1996) and may lead to tyrosine dephosphorylation of intracellular substrates at cell-cell contacts (Gebbink *et al* , 1995) Recently, members of the cadherin/catenin complex were found to colocalize and associate with RPTPμ and RPTPκ at adherens junctions (Brady-Kalnay *et al* , 1995, Fuchs *et al* , 1996) As β-catenin has been suggested as a substrate for RPTPκ PTPase activity, a functional role in the regulation of cell contact and adhesion controlled events is likely (Fuchs *et al* , 1996) Nevertheless, mice lacking RPTPμ (G Zondag and M Gebbink, personal communication) or RPTPκ (Skarnes *et al* , 1995) show no apparent defects in embryonal development nor histological abnormalities at the adult stage

**LAR and the LAR-like RPTPases, RPTPδ and RPTPσ**

The type IIA RPTPases LAR, RPTPδ, and RPTPσ (also known as BPTP-1, PTP-NE3, rPTP-2, LAR-PTP2, PTP-P1/-PS, PTP-NU3, CPTP-1/3, Crypα, and MPTP-H3/70 15) (Adachi *et al* , 1992, Boehm, 1993, Endo *et al* , 1996, Goldstein *et al* , 1992, Krueger *et al* , 1990, Mizuno *et al* , 1993, Ogata *et al* , 1994, Pan *et al* , 1993, Pot *et al* , 1991, Pulido *et al* , 1995a, 1995b, Sahin and Hockfield, 1993, Schaapveld *et al* , 1995, Stoker, 1994, Streuli *et al* , 1988, Wagner *et al* , 1994, Walton *et al* , 1993, Yan *et al* , 1993, Zhang *et al* , 1994) bear an extracellular part existing of three tandemly arranged Ig-like domains and eight Fn-III-like repeats with high homology to CAMs, like N-CAM, Ng-CAM/L1, and fasciclin II (Edelman and Crossin, 1991)

All three RPTPases are expressed on the cell surface as a complex of two noncovalently-associated subunits (denoted as E- and P-subunit, see Figure 3) derived from a pro-protein Processing of this pro-protein by a subtilisin-like endoprotease occurs intracellularly at a paired basic amino acid-site located in the extracellular region (Streuli

*et al.*, 1992; Yu *et al.*, 1992; Yan *et al.*, 1993; Pulido *et al.*, 1995a, 1995b). The E-subunit contains the CAM-like receptor-region, while the P-subunit contains a short segment of the extracellular region, the transmembrane peptide, and the cytoplasmic PTPase domains (see Figure 3) This arrangement is not only found for the type II RPTPases including RPTP$\mu$ and $\kappa$ (as described above), but also, for example, for the cell-cell adhesion molecule Ng-CAM/L1 and the *Drosophila* PTK Sevenless (Burgoon *et al.*, 1991; Simon *et al.*, 1989).




| | 1 | 2 |
|---|---|---|
| rat LAR | 98.2 | 100 |
| human LAR | 97.8 | 100 |
| *Drosophila* LAR | 71.8 | 82.7 |
| mouse RPTPδ | 79.4 | 91.6 |
| mouse RPTPσ | 85.1 | 93.3 |

**Figure 3.** LAR structure and conserved homology of the PTPase domains

The 150 kDa E-subunit contains the CAM-like region, whereas the 85 kDa P-subunit contains a short segment of the extracellular region, the transmembrane peptide, and the cytoplasmic PTPase domains a, site of proprotein processing resulting in the complex of two noncovalently-associated subunits, b, proteolytic cleavage at a second site within the P-subunit ectodomain near the transmembrane peptide necessary for shedding of the E-subunit, independent of the cleavage at a The same features have been reported for RPTPδ Proteolytic cleavage has also been shown for RPTPσ.

Comparison of the amino acid sequences of the PTPase domains 1 and 2 shows the striking conservation (in % homology) for the second PTPase domain not only between species, but also between the mouse homologs of the type IIA RPTPases For symbols see Figure 1

The E-subunit of LAR is shed during growth at high density (Streuli *et al* , 1992) This shedding results from a secondary proteolytic cleavage near the transmembrane peptide, is PMA-inducible, and independent of the first proteolytic cleavage (Serra-Pagès, *et al* , 1994) This suggests that the protease(s) responsible for the shedding is regulated via activation of PKC, reminiscent of the PMA-inducible shedding of other receptors, like the leukocyte adhesion molecule L-selectin, the colony stimulating factor (CSF)-1 receptor, and the tumor necrosis factor (TNF) receptor (Serra-Pagès *et al* , 1994) Shedding of the CAM-like ectodomain has also been reported for PTPδ, and is likely to be a common feature of the type II RPTPases (Pulido *et al* , 1995a, 1995b) It still remains to be established whether this shedding has an effect on the intracellular phosphatase activity and ligand reponsiveness The shed ectodomain might also have a distinct, cytokine-like function, thereby acting as a ligand itself or competing with the cell surface molecule for ligand binding More insight into the exact functional role of these features awaits characterization of the ligands and substrates

LAR, RPTPδ and RPTPσ are overall 70% identical at the amino acid level, and up to ~80-90% identical for both PTPase domains (Pulido *et al* , 1995b, Schaapveld *et al* , 1995) Using bacterially expressed proteins and artificial substrates it was found that the first, membrane proximal domain of LAR is catalytically active Domain 2 may have only a regulatory role in controlling the substrate specificity of domain 1 (Itoh *et al.*, 1992, Pot *et al.*, 1991, Streuli *et al* , 1990) In these assays, the catalytic activities of a recombinant LAR protein containing only the first domain or both domains were indistinguishable (Cho *et al* , 1992b, Itoh *et al.*, 1992), argueing against the model for cooperativity in catalytic PTPase activity (Pallen, 1993) Strikingly, among species the second domain of LAR shows a slower mutation rate than the first (Streuli *et al* , 1990), and also sequence comparison with the LAR like RPTPases PTPδ and PTPσ revealed a higher homology (up to 90% at the protein level) for the second domain when compared with the first domain (Schaapveld *et al.*, 1995, Figure 3) This is not seen for the type IIB RPTPases or for members of the other types of RPTPases (Schaapveld *et al* , 1995)

Multiple isoforms of LAR, RPTPδ, and RPTPσ (as shown in Figure 4) appear to be generated by tissue-specific and developmentally regulated alternative splicing (Endo *et al* , 1996, Longo *et al* , 1993, Mizuno *et al* , 1993, 1994, Ogata *et al* , 1994, O'Grady *et al* , 1994, Pan *et al* , 1993, Pulido *et al* , 1995a, 1995b, Stoker, 1994, Wagner *et al* , 1994, Walton *et al* , 1993, Yan *et al* , 1993, Zhang and Longo, 1995) In addition, alternative polyadenylation is involved in the formation of an RPTPσ isoform with a

truncated cytoplasmic segment lacking the second PTPase domain (Pan *et al* , 1993) One tissue can express multiple isoforms with varieties in the number of Fn-III-like repeat units, Ig-like domains, and presence or absence of mini-exon segments encoding small peptides This structural diversity might lead to functional diversity, i e ligand specificity, as was shown for N-CAM, where splicing of a 10-amino acid peptide located within the fourth Ig-like domain regulates the neurite outgrowth promoting activity of N-CAM (Doherty *et al.*, 1992) Within N-CAM, the Ig-like domains predominantly regulate cell adhesion and the Fn-III-like repeats contribute significantly to neurite outgrowth (Frei *et al* , 1992) Although the different isoforms are mainly found in brain, it is not known whether LAR, PTPδ or PTPσ affect neurite outgrowth

Yet, this would not be too surprising as many aspects of neural cell function are regulated via tyrosine phosphorylation For example the receptors for NGF and its related family of neurotrophins are all RPTKs of the Trk-family (for review see Barbacid, 1995) These receptors are expressed almost exclusively in neurons in a developmentally regulating pattern, and mutant animals have provided detailed information on the role they play in neuronal development The CAM-like RPTPases are good candidates to participate in such processes RNA *in situ* hybridization experiments demonstrate complex spatial and developmental regulation of LAR, RPTPδ, and RPTPσ expression during development in many areas of the brain For example, LAR is expressed in the forebrain, brainstem, dorsal root ganglia, and in hippocampal, pyramidal and dentate granule neurons, and cerebellar Purkinje cells (Longo *et al* , 1993, Schaapveld *et al* , unpublished results) Strikingly, high levels of RPTPσ were observed in the same structures (Yan *et al* , 1993), and also RPTPδ could be detected in the pyramidal cell layer of the hippocampus and the piriform cortex (Mizuno *et al* , 1993) In addition, LAR is expressed at high levels in the proliferative zone of the cortex, whereas RPTPσ shows highest levels of expression during the period of corticogenesis, in the external granule cell layer of the developing cerebellum (Sahin and Hockfield, 1993, Sahin *et al* , 1995, Yan *et al* , 1993) High levels of the brain-specific isoform of RPTPσ lacking four Fn-III like repeats (ΔFn4-7, see Figure 4) were also observed in the immature neurons of the olfactory neuroepithelium (Walton *et al* , 1993) Wang *et al* (1995), however, showed that the signal in the immature neuroepithelium represented the full-lenght transcript, whereas the brain-specific ΔFn4-7 isoform is expressed at the adult stage Comparison of the various expression data is, however, difficult because mostly different species or developmental stages were used Furthermore, due to the extensive alternative splicing, it

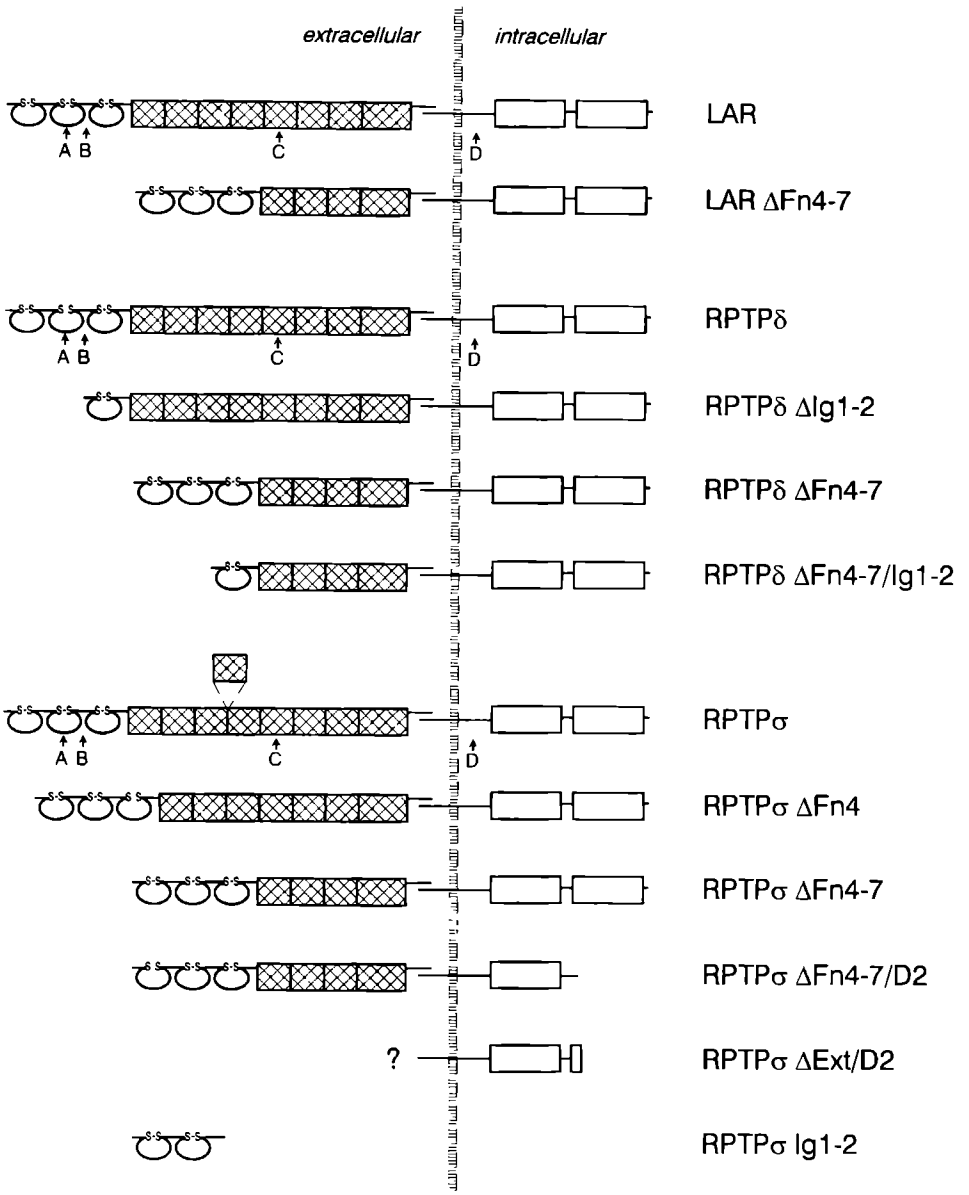

extracellular
intracellular
A B
C
D
LAR
LAR ΔFn4-7
RPTPδ
RPTPδ ΔIg1-2
RPTPδ ΔFn4-7
RPTPδ ΔFn4-7/Ig1-2
RPTPσ
RPTPσ ΔFn4
RPTPσ ΔFn4-7
RPTPσ ΔFn4-7/D2
?
RPTPσ ΔExt/D2
RPTPσ Ig1-2

**Figure 4.** Comparison of the structures and alternative splicing patterns of LAR, RPTPδ, and RPTPσ
A schematic representaion of LAR, RPTPδ, and RPTPσ isoforms is depicted The full lenght isoforms encode three Ig like domains, eight Fn-III-like repeats, the transmembrane peptide, and two cytoplasmic PTPase domains The positions of the mini-exon (me) A, meB, meC, and meD peptides are indicated by small arrows For LAR, meC represents the alternative spliced small exon 13 (O'Grady *et al.*, 1994) Alternative usage for the meA-meD peptides may also occur in the truncated isoforms No LAR isoforms containing the meB sequence were isolated In addition, a LAR-ΔFn4-7 isoform as reported by O'Grady *et al* (1994) was not found by others (Pulido *et al* , 1995b) Wagner *et al* (1994) reported the existence of a mouse RPTPσ isoform with nine Fn-III-like repeats due to a 132-bp insertion at the fourth Fn-III-like repeat The existence of a rat RPTPσ-ΔFn4 isoform has only been claimed by Ogata *et al* (1994) by sequence comparison with their mouse homolog The RPTPσ-ΔExt/D2 and -Ig1-2 isoforms have only been detected in chicken (Stoker, 1994). The RPTPσ-ΔExt/D2 might be a partial clone of RPTPσ-ΔFn4-7/D2 Sequence errors or PCR artifacts might explain some of these discrepancies For abbrevations and symbols see Figure 1 D2, PTPase domain 2, Ext, extracellular region

is hard to establish which isoform corresponds with a specific signal So, although a role in creating the neuronal architecture of the CNS is suggested, the precise physiological meaning of the isoform alteration during neuronal development as seen for all three RPTPases remains to be clarified

**The cell biological role of LAR?**

Alhough LAR was among the first PTPases to be identified and has been studied extensively at the cellular level, its physiological function remains unclear. Several possible physiological functions have been put forward Firstly, since PTPases may be involved in signals which inhibit cell growth upon cell-cell contact (Fischer *et al.*, 1991), LAR may function as a tumor suppressor gene The single copy gene for LAR (PTPRF) is located on human chromosome 1p32-34 1 (Disteche *et al.*, 1989, Jirik *et al.*, 1992; Streuli *et al.*, 1992; A. van den Maagdenberg, unpublished observation). This region is frequently deleted in tumors of neuroectodermal origin, as for example phaeochromocytoma, medullary thyroid carcinoma, and neuroblastoma (reviewed in Dracopoli *et al.*, 1994). Also, aberrant transcripts of LAR lacking the cytoplasmic phosphatase domains have been found in colon and breast tumors (Weinstein *et al.*, 1993) Furthermore, the resemblance of the LAR extracellular part to the tumor suppressor gene Deleted in Colorectal Cancer (DCC) (Fearon *et al.*, 1990) and other

CAM's which play a role in metastasis, is evident A possible role in breast carcinoma came from studies showing increased LAR expression in RPTK $p185^{neu}$ transformed breast epithelial cells and that overexpression of LAR can reduce the tumor growth of these transformed cells (Zhai *et al.*, 1993, 1995) It is still hypothetical whether LAR plays a suppressive regulatory role in tumor growth in these breast carcinoma cells as also candidate breast carcinoma tumor suppressor genes are located to the same chromosomal region as LAR (Genuardi *et al* , 1989, Weinberg, 1991) Furthermore, the involvement of other PTPases in the suppression of tumor growth of $p185^{neu}$ induced human breast carcinoma cells (Brown-Shimer *et al* , 1992, Elson and Leder, 1995a, Wiener *et al* , 1994) needs more clarification

Secondly, a role for LAR in insulin signalling has been suggested based on several findings For example, LAR expression is increased in adipose tissue of obese human subjects (Ahmad *et al* , 1995, Ahmad and Goldstein, 1995) Also, LAR has been found to be active against the autophosphorylated insulin receptor (IR) *in vitro*, preferentially dephosphorylating the phosphotyrosine residues that have been implicated in the regulation of the receptor kinase (Hashimoto *et al* , 1992a) Moreover, antisense inhibition of LAR led to increased ligand-dependent IR activation and signalling (Kulas *et al* , 1995, 1996b), whereas overexpression of membrane-bound LAR decreased the tyrosine phosphorylation of the IR upon insulin stimulation (Zhang *et al* , 1996) In addition, the IR and LAR could be co-immunoprecipitated upon chemical cross linking on the surface of rat hepatoma cells (Zhang *et al* , 1996) In these cells LAR may influence the phosphorylation status of the IR and its substrates IRS-1 and IRS 2 (Li and Goldstein, 1996) However, not only LAR but various other PTPases, such as the cytosolic PTP1B and SHP2 and the RPTPases CD45, RPTPα, and RPTPε are also capable of dephosphorylating the IR (Goldstein, 1993, Kulas *et al* , 1996a Møller *et al* , 1995) Also, the levels of LAR expression in the insulin sensitive tissues as liver and muscle are subject of debate (Hashimoto *et al* , 1992b, Longo *et al* , 1993, Pulido *et al* , 1995b, Streuli *et al* , 1992, Schaapveld *et al* , 1996, Zhang and Goldstein, 1991) Therefore, the identification of the PTPase family members that are critically involved in the *in vivo* regulation of insulin signalling needs further study

Finally, as LAR is colocalized with a coiled-coil protein, termed LAR-interacting protein 1 (LIP 1) at the ends of focal adhesions (FAs) LAR may be involved in cell-matrix interactions (Serra-Pagès *et al* , 1995) Strikingly, also RPTPδ and RPTPσ are able to interact with LIP 1 (Pulido *et al* , 1995b) LIP 1 binds to the second phosphatase

domain of LAR, as does also a second protein, termed TRIO, which was also identified using the two-hybrid system (Debant *et al* , 1996) TRIO contains N-terminally four spectrin like repeats (involved in intracellular targeting) followed by two functional GEF domains, guanine nucleotide exchange factors which promote the exchange of GDP for GTP One domain is specific for the small GTP-binding protein Rac which is involved in the formation of lamellipodia and membrane ruffles The second domain activates Rho which is involved in the formation of stress fibers and focal contacts (for review see Zigmond, 1996) Both GEF domains are followed by a pleckstrin homology (PH) domain, implicated in membrane association by interacting with membrane-bound proteins or by binding directly to phospholipids (for review see Lemmon *et al* , 1996, Mayer *et al* , 1993, Shaw, 1996) At its C terminus, TRIO contains adjacent to an Ig-like domain a protein serine kinase (PSK) domain, which is most similar to smooth muscle myosin light chain kinase Thus, the apparently inactive second PTPase domain of LAR may play a role in placing LAR in the proper cellular environment close to cytoskeletal proteins in the cell cortex and FAs The role of tyrosine phosphorylation in the maintenance and modulation of cell cell and cell-substratum interactions indeed strongly suggests a potential catalytical role for LAR in these regions An example is the formation of FAs by the changes in tyrosine phosphorylation of associated proteins (for review see Craig and Johnson, 1996 Parsons, 1996) FAs contain transmembrane proteins known as integrins, which link the ECM to an intracellular plaque associated with actin bundles (stress fibers) Attachment of cells to the ECM results in tyrosine phosphorylation of multiple intracellular proteins associated with these adhesions including paxillin, tensin, talin, $\beta$ integrin and focal adhesion kinase (FAK) Inhibition of cellular tyrosine kinases prevents the formation of FAs Taking into account that the expression of LAR is most widespread in epithelial cells, which are especially rich in FAs, and that LAR is also localized at other regions of association of cells and the basement membrane (Streuli *et al* , 1992), a functional role for LAR in integrin-mediated tyrosine phosphorylation and the formation of cellular structures like FAs is indeed most obvious

**Outline of this thesis**

The identification of many distinct RPTPase genes and the discovery of multiple isoforms resulting from alternative splicing and posttranslational modification has revealed a surprising complexity for this multiprotein family Studies on the biological significance

of the expression of RPTPase isoforms are hampered by the spatial and tissue regulation of isoform expression *in vivo* which can not be mimicked in individual cell lines *in vitro* Moreover, the study of structure-function relationships is intrinsically difficult because these genes and proteins are generally very large in size, and for most of the family members specific ligands and substrates are not at hand In addition, a direct read-out for activation or inhibition of RPTPases is not available The studies described in this thesis represent an approach that is independent of such knowledge, yet allows the study of the *in vivo* function of an individual RPTPase in signal transduction and growth regulation

The isolation, cloning, and characterization of the relevant segments of the structural and genomic DNAs of the mouse homolog of the CAM-like RPTPase LAR (Chapter II) provided us with the necessary tools to construct a replacement type targeting vector to generate mouse embryonic stem (ES) cell lines by homologous recombination lacking LAR exon sequences encoding both cytoplasmic PTPase domains (Chapter III) These ES cell lines were subsequently used to generate mice with a loss of function mutation, i e lacking LAR tyrosine phosphatase activity (Chapter IV) The observed effects of this mutation point to a role for LAR in mammary gland development and function (Chapter IV) In addition, in a collaborative effort the consequences of the lack of LAR PTPase activity with respect to metabolic responses induced by insulin were investigated (Chapter V) The conclusions which can be drawn from these studies and future directions to better define the complex interactions and functions of the RPTPase LAR within the signal transduction network controlling development and differentiation are discussed in the final chapter (Chapter VI)

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# Chapter II

## The Mouse Gene *Ptprf* Encoding the Leukocyte Common Antigen-Related Molecule LAR: Cloning, Characterization, and Chromosomal Localization

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**Summary**

The human receptor-like protein tyrosine phosphatase Leukocyte common Antigen-Related molecule (LAR, gene symbol PTPRF) closely resembles cell adhesion molecules, which suggests that it may be involved in the regulation of phosphotyrosine levels through cell-cell or cell-matrix interactions. To obtain a better understanding of LAR function, we have characterized the mouse *Ptprf* gene as a first step towards site-directed mutagenesis studies *in vitro* and *in vivo*. The cytoplasmic region of the mouse LAR (mLAR) protein is encoded by eleven exons that span only 4.5 kb of genomic DNA Compared to the known exon-intron structures of other mammalian receptor-like protein tyrosine phosphatase genes, such as *Ptpra* (encoding LRP) and *Ptprc* (coding for Ly-5), the *Ptprf* gene part encoding the cytoplasmic region of mLAR contains not only smaller, but also fewer introns. Sequence analysis of both phosphatase domains of mLAR and its homologs MPTPδ and mRPTPσ revealed a higher evolutionary conservation of the second, C-terminal domain in comparison to the first domain Fluorescence *in situ* hybridization was used to map the *Ptprf* gene to region C6-D1 on mouse Chromosome 4

## Introduction

Protein tyrosine phosphorylation is recognized as an important reversible reaction controlling many aspects of cellular function, including growth and differentiation, cell cycle control, and cytoskeletal integrity (Tonks *et al.*, 1992; Mourey and Dixon, 1994). The role of protein tyrosine kinases (PTKs) in these processes and the induction of their activity has been

extensively studied, but the recent identification and characterization of many protein tyrosine phosphatases (PTPases) suggests that dephosphorylation of signal transduction proteins is also a crucial event in signal transduction (Fischer *et al* , 1991, Brady-Kalnay and Tonks, 1994) To date more than 30 PTPases have been identified (Mourey and Dixon, 1994) Sequence comparisons have revealed that there are two major classes of PTPases (for review see Tonks *et al* , 1992, Mourey and Dixon, 1994), namely (i) those found in the cytoplasm or nucleus that contain only one tyrosine phosphatase domain and (ii) membrane-bound, receptor-like PTPases (RPTPases) that have two tandemly repeated catalytic domains There also exist intermediate forms such as HPTPβ (Krueger *et al.*, 1990) and PTP-SL (Hendriks *et al* , 1995) that are transmembrane proteins with only one catalytic domain

The RPTPases can be subdivided into five types based on the structure of their extracellular segments (Mourey and Dixon, 1994) Type I represents the CD45 family, which is comprised of multiple isoforms arising from differential splicing of the RNA of a single gene Type II members (e g LAR, HPTPδ, RPTPσ, RPTPμ, RPTPκ) are characterized by the presence of multiple immunoglobulin (Ig) and fibronectin (FN) type III-like domains Type III carry multiple FN-III domains (e g HPTPβ), while type IV members (e g HPTPα, HPTPε) possess small glycosylated segments Finally, type V members (e g HPTPζ, HPTPγ) have an amino-terminal motif with homology to carbonic anhydrase

The extracellular region of the type II RPTPases resembles that of cell adhesion molecules (CAMs) such as N-CAM (Edelman and Crossin, 1991) Recently, homophilic adhesive properties have been attributed to RPTPμ and RPTPκ (Gebbink *et al* , 1993, Brady-Kalnay *et al* , 1993, Sap *et al* , 1993) In combining cell adhesion motifs at the cell surface with intracellular phosphatase activity, these RPTPases may be invloved in the control of cell growth, motility and differentiation in a direct response to cell-cell interaction

The cytoplasmic region of most type II RPTPases contains two tandem phosphatase domains of approximately 260 amino acid residues each, but it is not clear at present whether both domains are enzymatically active *in vivo* or exhibit different substrate specificities Structure function relationships have only been established for phosphatase domains expressed in bacteria and therefore outside their natural context Although site-directed mutagenesis studies have shown the significance of the 'signature sequence' (I/V)HCXAGXXR(S/T)G for tyrosine phosphatase activity of the first, transmembrane proximal phosphatase domain (Mourey and Dixon, 1994), it remains to be determined whether the distal phosphatase domain also exhibits catalytic activity (Wang and Pallen, 1991, Tan *et al.*, 1993), or plays only a regulatory role in modulating the activity of the first domain (Streuli *et al* , 1990, Pot

*et al.*, 1991; Krueger and Saito, 1992). However, *in vitro* studies are inadequate to address such questions because specific inhibitors of PTPases are not yet at hand and substrates and ligands are unknown.

To initiate studies into the role of LAR domains in an organismal context, we set out to isolate structural and genomic mouse LAR (mLAR) DNAs Here we describe the organization of the *Ptprf* gene region encoding the cytoplasmic part of mLAR. Compared with known exon-intron structures of other mouse PTPase genes, such as *Ptpra* (Wong *et al.*, 1993) and *Ptprc* (Saga *et al.*, 1988), the *Ptprf* gene exhibits a more compact structure.

Comparison of both phosphatase domains of mLAR with closely homologous sequences of MPTPδ (Mizuno *et al.*, 1993) and mRPTPσ (Ogata *et al.*, 1994) revealed a higher evolutionary conservation of the second, C-terminal domain compared to the proximal 'catalytically active' domain.

Finally, we report the chromosomal localization of the *Ptprf* gene to region C6-D1 of mouse Chromosome 4.

## Materials & Methods

### *Isolation of mouse LAR genomic and cDNA clones*

Cosmid clones were isolated from a mouse 129/SvEv genomic DNA cosmid library (kindly provided by Dr. M Hofker, University of Leiden, Leiden) using a random-primed (Feinberg and Vogelstein, 1983) 1 5 kb *Eco*RI-*Sal*I human LAR (hLAR) cDNA fragment (positions 4444-5933, Streuli *et al.*, 1988) as a probe Hybridizations were carried out according to Church and Gilbert (1984) and filters were washed for 5, 10, and 30 min at 65°C in 0.25 M sodium phosphate/1% SDS/1 mM EDTA Positive clones were purified by subsequent rounds of low density screening

The same hLAR cDNA probe was used to screen an oligo dT-primed mouse brain cDNA library in λZAPII (Stratagene) Filters were washed three times for 30 min at 65°C in 2 × SSC/0 2 % SDS Positive phages were plaque-purified and inserts were rescued as pBluescript SK plasmids according to the manufacturers protocol

### *Sequence analysis*

Nested deletion mutants of cDNA clone mLAR#9 were generated using Exonuclease III (Promega) and nucleotide sequences were determined using the double-stranded DNA dideoxy sequencing method (Hattori and Sakaki, 1986) A 6 5 kb *Sst*I-*Bam*HI *Ptprf* genomic fragment was used for exon-intron determination according to the shotgun sequencing strategy of Deininger (1983) Briefly,

fragments of around 500 bp were generated by sonication, blunt-ended using Klenow (Boehringer) and subcloned into the *Sma*I site of vector pGEM4 (Promega) Clones, grown in microtiter plates, were replica plated onto nitrocellulose filters and screened with the mLAR cDNA#9 insert. Thirty positive clones were used for sequence determination (Hattori and Sakaki, 1986)

DNA sequence gel readings were recorded, compared, edited, and assembled using the IGSUITE 5 35 package (Intelligenetics Inc , Moutain View, California). Sequences were aligned using the GCG Wisconsin programs PILEUP and LINEUP (Devereux *et al.*, 1984) Evolutionary reconstruction was done using the program FITCH supplied in the phylogeny inference package PHYLIP (distributed by Dr J Felsenstein, University of Washington, Seattle) FITCH uses distance matrices to construct trees without allowing negative branch length (Fitch and Margoliash, 1967)

***Fluorescence in situ hybridization***

Fluorescence *in situ* hybridization was performed on mouse strain 129-derived E14 embryonic stem cell (Handyside *et al.*, 1989) metaphase spreads for the regional localization of the *Ptprf* gene, using mouse cosmid #5 as a probe. Biotinylated cosmid DNA was dissolved in 10 $\mu$l hybridization solution containing 2 × SSC, 10% dextran sulphate, 1% Tween-20, and 50% formamide The probe mixture was heat-denatured at 80°C for 10 minutes, followed by incubation at 37°C to allow re-annealing of highly repetitive sequences Hybridization of this probe to heat-denatured chromosome spreads, under a cover slip, was carried out overnight at 37°C Hybridizing probe was detected immunohistochemically using fluorescein isothiocyanate conjugated to avidin-D (Suijkerbuijk *et al.*, 1991) Images of metaphase preparations were captured by a cooled high-performance CCD camera (Photometrics) coupled to a Macintosh II computer Separate images of both mLAR hybridizing signals and DAPI-counterstained chromosomes were transformed into pseudocoloured images using image analysis software.

## Results & Discussion

***Isolation and characterization of the mLAR-encoding Ptprf gene***

We are currently investigating the biological role of LAR and the functions of the tyrosine phosphatase domains in the LAR signal transduction pathway using reverse genetic approaches As a first step we isolated and characterized mouse LAR genomic and cDNA clones, with special emphasis on the region encoding the phosphatase domains

A 1.5 kb *Eco*RI-*Sal*I hLAR cDNA probe encoding both phosphatase domains (Streuli *et al.*, 1988) was used to isolate cosmid clones, two of which, #5 and #11, repeatedly showed stronger hybridization signals under more stringent washing conditions. These clones were

characterized further. Southern blot and restriction enzyme analyses revealed these two clones to overlap and span some 50 kb of the mouse *Ptprf* locus, as shown in Fig 1 We assume that the other, more weakly hybridizing cosmid clones contained either *Ptprf* pseudogenes or genes with homology to *Ptprf*, such as *Ptprd* (encoding MPTPδ, Mizuno *et al.*, 1993) or *Ptprs* (coding for mRPTPσ, Ogata *et al.*, 1994).

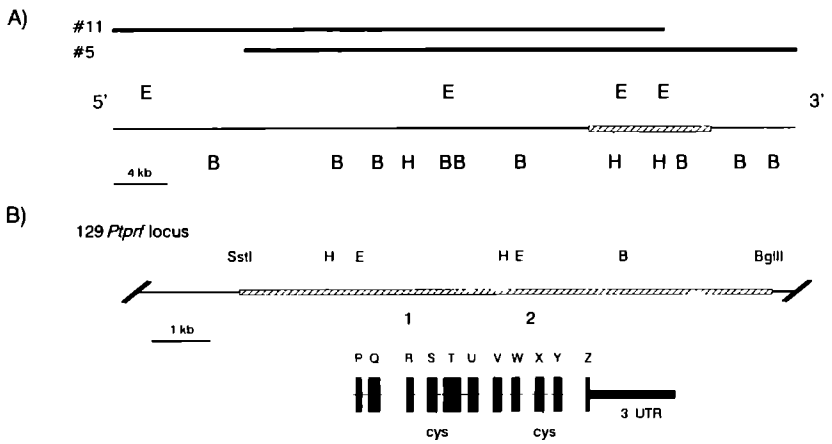


**Figure 1.** Schematic representation of the *Ptprf* locus (**A**) and exon-intron structure of the region of the gene encoding the phosphatase domains of the mLAR protein (**B**) **A**) Thick solid bars indicate the positions of the cosmid clones relative to the *Ptprf* locus A hatched bar marks the *Sst*I-*Bgl*II fragment that is shown in more detail in panel B B, *Bam*HI,E, *Eco*RI,H, *Hin*dIII **B**) Introns (thin lines) and exons (filled boxes) are drawn to scale Exons are numbered P thru Z The region encoding the two phosphatase domains are indicated by brackets and numbered 1 and 2, respectively. Cys indicates the position of the codon for the conserved cystein residue within the signature sequence of each phosphatase domain 3' UTR, 3' untranslated region

We compared genomic and cDNA sequences to position exon-intron boundaries within the *Ptprf* gene. To this end, ten cDNA clones were isolated from a λZAPII mouse brain cDNA library using the hLAR cDNA probe mentioned above End-in sequencing and comparison with the cognate positions in hLAR (Streuli *et al.*, 1988), rat LAR (rLAR, Pot *et al.*, 1991), and fragments of mLAR (Hendriks *et al.*, 1995) revealed that three clones contained genuine mLAR cDNAs The other cDNA clones contained mLAR homologous sequences In Fig 2, the sequence of the largest mLAR cDNA clone, mLAR#9, is depicted alongside the deduced amino acid sequence.

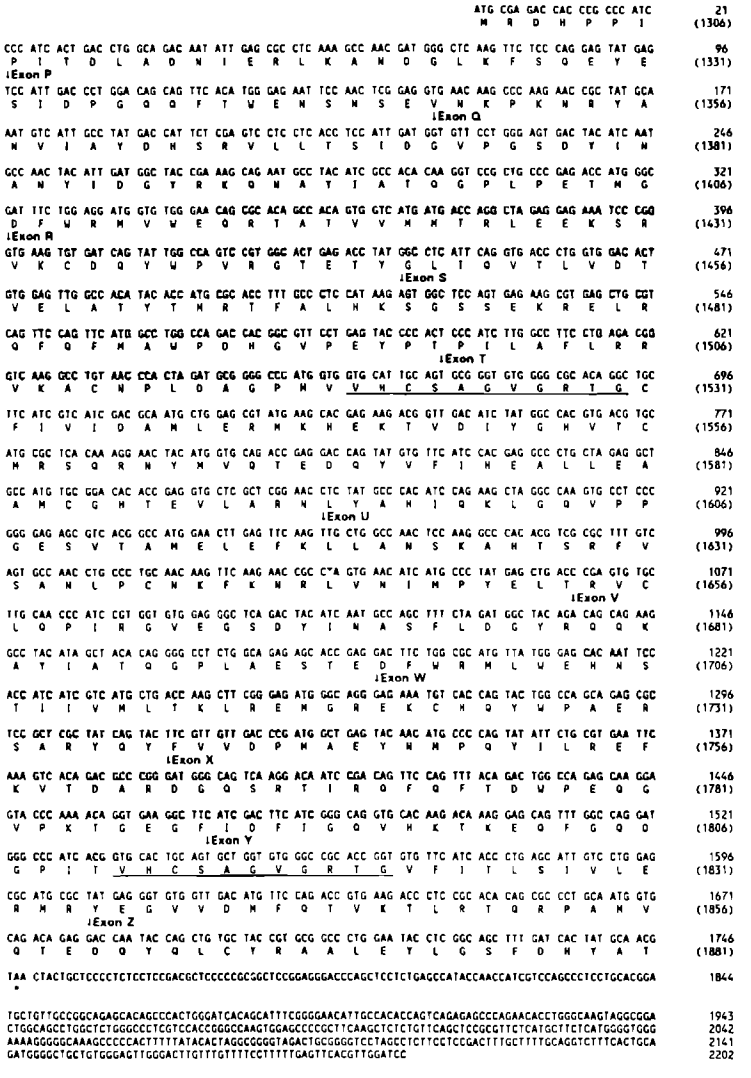

**Figure 2.** Definition of the exon intron boundaries within the partial mLAR cDNA sequence
Exon intron boundaries are indicated by arrows at the first nucleotide of each exon The 3' UTR sequence is given up to the *Bam*HI site as indicated in Fig 1 Numbers to the right indicate nucleotides as present in clone mLAR#9 and in brackets, amino acids according to the numbering system of Streuli *et al* (1988) Regions which exhibit 100% identity with previously published PCR derived mLAR fragments (Hendriks *et al* , 1995), are shown on a grey background Underlined sequences indicate the 'active site' signature motif

All three mLAR cDNAs ended in an A-rich stretch in the 3' untranslated region (3' UTR) also found in the hLAR mRNA message (position 6900, Streuli *et al.*, 1988). Screening of the mouse cDNA library with a more 3' UTR hLAR probe (*Sal*I-*Eco*RI, positions 5933-7700, Streuli *et al.*, 1988), yielded additional clones which terminated at the genuine polyadenylation site as predicted from hLAR sequence data (not shown) We therefore conclude that clone mLAR#9 represents a mLAR cDNA in which the reverse transcription reaction started at the A-rich stretch within the 3' UTR.

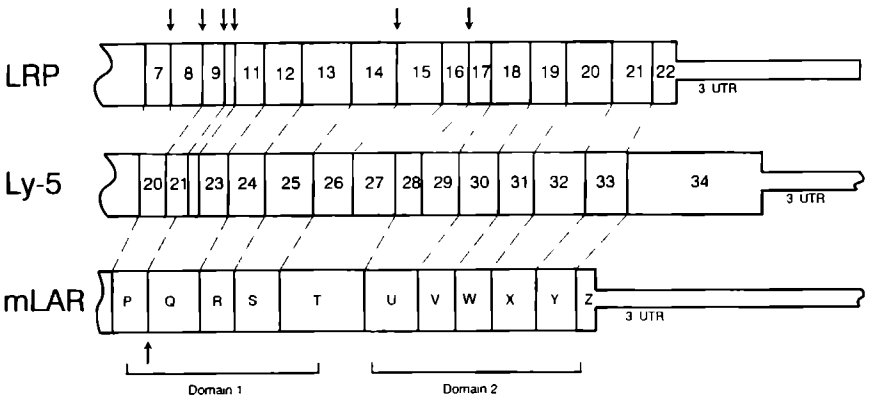


**Figure 3.** Comparison of exon segmentation as observed in the *Ptpra*, *Ptprc*, and *Ptprf* genes within the region encoding the phosphatase domains of RPTPases LRP (Wong *et al* , 1993), Ly 5 (Saga *et al* , 1988), and mLAR, respectively Dashed lines indicate identical boundaries Downward arrows indicate introns present in *Ptpra* and *Ptprc* but missing in the *Ptprf* gene The upward arrow points to an intron present in *Ptprf* but absent in *Ptpra* and *Ptprc* Exon numbers for LRP and Ly-5 are according to the literature

Southern blot analysis using cDNA clone mLAR#9 as a probe revealed that the sequence encoding the mLAR cytoplasmic region resided in a 6 5 kb *Sst*I-*Bam*HI genomic fragment. Sequence analysis showed both phosphatase domains to be encoded by eleven exons contained within a 4 5 kb *Eco*RI-*Bam*HI fragment (Fig. 1). The most 3' exon contains the last 22 codons for the protein and the entire 3'UTR of the mRNA The intron lengths in the gene segment shown in Fig 1 vary from only 80 bp up to 0 5 kb Comparison of the deduced organization of the *Ptprf* gene with that of the *Ptpra* and *Ptprc* RPTPase genes (encoding for LRP and Ly-5, respectively, Wong *et al.*, 1993, Saga *et al.*, 1988), revealed conspicuous differences (Fig. 3) Whereas the genomic organization of the first phosphatase domain is similar but not identical to the second domain in all three PTPase family members,

both mLAR phosphatase domains together are encoded by only eleven exons, in contrast to the seventeen (spanned within ~34 kb) and sixteen exons (spanned within ~27 kb) for LRP and Ly-5, respectively. This finding is at variance with the conclusions of Wong *et al.* (1993), who postulated that all RPTPases exhibit a similar genomic organization of the region encoding the phosphatase domains. Our results show clear differences in the gene structure of RPTPases. We propose that an ancestral gene originally contained a single tyrosine phosphatase domain encoded by at least nine exons. This may have been duplicated later to produce RPTPases with two phosphatase domains. Subsequently, several intron losses may have occurred for both the phosphatase domains as well as for the various types of RPTPases. One intron (between exon P and Q), still present in the *Ptprf* gene has been removed from the *Ptpra*/*Ptprc* ancestor. In the *Ptprf* gene, five introns have been removed that are still present in the other two RPTPase genes (Fig. 3). The reason why the gene structure of the *Ptprf* gene is so much more compact than other RPTPase genes remains, however, obscure for the moment.

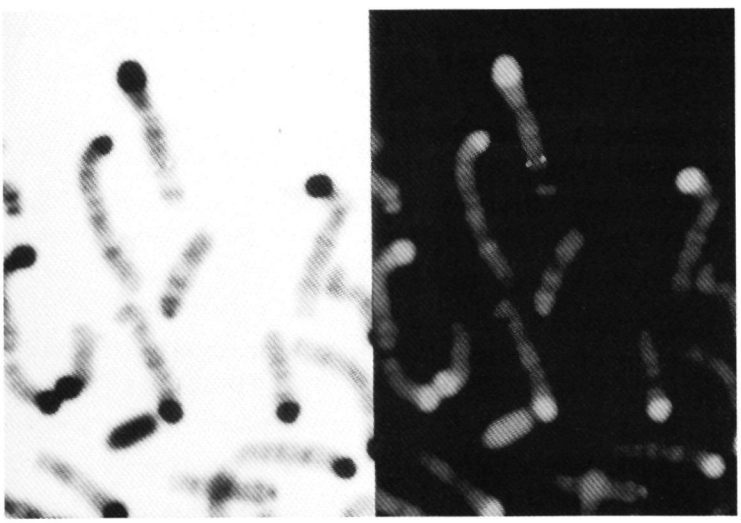

**Figure 4.** Chromosomal localization of the *Ptprf* gene.
Unique hybridizing signals of cosmid mLAR#5 were recorded in more than 30 metaphase spreads (right panel). Positively imaged chromosomes were counterstained with DAPI for the identification of the individual chromosomal sub-bands (left panel).

***Chromosomal localization of the Ptprf gene***

Mouse cosmid clone mLAR#5 was used as a probe for fluorescence *in situ* hybridization in order to determine the chromosomal localization of the *Ptprf* locus Alignment to G banded chromosome images showed the *Ptprf* gene to localize within the region C6-D1 of mouse Chromosome 4 (Fig 4) Based on data on evolutionary conservation of chromosome segments in man and mouse (Lyon and Kirby, 1993), this is in agreement with the localization of the PTPRF gene within the p32-33 region of human Chromosome 1 (Streuli *et al* , 1992) This localization is distinct from those of the *Ptprd* and *Ptprs* loci, which have been assigned to the region around the *b* locus on mouse Chromosome 4 (Mizuno *et al* , 1993) and distal mouse Chromosome 17 (Yan *et al* , 1993), respectively Together with RNA *in situ* hybridization data, which reveal different expression patterns for mLAR, MPTPδ, and mRPTPσ (R Q J Schaapveld, J T G Schepens, and W J A J Hendriks, unpublished results), these mapping data are in agreement with Mizuno *et al* (1993) and demonstrate that mLAR, MPTPδ, and mRPTPσ are indeed separate entities

***Homology to other receptor-like PTPases***

The mLAR cDNA sequence presented here is nearly identical to rat (Pot *et al* , 1993) and human LAR (Streuli *et al* , 1988) the overall homology at the amino acid level is 99 6% and 98 2%, respectively The homology (up to 90% at the amino acid level) to other type II RPTPases, MPTPδ and mRPTPσ (Mizuno *et al* , 1993, Ogata *et al* , 1994), which also contain two catalytic domains, a single transmembrane domain and, extracellularly, eight FN III and three Ig-like domains, is also striking Indeed, we also isolated clones representing MPTPδ and mRPTPσ by screening both the genomic and cDNA libraries at low stringency (not shown) Nucleotide sequence alignment of the phosphatase domains was used to gain insight into their evolutionary history A phylogenetic tree, as depicted in Fig 5, suggests that a common ancestor for PTPδ and RPTPσ diverged from the LAR lineage most probably before the time of mammalian radiation These results suggest that the mutation rate for LAR is lower than those of both PTPδ and RPTPσ, reflecting the maintenance of selective pressure on the LAR lineage and the adaptation to new roles in growth and development for PTPδ and RPTPσ

In addition, the homology between these three proteins is more conserved in phosphatase domain 2 than in domain 1 This is not seen for the other, more distant, members of the type II RPTPases (i e RPTPμ, RPTPκ), or among type IV (e g HPTPα HPTPε) and type V (e g HPTPζ, HPTPγ) RPTPases (Jiang *et al* , 1993, Krueger *et al* , 1990, Krueger and

Saito, 1992; Kaplan *et al.*, 1990). Further studies on the biological significance of each individual phosphatase domain in RPTPases are now in progress.

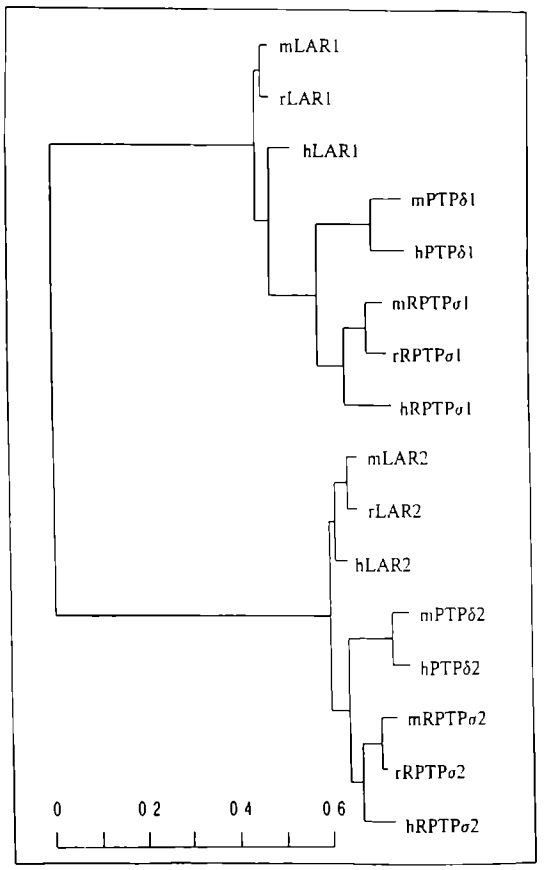


**Figure 5.** A proposed phylogenetic tree of the phosphatase domains 1 and 2 of the human, rat, and mouse PTPases LAR, PTPδ, and RPTPσ Nucleotide sequences encoding the region which starts immediately after the sequence DFWRM(I/V)W(E/D) and ends before the stretch HCSAG(V/I)GR were used for both domains of mLAR (accession number Z37988), rLAR (M60103), hLAR (Y00815), mPTPδ (D13905), hPTPδ (X54133), mRPTPσ (D28530), rPTPσ (A49104), and hRPTPσ (S78080) This unrooted tree was constructed on the basis of a distance matrix created according to the Kimura 2-parameter model (Kimura, 1980) with a transition/transversion ratio of 2, using the program FITCH and performing global rearrangements to verify the obtained topology Changes in sequence input order did not alter the tree topology The scale for branch lengths (bottom) represents the expected underlying number of changes per site

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# Chapter III

## Gene Targeting of the Receptor-like Protein Tyrosine Phosphatase LAR by Homologous Recombination in Mouse Embryonic Stem Cells

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# Gene Targeting of the Receptor-like Protein Tyrosine Phosphatase LAR by Homologous Recombination in Mouse Embryonic Stem Cells

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**Summary**

Receptor-like protein tyrosine phosphatases (RPTPases) comprise an extracellular ligand-binding region, a transmembrane domain, and as a rule two cytoplasmic tyrosine phosphatase domains *In vitro* studies using the cytoplasmic parts of RPTPases and artificial substrates have suggested that the first, membrane proximal phosphatase domain exhibits catalytic activity, whereas the second, C-terminal phosphatase domain may regulate the phosphatase activity of the first domain Further studies, however, are hampered by the fact that RPTPase-specific ligands and substrates still remain to be identified Moreover, the complexity of transmembrane signalling is difficult to mimick *in vitro* To circumvent these problems, the individual functions of the two phosphatase domains in RPTPases can be studied *in vivo* by means of homologous recombination in mouse embryonic stem (ES) cells Here, we decribe the use of 'double replacement' gene targeting in mouse embryonic stem cells to generate cell and animal models for studying the individual role of both phosphatase domains of the RPTPase Leukocyte common Antigen-Related molecule LAR In addition, exploiting the process of gene conversion, LAR-negative ES cells were generated to analyse structure-function relationships of LAR mutants on a null background

## Introduction

### *Receptor-like protein tyrosine phosphatases*

Protein-tyrosine phosphatases (PTPases) are the natural antagonists of the well-known protein tyrosine kinases (PTKs) and play an important role in the control of diverse cellular signalling pathways, cell growth, and differentiation (Fischer *et al.*, 1991; Tonks *et al.*, 1992; Mourey and Dixon, 1994). The identification of many distinct PTPase genes, and the discovery of multiple isoforms resulting from alternative splicing and post-translational modifications, have revealed a suprising degree of complexity in this multiprotein family. On the basis of their overall structures, two classes of PTPases can be distinguished, namely: (i) those found in the cytoplasm and the nucleus that have only one tyrosine phosphatase domain and (ii) membrane-bound, receptor-like PTPases (RPTPases) that have an extracellular ligand-binding region, a transmembrane domain, and, with a few exceptions, two repeated cytoplasmic phosphatase domains (Fig 1)

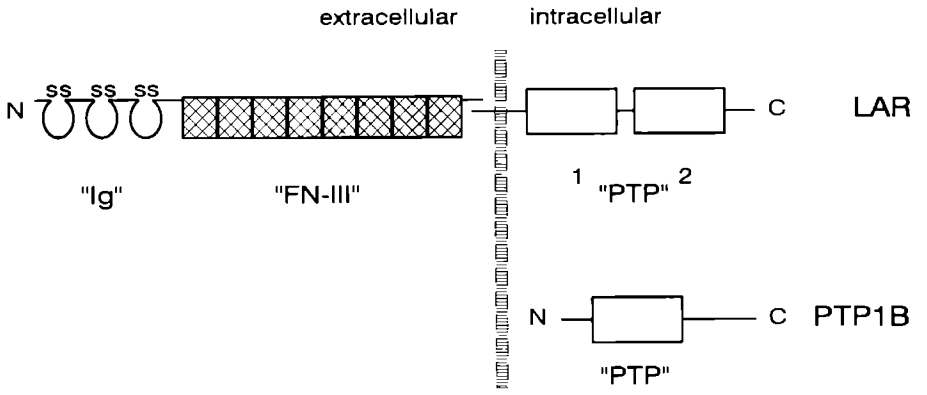



**Figure 1.** Structural organization of the two distinct PTPase classes, as illustrated by PTP1B (Tonks *et al* , 1988) and LAR (Streuli *et al* , 1988) Dashed lines indicate the plasmamembrane Ig, Immunoglobulin like domain, FN-III, fibronectin type III repeat; PTP, protein tyrosine phosphatase domain; N, amino terminus, C, carboxy-terminal end

One of the best studied RPTPases is the Leukocyte common Antigen Related molecule LAR (Streuli *et al* , 1988) LAR is composed of two cytoplasmic phosphatase domains, a transmembrane segment, and an extracellular region which shares homology to immunoglobulin (Ig)-like and fibronectin type III (FN-III) domains (Fig 1) Such an arrangement of these extracellular motifs is commonly found in cell adhesion molecules like N-CAM (Edelman and Crossin, 1991) It is tempting to speculate that LAR may play a role in cell adhesion, through ligand-mediated dephosphorylation of intracellular substrates

LAR is expressed on the cell surface as a complex of two non-covalently associated subunits derived from the same pro protein (Streuli *et al* , 1992, Yu *et al* , 1992) The ~200 kDa precursor is cleaved within the cell at a paired basic amino acid site by a subtilisin-like endoprotease to generate 150 kDa and 85 kDa fragments (Fig 1) The N linked glycosylated 150 kDa fragment represents the amino terminus of the protein, and is shed from the cell membrane during growth Whether this shedding has an effect on the intracellular phosphatase activity remains to be investigated The 85 kDa fragment contains the transmembrane segment and the two phosphatase domains of approximately 260 amino acid residues each *In vitro* site directed mutagenesis studies have suggested that the first phosphatase domain in RPTPases exhibits catalytic activity, for which a cysteine residue in the 'signature sequence' (I/V)HCXAGXXR(S/T)G is essential (reviewed in Mourey and Dixon, 1994) It is still a point of controversy whether the second, C-terminal phosphatase domain has only a regulatory function in modulating the substrate specificity of the first domain (Streuli *et al* , 1990, Pot *et al* , 1991, Krueger and Saito, 1992), or has catalytic activity by itself (Wang and Pallen, 1991, Tan *et al* , 1993) This problem is difficult to address because of the complexity of transmembrane signalling Furthermore, extracellular ligands and downstream substrates, that are specific for a single RPTPase, are currently not known Therefore, we set out to study the loss of function of the two individual phosphatase domains of LAR *in vivo* by means of gene targeting using homologous recombination in mouse embryonic stem (ES) cells

***Gene targeting by homologous recombination***

Gene targeting, the homologous recombination of chromosomal DNA sequences with newly introduced DNA sequences (Thomas and Capecchi, 1987), is now widely used to study gene function *in vivo* (for review see Koller and Smithies, 1992) Pluripotent ES cells, containing the desired genomic alteration, are microinjected into blastocysts and

subsequently transferred into pseudo-pregnant foster mothers These ES cells can contribute to the germline of the resulting chimaeric mice, which then can transmit the mutated allele to their offspring In this way, mutations can be transferred from the culture dish to the whole animal Subsequent inbreeding can give rise to mice carrying the mutation in both alleles, allowing the analysis of the mutation in the heterozygous and homozygous state

Thusfar, gene inactivation is the major application of gene targeting, and more than a hundred different so-called knock out mice have been created and studied Since phenotypic alterations in null mutants are often very complex or embryonically lethal, gene function may be better understood by introducing subtle mutations in functional domains of a protein while leaving the remainder of the protein intact To date, three procedures have been described to introduce specific mutations in the genome, namely (i) the 'hit and run' or 'in-out' procedure (Hasty *et al* , 1991, Valancius and Smithies, 1991), (ii) a one-step recombination strategy using the Cre *lox*P recombination system (Gu *et al* , 1993), and (iii) the 'double replacement', 'two-step', or 'tag-and exchange' strategy (Askew *et al* , 1993, Gondo *et al* , 1994, Stacey *et al* , 1994, Wu *et al* , 1994) This latter procedure requires two rounds of homologous recombination using replacement type targeting vectors In the first step, a cassette containing both a positive and a negative selectable marker is introduced into the gene locus to mark the region of interest by selection for the positive selectable marker In the second step, using the negative selectable marker to enrich for recombinants, the cassette is replaced again by the original endogenous sequences carrying any desired subtle mutation (see also Fig 2) This procedure can be used to generate cell lines and subsequently mice in which either the LAR phosphatase domains 1 or 2, or both, are inactivated

## Materials & methods

***'Double replacement' targeting at the LAR locus***

The isolation and characterization of mouse 129 genomic LAR sequences were described previously (Schaapveld *et al* , 1995) A 7 5 kb *Bam*HI-*Eco*RI fragment just upstream of the exons encoding the cytoplasmic phosphatase domains was subcloned into pBluescript KS+ (Stratagene) From this, the 3' segment was subcloned as a 3 2 kb *Pst*I *Sal*I fragment into a modified pGEM3 vector having a *Xho*I linker inserted in the *Sma*I site, resulting in pLAR5' From the 4 5 kb

*Bam*HI clone just 448 bp downstream the stop-codon of LAR, the 5' 2 3 kb *Bam*HI-*Bgl*II fragment was subcloned into the *Bam*HI-site of pLAR5', generating pLAR5'-3' Finally, a hygromycin phosphotransferase/thymidine kinase fusion gene (HyTK, Lupton *et al.*, 1991) or a neomycin phosphotransferase expression cassette (Neo, van Deursen *et al* , 1991) were inserted as a *Xho*I fragment into the *Sal*I-site of pLAR5'-3' for step 1 or step 2, respectively Prior to electroporation, targeting constructs (pLARHyTK or pLARNeo) were linearized using the unique *Xho*I-site from the introduced linker

E14 ES cells were cultured on irradiated SNLH9 feeder cells as described (van Deursen and Wieringa, 1992) Exponentially growing ES cells were collected and mixed with 20 μg of linearized pLARHyTK (step 1) or pLARNeo (step 2), electroporated at 250 V/500 μF (Gene Pulser, Biorad), and plated at $5.10^6$/9 cm dish onto irradiated feeder cells Selection was applied 24 h later using 300 μg/ml Hygromycin B (HygroB, ICN) for step 1 or 300 μg/ml G418 (Gibco/BRL) whether or not in combination with 0.2 μM 1-[2 deoxy, 2-fluoro-ß-D-arabinofuranosyl] 5-iodo-uracil (FIAU, Bristol Myers Squibb) for step 2 After 9-11 days of selection, drug resistant ES clones were picked, expanded, and used for genotyping

***Production of homozygous mutant ES cells***

$HygrB^r/G418^r/FIAU^s$ ES clones were plated at 1.105 cells/9 cm dish onto 0 1 % gelatin in the presence of Leukemia Inhibiting Factor (LIF, 1,000 U in CHO conditioned medium) to keep them pluripotent Selection was applied 24 h later using 0-2 mg/ml G418 in combination with 0-0 2 μM FIAU After 8-13 days of selection, ES clones were picked and expanded for further analysis

***Genotyping***

Genomic DNA from individual ES cell clones was extracted and analyzed by Southern blot analysis as described (Steeghs *et al.*, 1995). Initial screening was performed using a 3'-flanking probe (a *Sau*3A fragment derived from a 2 5 kb *Bam*HI fragment just downstream of the 4.5 kb *Bam*HI clone mentioned above) Positive clones were also analyzed with a 5'-flanking probe (a 1 0 kb *Bam*HI-*Xba*I fragment representing the 5' end of the 7.5 kb *Bam*HI-*Eco*RI fragment mentioned above), a 1 8 kb *Eco*RI-*Hin*dIII fragment from the HyTK cassette, a 0.8 kb *Pst*I fragment from the Neo cassette, and a 1 8 kb *Bam*HI mouse LAR cDNA fragment comprising all exons encoding both cytoplasmic phosphatase domains (pos. 420-2202, Schaapveld *et al.*, 1995) to check for proper homologous recombination.

***RNA analysis***

A RT-PCR reaction using LAR-specific primers was designed to check for expression of both LAR phosphatase domains in wildtype (+/+), heterozygous (+/-), and homozygous mutant (-/-) ES cells Total RNA from the diverse clones was isolated using RNAzol B (Cinna/Biotecx),

according to the manufacturers protocol 2 μg of RNA was reverse transcribed using random hexamers (2 μg, Pharmacia) and Superscript reverse transcriptase (100 U, Gibco/BRL) One-tenth of the cDNA was subsequently analyzed by PCR (Hoffmann-La Roche) using *Taq* polymerase and the LAR-specific primers LAR5s (5'-GAG CTG CGT CAG TTC CA-3', sense), located 129 bp upstream of the codon for the conserved cysteine residue in the first phosphatase domain and LAR2as (5'-GAC TTG CTC AGG TAG GGC-3', antisense), located 532 bp downstream of the stop-codon of LAR, resulting in a 1,745 bp amplicon Each cDNA preparation was tested with a ß-Actin PCR to ensure the integrity of the cDNA synthesis The ß-Actin primers (1) 5' GCT AT/CG AGC TGC CTG ACT G 3' (sense) and (2) 5' GAG GCC AGG ATG GAG CC-3' (antisense) detect a 338 bp amplicon The PCR cycle profile consisted of 94°C for 1 min, 50°C for 1 min (ß-Actin 58°C) and 72°C for 3 min (ß-Actin 1 min), for 35 cycles, and was performed in a programmable thermocycler (Perkin-Elmer Cetus) Each 50 μl reaction contained 10 mM Tris-HCl (pH 8,3), 50 mM KCl, 2 5 mM $MgCl_2$ (ß-Actin 1 5 mM $MgCl_2$), 0 01% BSA, 1 mM dNTPs, 50 ng of each primer, and 1 U *Taq* polymerase In addition to template minus (-cDNA) controls, reverse transcriptase-minus (-RT) controls were included One-fifth of the reaction products was separated on 1% (LAR) or 2% (ß-Actin) agarose gels and visualized by ethidium bromide staining

To ensure LAR double knock-out ES cells were really negative, the LAR-specific PCR products were transferred to nylon membrane (Biotrace, Gelman Sciences) in 0 4 N NaOH and analyzed by hybridization to the mLAR cDNA probe encompassing the exons encoding both phosphatase domains

***Generation of a LAR polyclonal antiserum***

The 1 8 kb *Bam*HI LAR cDNA fragment was subcloned into the bacterial expression vector pGEX-3X (Pharmacia) The construct was introduced into *E coli* strain DH5α and expression of fusion protein was induced by 0 1 mM IPTG The expressed fusion protein was isolated using standard protocols (Sambrook *et al* , 1989) followed by preparative SDS-PAGE and electroelution (Biotrap, Schleicher & Schuell) A New Zealand white rabbit was immunized intraperitoneally with ~250 μg fusion protein in the presence of Freund's complete adjuvants and boosted for three times (~250 μg fusion protein with incomplete Freund's adjuvants) with three weeks intervals Two weeks after the last boost serum (αLAR-cyt) was collected

***Immunoprecipitation and immunoblotting***

Wildtype and double knock-out ES cells were plated at 2 $10^5$/well on a 6-well dish 48 h later cells were washed with PBS containing 1 mM $CaCl_2$, incubated for 10 min on ice in 1 ml NP-40 lysis-buffer (50 mM Tris-HCl, pH 8 0, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 1 mM PMSF, 1 mM $Na_3VO_4$, and 10 mM NaF), and scraped After centrifugation, the supernatants were precleared with CL2B-Sepharose beads (Pharmacia)

and incubated O/N with αLAR-cyt (1:100) and GammaBind plus Sepharose beads (Pharmacia) Immune complexes were washed four times with modified lysis-buffer (0.1% NP-40), transferred to a new tube, and washed one more time Immunoprecipitates were released from the beads by boiling for 5 min in SDS sample buffer, resolved on a 8% SDS-PAGE gel, and blotted to a nitrocellulose filter The immunoblot was blocked for 1 h with 1% BSA (Sigma) in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20), probed O/N with αLAR-cyt (1:1000) in TBST, washed three times with TBST, incubated for 1 h with Prot A/G-PO (1:10,000, Pierce), washed again, and developed using chemiluminiscence (NEN-DuPont)

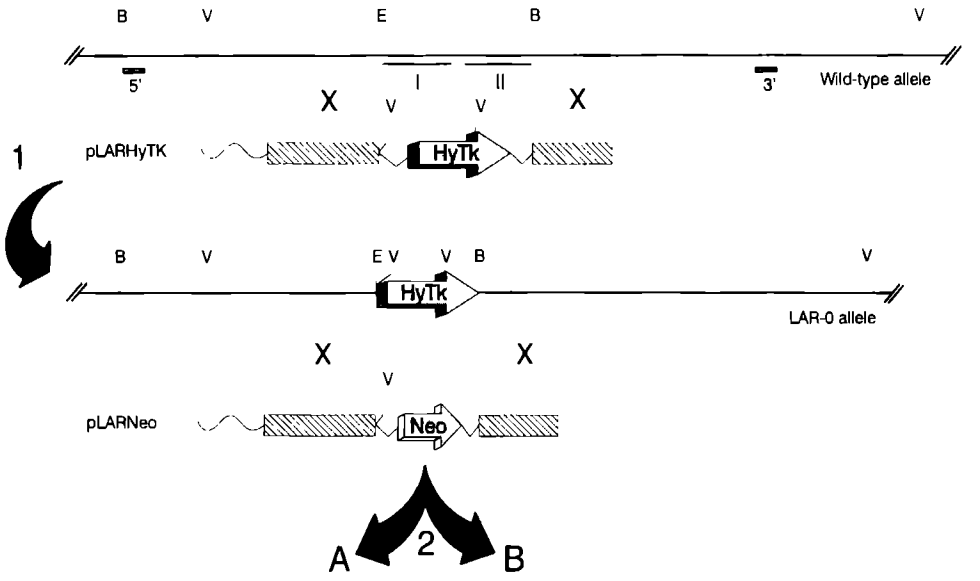
## Results

### *Targeting of the RPTPase LAR gene*

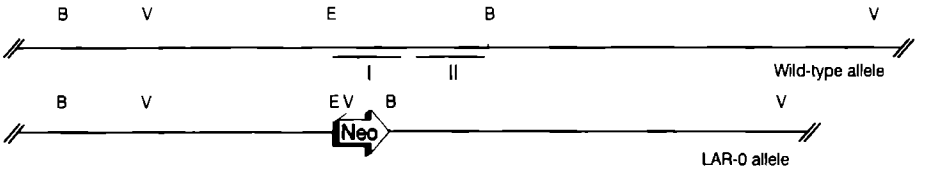
The relevant segments of the structural and genomic murine LAR DNAs were isolated using a human LAR cDNA clone as a probe (Schaapveld *et al.*, 1995) A replacement-type targeting vector was constructed in which a dominant positive/negative selection marker, a hygromycin phosphotransferase-thymidine kinase (HyTK) fusion gene (Lupton *et al.*, 1991), was flanked by 3.2 and 2.3 kb of endogenous LAR genomic segments to provide the necessary homology for targeted integration Upon homologous recombination, the HyTK cassette replaces a 4.5 kb genomic segment containing all exons encoding the cytoplasmic part of LAR (Fig 2, step 1)

**Figure 2.** Disruption of the LAR gene by 'double replacement' targeting

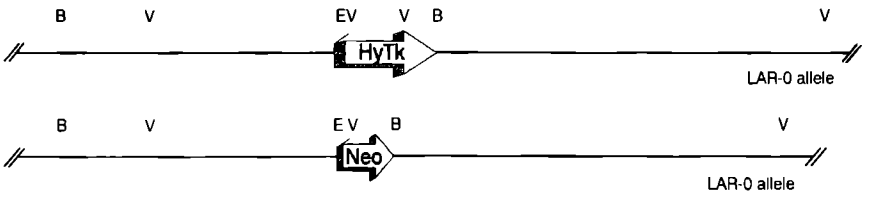
A recombination event replaces the genomic sequences comprising both phosphatase domains by the HyTK fusion cassette (step 1) The resulting HygroB$^r$/FIAU$^s$ homologous recombinant cell line is then used in a second round of targeting (step 2) in which either the HyTK fusion cassette (**A**) or the LAR phosphatase domains of the remaining wild-type allele (**B**) are replaced by the Neo gene Hatched bars represent the endogenous LAR genomic segments and the grey arrows symbolize the selection cassettes Small grey bars indicate the 5'- and 3'-diagnostic probes, and the solid bars I and II mark the genomic segments encoding the phosphatase domains 1 and 2, respectively. B, *Bam*HI; V, *Eco*RV; E, *Eco*RI




A: REPLACEMENT




B: DOUBLE KNOCK-OUT



The targeting construct was introduced into E14 ES cells by electroporation and 272 Hygromycin B resistant (HygroB$^r$) clones were screened for homologous recombination by Southern blot analysis using 5'- and 3'-diagnostic probes derived from genomic sequences flanking the targeting vector region. Using the 3'-probe, ten clones were found to display the diagnostic *Eco*RV fragment, indicative of a targeting event. Eight out of the ten clones also revealed the proper *Bam*HI digestion pattern with the 5'-probe, demonstrating correct homologous recombination at both ends (Fig. 3). Unfortunately, these cell lines were unsuitable for germline transmission, probably due to high expression of the thymidine kinase fusion protein in germ cells (Braun *et al.*, 1990; Ramirez-Solis *et al.*, 1993). However, they can serve as starting cell lines for the introduction of inactivating point mutations in the phosphatase domains by a second recombination step.

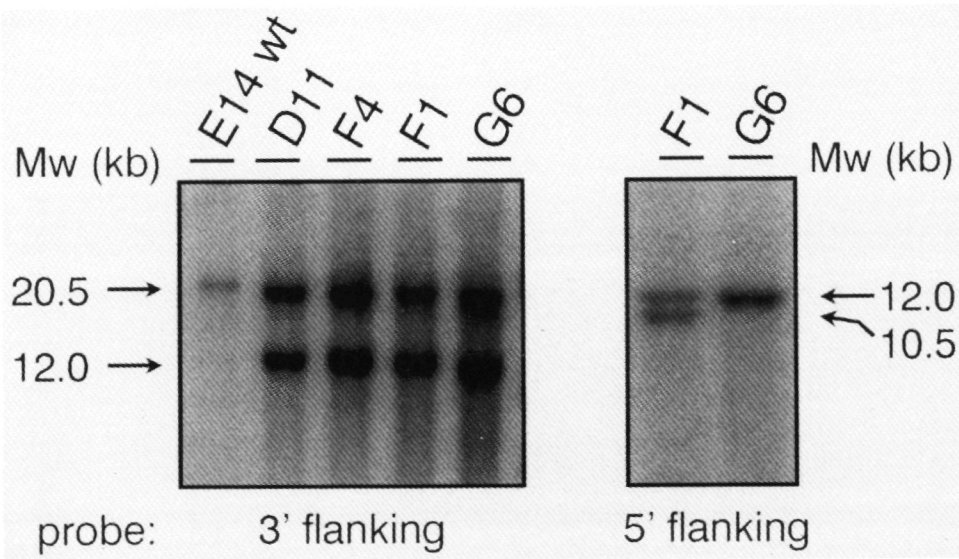



**Figure 3.** Screening for homologous recombinants.
Southern blot autoradiograms of ±10 μg genomic DNA from individual HygroB$^r$ ES clones. The 3'-flanking probe reveals the diagnostic 12.0 kb fragment in *Eco*RV digested DNA (left panel), indicative of targeting at the LAR locus. Use of a 5'-flanking probe on *Bam*HI digested DNA (right panel) is necessary to confirm correct homologous recombination (clone G6 is a result of an integration event at the LAR locus).

To test the feasability of this 'double replacement' strategy and to obtain germline transmission of a LAR null allele for the generation of LAR knock-out mice, one of the targeted ES cell lines (E14.F4) was again transfected, but now with a gene targeting vector in which all exons encoding the cytoplasmic part of LAR were replaced by the neomycin phosphotransferase (Neo) gene (Fig 2, step 2) Applying different combinations of the selective agents HygroB, G418 and 1-[2 deoxy, 2-fluoro-ß-D-arabinofuranosyl] 5-iodo-uracil (FIAU) resulted in 1) $G418^r/FIAU^r$ ES cells, in which the HyTK cassette is exchanged for the Neo cassette, and 2) $G418^r/HygroB^r$ ES cells, in which both LAR alleles are targeted (Table 1, Fig 4) Highly chimaeric mice were generated upon injection into blastocysts of either 'replacement' clones or 'double knock-out' clones Chimaeras resulting from the replacement clones were tested in a breeding program and germline transmission of the desired genotype has been obtained (Schaapveld *et al* , manuscript in preparation), demonstrating that a second round of homologous recombination in the E14 F4 cell line is possible without affecting the germline competence This opens the way for the introduction of inactivating point mutations in each of the two LAR phosphatase domains using a replacement-type targeting vector carrying the mutated genomic segment

**Table 1.** Targeting of the LAR gene in E14 F4 cells (step 2)

| Selection | # clones screened | Targeting events | Homologous recombinants |
|---|---|---|---|
| G418 & HygroB | 185 | 3 | 1 (DK) |
| G418 | 189 | 2 | 2 (DK & RC) |
| G418 & FIAU | 17 | 2 | 2 (RC) |

DK = Double Knock out RC = Replacement Clone

As mentioned above, cell lines were also obtained in which both LAR alleles were inactivated as a result of the second replacement step Such a double knock-out cell line may be a useful tool to assess the function of a gene, especially when the mutation is lethal in early development Other groups have used double knock-out cell lines, generated by two consecutive rounds of targeting with two different vectors, to study

genes involved in cell growth and differentiation (te Riele *et al.*, 1990; Mortensen *et al.*, 1991).

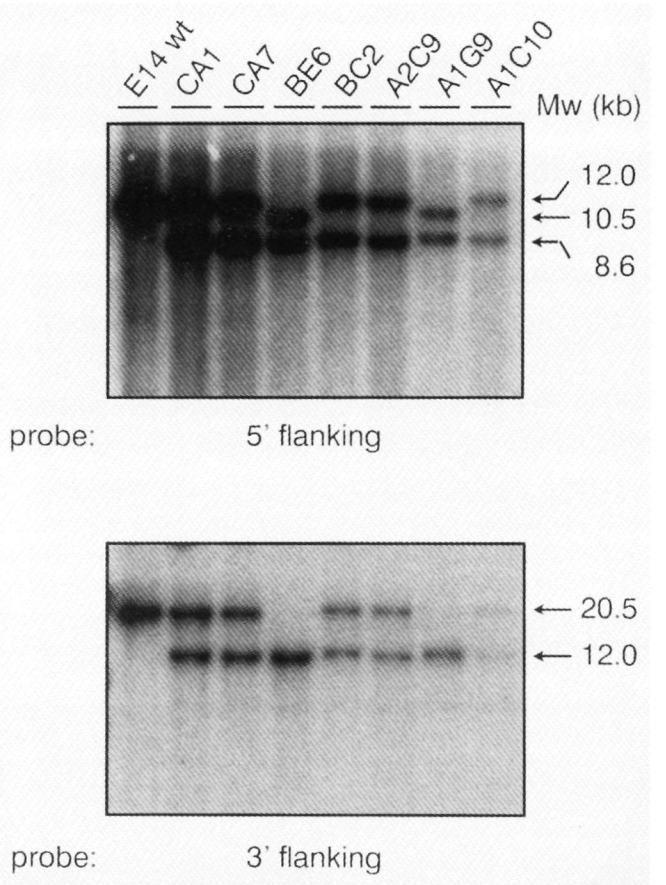



**Figure 4.** Detection of 'replacement' and 'double knock-out' clones.
Southern blot autoradiograms of $\pm 10$ $\mu$g genomic DNA from individual clones resulting from the second round of targeting in the E14.F4 cell line (see Fig. 3). In DNA digested with *Bam*HI (upper panel), the 5'-flanking probe reveals the diagnostic 8.6 kb and/or 10.5 kb fragments indicating targeting at the LAR locus. Replacement (BC2, CA1, and CA7) and double knock-out (A1G9, and BE6) cell lines are shown. Use of a 3'-flanking probe on *Eco*RV digested DNA (lower panel) reveals the 12 kb diagnostic fragment for both events. Screening with HyTK and Neo probes was necessary to discriminate between integration (A1C10, and A2C9) and recombination events (data not shown).

***Production of LAR double knock-out ES cells***

Recently, a method has been described that requires only a single targeting construct to generate double knock-out cell lines (Mortensen et al , 1992) It is based on the phenomenon that cells heterozygous for a given locus can be rendered homozygous by mitotic recombination or gene conversion Since cells bearing two copies of a Neo gene are more resistant to G418, homozygous cells can be generated from a heterozygous cell line (containing only one copy of the Neo gene) by culturing in sublethal concentrations of G418 We tested this approach by taking advantage of the presence of the HyTK fusion cassette and the Neo gene in the LAR double knock out HygroB$^r$/G418$^r$/FIAU$^s$ cell line, E14 BE6 This cell line was cultured not only in the presence of a high G418 concentration, but also in the presence of FIAU As a result, only cells that mutate or delete the HyTK cassette and at the same time increase their neomycin phosphotransferase levels will survive Gene conversion resulting in the replacement of the HyTK allele by a copy of the Neo allele will have both these effects In this way, we were able to generate double knock-out cell lines containing two copies of the Neo gene (Table 2, Fig 5)

**Table 2.** Use of high G418 concentrations to generate homozygous mutant ES cells

| Selection | | # Clones | % Homozygous |
|---|---|---|---|
| G418 (mg/ml) | FIAU ($\mu$M) | picked | (# analyzed) |
| 1 0 | 0 2 | 19 | 14 (7) |
| 1 5 | 0 2 | 15 | 16 (12) |
| 2 0 | 0 2 | 2 | - (0) |

Next we investigated the consequences of double replacement gene targeting and gene conversion at the RNA level in these double knock out cell lines Total absence of LAR transcripts containing sequences encoding both phosphatase domains was analyzed by RT-PCR using mouse LAR specific primers, followed by Southern blot analysis using a mouse LAR cDNA probe encoding both phosphatase domains Double knock-out cell lines containing two copies of the Neo gene (C1) or one copy of the HyTK fusion cassette and one copy of the Neo gene (BE6) show total absence of LAR mRNA, whereas heterozygote cell lines containing only one copy of the Neo gene (CA1) or only one copy

of the HyTK fusion cassette (F4) still show expression of LAR (Fig. 6).

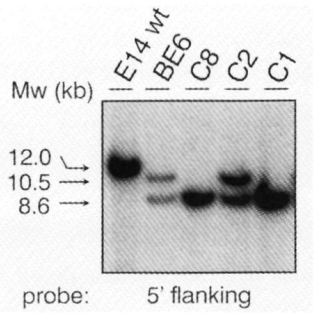



**Figure 5.** Detection of gene conversion events in LAR-negative ES cells.
Southern blot autoradiograms of $\pm 10$ $\mu$g genomic DNA from individual clones surviving high concentration of G418. In DNA digested with *Bam*HI the 5'-flanking probe reveals the two diagnostic 8.6 kb fragments indicating loss of the HyTK cassette by gene conversion using the Neo allele as template (cell lines C1, and C8).

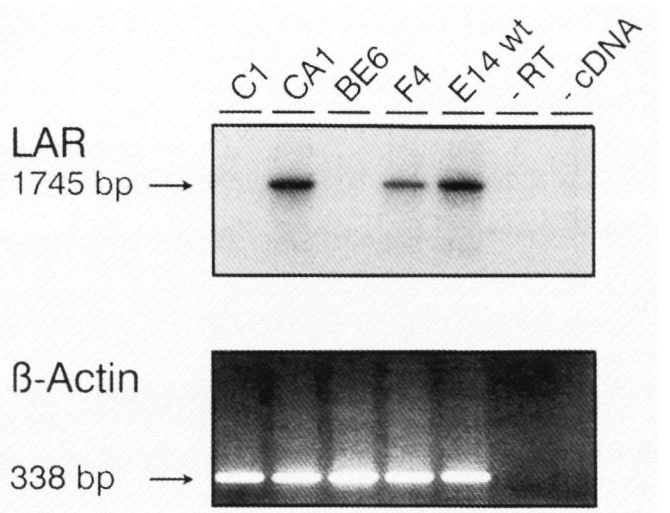



**Figure 6.** Detection of RNA expression in LAR-negative ES cells.
RT-PCR analysis followed by Southern blot analysis of individual clones generated by the various ways described above. Double knock-out cell lines (C1, and BE6) show complete absence of LAR expression (upper panel). RT-minus and cDNA-minus controls were included to show specificity of the RT-PCR. ß-Actin primers were used as a control for the cDNA synthesis (ethidium bromide picture, lower panel). Amplicon sizes are shown on the left.

To confirm the absence of the LAR phosphatase domains at the protein level, protein lysates of the double knock-out cell lines were prepared, and analyzed by immunoprecipitation followed by Western blot analysis. A rabbit polyclonal antiserum raised against a GST-LAR fusion protein containing both phosphatase domains failed to detect the 85 kD subunit of LAR (Streuli *et al.*, 1992) in the double knock-out cell line (Fig. 7). This demonstrates the efficient inactivation of both LAR alleles in ES cells by initially targeting a single allele followed by selection of double knock-outs.

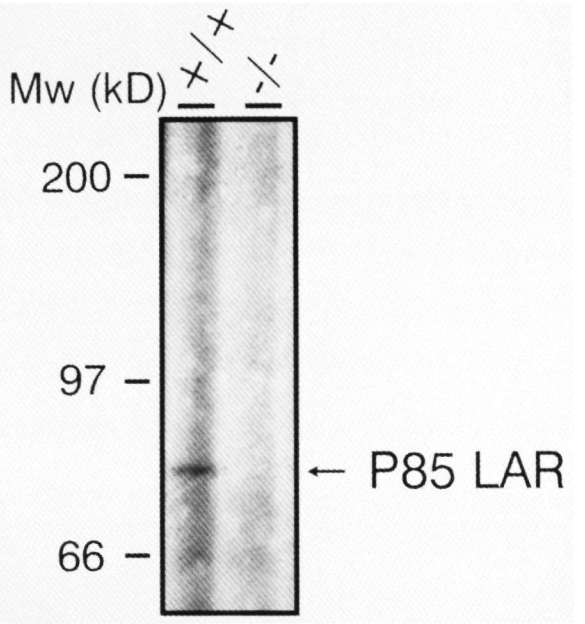



**Figure 7.** Absence of the LAR phosphatase doamins in double knock-out ES cells.
Protein lysates of wildtype (E14, +/+) en double knock-out (C1, -/-) ES cells were prepared and analyzed by immunoprecipitation followed by Western blot analsyis, using a rabbit polyclonal antiserum against both phosphatase domains of mouse LAR. P85 LAR represents the 85 kD subunit of LAR containing both phosphatase domains. Protein size markers are indicated on the left.

**In Conclusion**

Using replacement-type targeting vectors we have shown various ways of generating cell and animal models to study the role of the RPTPase LAR in signal transduction. Firstly,

ES cell lines lacking the gene sequences essential for LAR phosphatase activity were generated using a double replacement strategy involving two subsequent homologous recombination events. Secondly, the feasability of selecting for gene conversion events to mutate the remaining allele was confirmed. Thirdly, the E14.F4 line that resulted from the first targeting step involving the HyTK positive/negative selection cassette was succesfully used for a second round of homologous recombination without the loss of germline competence.

We have observed homologous recombination events involving the region that spans both phosphatase domains of LAR at an average frequency of about 1 in 50 clones for both steps 1 and 2 An insertion rather than a replacement mutation was seen in 20% of our clones. In agreement with others, this underlines the necessity of using probes not only from both sides of the targeted locus, but also probes for the selectable markers and the deleted region, to screen for genuine homologous recombination events However, this frequency of illegtimate targeting events may not be general and might depend on intrinsic properties of the locus under study.

Future experiments will be aimed at the reconstitution of the LAR-negative ES cell line with wild-type human LAR as well as with several mutants Generated cell lines can then be used to study the role of LAR in cell proliferation and differentiation by analyzing, for example, their cell growth properties, phosphotyrosine protein patterns, and the LAR phosphatase activity itself during ES cell differentiation The strategies and techniques used here to create $LAR^{-/-}$ models are generally applicable and provide a unique means to study many 'orphan' signal transduction molecules

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# Chapter IV

## Impaired Mammary Gland Development and Function in Mice Lacking LAR Receptor-like Tyrosine Phosphatase Activity

Roel Q.J Schaapveld, Jan T.G. Schepens, Gertraud W. Robinson, Joline Attema, Frank T.J.J. Oerlemans, Jack A.M. Fransen, Michel Streuli, Bé Wieringa, Lothar Hennighausen, and Wiljan J A.J Hendriks

*Developmental Biology,* in press (1997)

## Impaired Mammary Gland Development and Function in Mice Lacking LAR Receptor-like Tyrosine Phosphatase Activity

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**Summary**

The LAR receptor-like protein tyrosine phosphatase is comprised of two intracellular tyrosine phosphatase domains and a cell adhesion molecule-like extracellular region containing three immunoglubulin-like domains in combination with eight fibronectin type-III-like repeats This architecture suggests that LAR may function in cellular signalling by the regulation of tyrosine phosphorylation through cell cell or cell-matrix interactions We used gene targeting in mouse embryonic stem cells to generate mice lacking sequences encoding both LAR phosphatase domains Northern blot analysis of various tissues revealed the presence of a truncated LAR mRNA lacking the cytoplasmic tyrosine phosphatase domains and indicated that this LAR mutation is not accompanied by obvious changes in the expression levels of one of the LAR-like receptor tyrosine phosphatases PTPδ or PTPσ LAR$^{-/-}$ mice develop and grow normally and display no appreciable histological tissue abnormalities However, upon breeding we observed an abnormal neonatal death rate for pups from LAR$^{-/-}$ females Mammary glands of LAR$^{-/-}$ females were incapable of delivering milk due to an impaired terminal differentiation of alveoli at late pregnancy As a result, the glands failed to switch to a lactational state and showed a rapid involution postpartum In wildtype mice, LAR expression is regulated during pregnancy reaching maximum levels around day 16 of gestation Taken together, these

findings suggest an important role for LAR mediated signalling in mammary gland development and function

**Introduction**

Protein tyrosine phosphorylation, a key event in signal transduction, is a post-translational modification controlled by the agonistic or antagonistic action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases, Mourey and Dixon, 1994, Sun and Tonks, 1994) The PTPases comprise a diverse family of receptor like and cytoplasmic-type enzymes including multiple isoforms resulting from alternative splicing and post translational modifications (for review see Saito, 1993)

Receptor-like PTPases (RPTPases) contain one or two homologous PTPase domains, a transmembrane segment, and diverse combinations of domains with possible ligand-binding properties in the extracellular part For example, the closely related RPTPases RPTP$\mu$ and PTP$\kappa$ mediate cell-cell interactions in a homophilic, but not heterophilic manner (Brady Kalnay *et al* , 1993, Gebbink *et al* , 1993, Sap *et al* , 1994, Zondag *et al* , 1995) In RPTP$\beta$ the extracellular moiety binds to a non-related cell-surface protein, the neuronal recognition molecule contactin (Peles *et al* , 1995) It has not been clarified if and how such interactions modulate tyrosine phosphatase activity and whether RPTPases play a role early in signal transduction or later in adaptation or response cessation (Ninfa and Dixon, 1994)

Although LAR was among the first RPTPases to be identified (Streuli *et al* , 1988) and has been studied extensively at the cellular level, its physiological function remains elusive LAR is composed of two cytoplasmic phosphatase domains in tandem, a transmembrane segment, and an extracellular part which shares homology to immunoglobulin (Ig) like and fibronectin type III (Fn-III) domains, commonly found in cell adhesion molecules (Edelman and Crossin, 1991) This architecture suggests a physiological function for LAR in cellular signalling by tyrosine dephosphorylation as a response to cell-cell or cell-matrix interactions Recently, an intracellular LAR-interacting protein (LIP 1) was identified, which binds to the second PTPase domain and colocalizes with LAR to the ends of focal adhesions most proximal to the cell nucleus, suggesting a role for LAR in disassembly of focal adhesions (Serra Pages *et al* , 1995)

LAR has a broad tissue distribution, and is expressed on the cell surface as a complex of two noncovalently associated subunits of 150 kDa and 85 kDa resulting from cleavage

of the pro-protein (Streuli *et al.*, 1992; Yu *et al.*, 1992) The N-linked glycosylated 150 kDa fragment is shed during growth Whether this shedding, which is PMA-inducible (Serra-Pagès *et al.*, 1994), has an effect on the intracellular phosphatase activity remains to be established The 85 kDa fragment contains a short ectodomain, the transmembrane segment, and the two PTPase domains of ~280 amino acid residues each Two closely-related family members do exist that show a similar intracellular processing and cell surface expression, namely the LAR-like RPTPases PTPδ and PTPσ (Pulido *et al*, 1995a; Yan *et al.*, 1993)

*In vitro* site-directed mutagenesis studies suggested that the first, membrane-proximal phosphatase domain of LAR exhibits catalytic activity whereas, the second, membrane distal phosphatase domain may have only a regulatory function in modulating the substrate specificity of the first domain (Pot *et al.*, 1991; Streuli *et al.*, 1990). However, the extracellular ligand(s) and downstream substrate(s) of LAR have not been identified Therefore, we set out to study the consequences of the loss of LAR PTPase function in vivo by means of gene targeting using homologous recombination in mouse embryonic stem (ES) cells. Characterization of the resulting LAR$^{-/-}$ mice points to a role for LAR PTPase activity in mammary gland development during pregnancy

## Materials & Methods

### *Generation of LAR deficient mice*

The isolation and characterization of mouse 129 genomic LAR sequences were described previously (Schaapveld *et al.*, 1995) A 7 5 kb *Bam*HI-*Eco*RI fragment just upstream of the exons encoding the cytoplasmic phosphatase domains was subcloned into pBluescript KS+ (Stratagene) From this, the 3' segment was subcloned as a 3.2 kb *Pst*I-*Sal*I fragment into a modified pGEM3 vector having a *Xho*I-linker inserted in the *Sma*I-site, resulting in pLAR5' From the 4 5 kb *Bam*HI clone just 448 bp downstream the stop-codon of LAR, the 5' 2 3 kb *Bam*HI-*Bgl*II fragment was subcloned into the *Bam*HI-site of pLAR5', generating pLAR5'-3'. Finally, a neomycin phosphotransferase expression cassette (Neo) (van Deursen *et al*, 1991) was inserted into the *Sal*I-site of pLAR5'-3'. Prior to electroporation, the targeting construct (pLARNeo) was linearized using the unique *Xho*I-site from the introduced linker

E14 ES cells were cultured on irradiated SNLH9 feeder cells as described (van Deursen and Wieringa, 1992). Exponentially growing ES cells were collected and mixed with 20 μg of linearized pLARNeo, electroporated at 250 V/500 μF (Gene Pulser, Biorad), and plated at 5 x $10^6$

per 9 cm dish onto irradiated feeder cells Selection was applied 24 hours later using 300 μg/ml G418 (Gibco/BRL) After 9-11 days of selection, G418 resistant ES clones were picked and expanded

Genomic DNA from individual ES cell clones was extracted and analyzed by Southern blot analysis as described (Steeghs *et al* , 1995) Initial screening was performed using a 3'-flanking probe (a *Sau*3A fragment derived from a 2 5 kb *Bam*HI fragment just downstream of the 4 5 kb *Bam*HI clone mentioned above) Positive clones were also analyzed with a 5' flanking probe (a 1 0 kb *Bam*HI-*Xba*I fragment representing the 5' end of the 7 5 kb *Bam*HI-*Eco*RI fragment mentioned above), a 0 8 kb *Pst*I fragment from the Neo cassette, and a 1 8 kb *Bam*HI mouse LAR cDNA fragment comprising all exons encoding both cytoplasmic phosphatase domains (Schaapveld *et al* , 1995) to check for proper homologous recombination

Three correctly targeted ES cell clones that also displayed the correct number of 40 chromosomes (CA1, CA7, and BC2) were used for injection into C57BL/6 recipient blastocysts and implanted into the uterine horns of pseudopregnant (C57BL/6 x CBA/Ca)F1 foster mothers (Bradley, 1987) Resulting male chimaeras were mated with C57BL/6 females to check for germline transmission DNA obtained from tail biopsies of agouti offspring and subsequent generations was screened for the presence of the mutant LAR allele by Southern blot analysis and/or polymerase chain reaction (PCR, Hoffmann-La Roche)

***Genotyping***

A PCR reaction using four primers was designed to discriminate between wildtype ($LAR^{+/+}$), heterozygous ($LAR^{+/-}$), and homozygous mutant ($LAR^{-/-}$) mice The wildtype allele was detected as a 542 bp amplicon using the primers LAR5s (5'-GAG CTG CGT CAG TTC CA-3', sense), located 129 bp upstream of the codon for the conserved cysteine residue in the first phosphatase domain, and LAR4as (5' GAC AGG CTT CCC ACG AC-3', antisense), a genomic primer located 410 bp downstream of the same cysteine codon The mutant allele was detected as a 394 bp amplicon using the primers Neo3' (5'-CTA TCG CCT TCT TGA CGA GTT 3', sense), located 25 bp upstream of the stop codon in the Neo cassette, and LAR2as (5' GAC TTG CTC AGG TAG GGC 3 , antisense), located 532 bp downstream of the stop-codon of LAR The PCR cycle profile consisted of 94°C for 1 minute, 59°C for 1 minute and 72°C for 1 minute, for 35 cycles, and was performed in a programmable thermocycler (Perkin-Elmer Cetus) Each 50 μl reaction contained 10 mM Tris-HCl (pH 8 3), 50 mM KCl, 2 5 mM $MgCl_2$, 0 01% BSA, 0 01% gelatin, 0 05% NP 40, 0 05% Tween-20, 1 mM dNTPs, 100 ng genomic DNA, 100 ng of each primer, and 1 U *Taq* polymerase One-fifth of the reaction products was separated on 2% agarose gels and visualized by ethidium bromide staining

***Breeding of mice***

Mice were kept at the Central Animal Facility of the University of Nijmegen in a standard room with a day/night rhythm of 06 00/18 00 hours at a temperature of 21 °C and a humidity of 50-60% Males were housed together with four females in a macrolon type I cage and fed ad libitum Upon pregnancy, females were separated to give birth to their pups The first, second, and third litter of each female was counted and pups were observed every day Experiments were performed on F2 mice with a 129 x C57BL/6 hybrid genetic background Breeding results were statistically analyzed using MANOVA (SPSS) and Student's *t* test

***RNA analysis***

Poly(A)$^+$ RNA from kidney, brain, heart, liver, and lung (pooled from four three-months-old male LAR$^{+/+}$, LAR$^{+/-}$, and LAR$^{-/-}$ mice, respectively) was isolated as described previously (Hendriks *et al* , 1995) For Northern blot analysis, 3 $\mu$g of each sample was fractionated on a 1% (w/v) agarose/2 2 M formaldehyde gel, transferred to a Hybond N$^+$ membrane (Amersham), and linked by UV-radiation (UV Stratalinker 1800, Stratagene) Blots were subsequently probed with the 1 8 kb *Bam*HI mouse LAR cDNA fragment described above, a 162 bp *Eco*RI fragment from mouse LAR cDNA#9 upstream of the deleted region (Schaapveld *et al* , 1995), a 1 1 kb *Xho*I fragment from the Neo cassette, and mouse cDNA probes (Schaapveld *et al* , 1995) spanning the phosphatase domains of the LAR-like RPTPases PTPδ (pos 2486 3815, Mizuno *et al* 1993) and PTPσ (pos 4518-3' UTR, Ogata *et al* , 1994), respectively In addition, a rat 1 3 kb *Pst*I glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Fort *et al* , 1985) was used to enable comparison of RNA loading Hybridization signals on autoradiograms were analyzed on a GS-670 imaging densitometer (Bio Rad) Hybridized probes were stripped for 10 min in boiling 0 1xSSC/0 1% SDS Removal of probes was checked by exposing filters overnight between hybridizations

For analysis of LAR expression during mammary gland postnatal development, the first inguinal glands of 3-4 wildtype mice were collected (pregnancy staged by detection of vaginal plug) and RNA was prepared according to Chomzynski and Sacchi (1987) Total RNA (20 $\mu$g) was separated on a 1 5% formaldehyde-agarose gel, transferred to Nylon membrane (GeneScreen Plus) and hybridized with the LAR, PTPδ, PTPσ and GAPDH probes described above and with a 28S rRNA probe Phosphor image analysis was performed using a Model GS-363 Molecular Imager System (Bio-Rad) and Molecular Analyst software (Bio Rad) to quantify the relative expression levels

Analysis of milk protein expression was essentially done as described previously (Robinson *et al* , 1995) Briefly, the first inguinal glands of non lactating LAR$^{-/-}$ and wildtype mice at 18 5 days of pregnancy and at parturition were collected and RNA isolated For hybridizations oligonucleotide probes specific for WDNM1, WAP, $\beta$ casein, and $\alpha$-lactalbumin were used

***Histological analysis***

Various tissues from 7, 14, and 21 weeks old mice (male and females) were dissected, fixed in buffered formaldehyde, dehydrated, and embedded in paraffin Sections of 6 μm thickness were stained with haematoxylin-eosin according to standard histological procedures Brains were analyzed using parasagittal and coronal sections stained with cresyl violet

For analyses of mammary glands only female mice with a known history (2-3 litters) regarding neonatal death were used At the appropiate time point during pregnancy mice were anaesthetized with Avertin (Aldrich) and glands were taken surgically Mice were subsequently allowed to recover and to give birth to their pups At parturiton and the appropiate time point during the lactation period mice were sacrificed by cervical dislocation

For histological analyses of mammary glands, the right first inguinal mammary glands were dissected, fixed and embedded in paraffin as described above 5 μm sections were stained with haematoxylin eosin or used for immunohistochemical detection of WAP protein as described earlier (Robinson *et al* , 1995) Mammary glands were also frozen in liquid $N_2$ and embedded in Tissue-Tek O C T Compound (Miles) Serial cryostat-sections of 10 μm were stained with oil-red O to enable detection of lipid vesicles, or with haematoxylin-eosin

For whole mount preparations of mammary glands the left second thoracic and first inguinal mammary fat pads were dissected, mounted on glass slides, fixed, and stained with Delafield's haematoxylin or carmine as described (Edwards *et al* , 1988) Subsequently, they were made transparant by immersing in methyl salicylate, analyzed using a dissecting microscope, and photographed As controls for mouse strain-specific differences, mammary glands from 129, C57BL/6, and (129 x C57BL/6)F1 females were included in the analysis

***Milk protein analysis***

Milk collection was done essentially as described (Simons *et al* , 1987) At day 2 of lactation (day of birth of pups is taken as day 0), female mice were separated from their pups and 2 h later injected intraperitoneally with 1 or 2 1 U oxytocin (Intervet) After 10 minutes milk collection was started

## Results

### *Targeted deletion of the LAR phosphatase domain gene sequences*

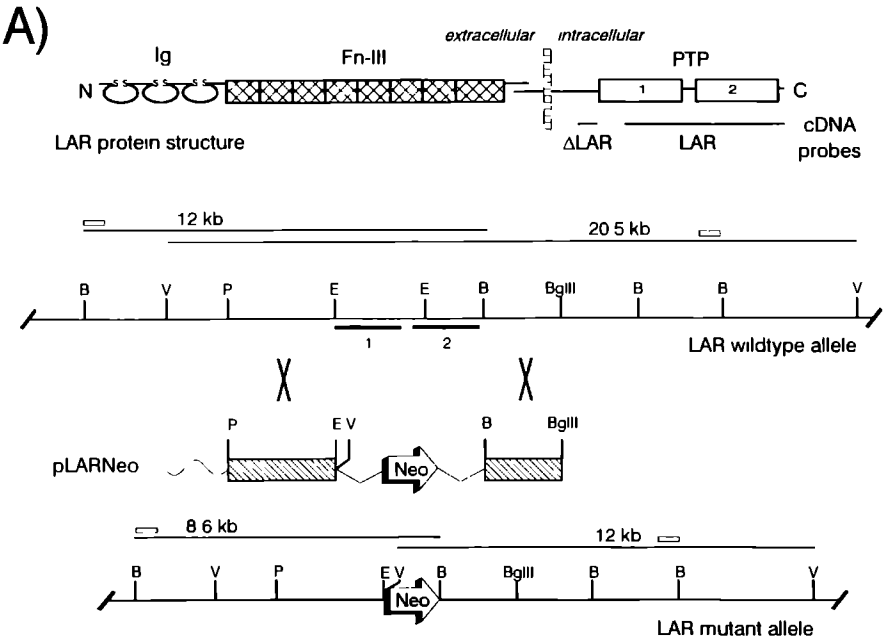
To study LAR function in an organismal context we used gene targeting by homologous recombination in ES cells to produce LAR$^{-/-}$ mice A replacement-type targeting vector was constructed in which a positive selection marker, a 1 1 kb neomycin

phosphotransferase expression cassette (Neo), is flanked by 3.2 and 2.3 kb of endogenous LAR genomic segments (Schaapveld *et al.*, 1995) which provide the necessary homology for targeted integration. Upon correct homologous recombination, a 4 5 kb genomic segment containing all exons encoding the phosphatase domains of LAR, is replaced by the Neo cassette in the same transcriptional orientation (Fig. 1A). This event results in a shorter *Bam*HI fragment (12.0 to 8.6 kb) and *Eco*RV fragment (20.5 kb to 12.0 kb) spanning the 5' and 3' regions of homology, respectively This targeting construct was introduced into E14 ES cells by electroporation and 365 G418 resistant clones were screened for homologous recombination Southern blot analysis using 5'- and 3'-diagnostic probes derived from genomic DNA flanking the targeting vector region revealed a targeting frequency of about 1 in 50 clones Three cell lines displaying correct homologous recombination and a normal karyotype were injected into blastocysts. Chimaeric males derived from all three cell lines were mated with C57BL/6 females and shown to transmit the ES cell genome. Offspring carrying the desired mutation were then sibling mated and used to produce heterozygous and homozygous mutant mice in a mixed 129 x C57BL/6 background

**Figure 1.** Disruption of the LAR gene by homologous recombination

(**A**) LAR protein structure showing the relevant functional domains Ig, Immunoglobulin-like domains, Fn-III, fibronectin type-III-like domains, PTP, tyrosine phosphatase domains 1 and 2 The LAR cDNA probes as used for Northern blot analysis (see Fig 2) are indicated by solid bars Below, a schematic diagram of the relevant part of the mouse genomic LAR locus and the targeting strategy are shown. Hatched bars in pLARNeo represent the fragments homologous to endogenous LAR genomic segments and the gray arrow symbolizes the neomycin phosphotransferase selection cassette Small gray bars indicate the 5'- and 3'-diagnostic probes, and the solid bars 1 and 2 mark the genomic segments encoding the phosphatase domains 1 and 2, respectively B, *Bam*HI; E, *Eco*RI: V, *Eco*RV; P, *Pst*I.

(**B**) Southern blot analysis of tail DNA from wildtype (+/+), mice heterozygous (+/-), and mice homozygous (-/-) for the mutant LAR allele The LAR 5'- and 3'-flanking probes (panels A and B) reveal the mutant diagnostic 8.6 kb *Bam*HI and 12 0 kb *Eco*RV fragments next to the wildtype 12 0 kb *Bam*HI and 20 5 kb *Eco*RV fragments, respectively Hybridization with a LAR cDNA probe encompassing both phosphatase domains confirms the total absence of these sequences in LAR$^{-}$ mice (panel C) A Neo cDNA probe reveals the absence of additional integrations of the targeting vector (panel D)


A)
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Ig
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LAR protein structure
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B V P E E B BglII B B V
1
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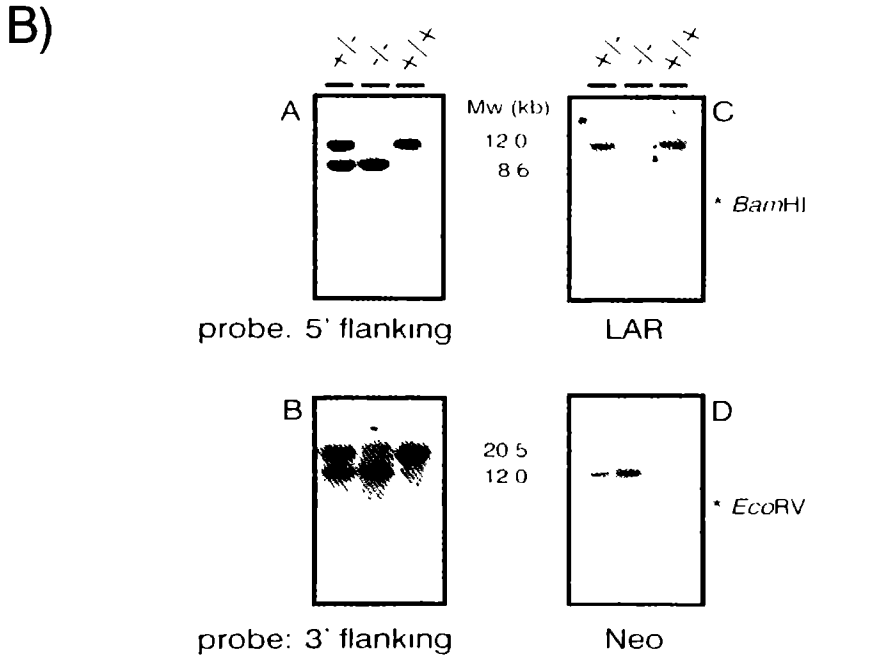
B)
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probe. 5' flanking
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D
* EcoRV
probe: 3' flanking
Neo

To verify the presence of the correct replacement mutation in our lines, offspring born from F1 heterozygote crosses were genotyped by Southern blot (Fig 1B) or PCR analysis of genomic DNA isolated from tail biopsies The 5' and 3' flanking probes revealed the correct targeting of the LAR locus (Fig 1B, panels A and B) A LAR cDNA probe encompassing both phosphatase domains (Schaapveld *et al* , 1995) failed to detect the wildtype 12 0 kb *Bam*HI fragment in DNA isolated from homozygous mutant mice, as anticipated (Fig 1B, panel C) Using the neomycin resistance gene as a probe, only the 12 0 kb *Eco*RV fragment and no additional integrations of the targeting construct were detected (Fig 1B, panel D)

The mutant LAR allele was identified in 145 heterozygous ($LAR^+$ , 49 7%) and 68 homozygous (LAR , 23 3%) mice out of a total of 292 mice tested Thus, the mutant allele segregated essentially according to Mendelian laws, indicating that the removal of the LAR PTPase domains is not lethal

***The mutant LAR allele produces a truncated mRNA***

To investigate the consequences of LAR gene targeting at the RNA level, poly(A) RNA was isolated from several tissues and analyzed by Northern blot analysis (Fig 2) Using the mouse LAR cDNA encompassing both LAR PTPase domains as a probe, no wildtype LAR mRNA was detected in LAR mice (Fig 2, panel A) The faint signals present in the $LAR^-$ brain isolate are due to cross hybridization to the highly homologous RPTPase PTPσ (Schaapveld *et al* , 1995, see also below) Total absence of LAR mRNA containing the PTPase domains was confirmed by RT-PCR using mouse LAR specific primers and poly(A)$^+$ RNA isolated from various tissues (data not shown) In mRNA isolated from wildtype brain, an additional mRNA isoform of ~6 5 kb is seen along with the ubiquitously expressed messenger of ~8 kb

The mutant LAR allele is expected to give rise to shorter transcripts that contain the sequences encoding the LAR extracellular region and ending at the poly(A) addition site present in the Neo cassette Indeed both an *Eco*RI mouse LAR cDNA fragment located just upstream of the deleted region and the neomycin resistance gene probe detect a ~5 5 kb mRNA (Fig 2, panel B and C) In brain a ~4 kb transcript is also found in $LAR^+$ and $LAR^-$ mice that likely encodes a shorter LAR isoform lacking extracellular region sequences (O'Grady *et al* , 1994, Zhang and Longo, 1995) The truncated transcript(s) in the $LAR^-$ mice, if translated at all, encode(s) a mutated LAR molecule that lacks PTPase activity

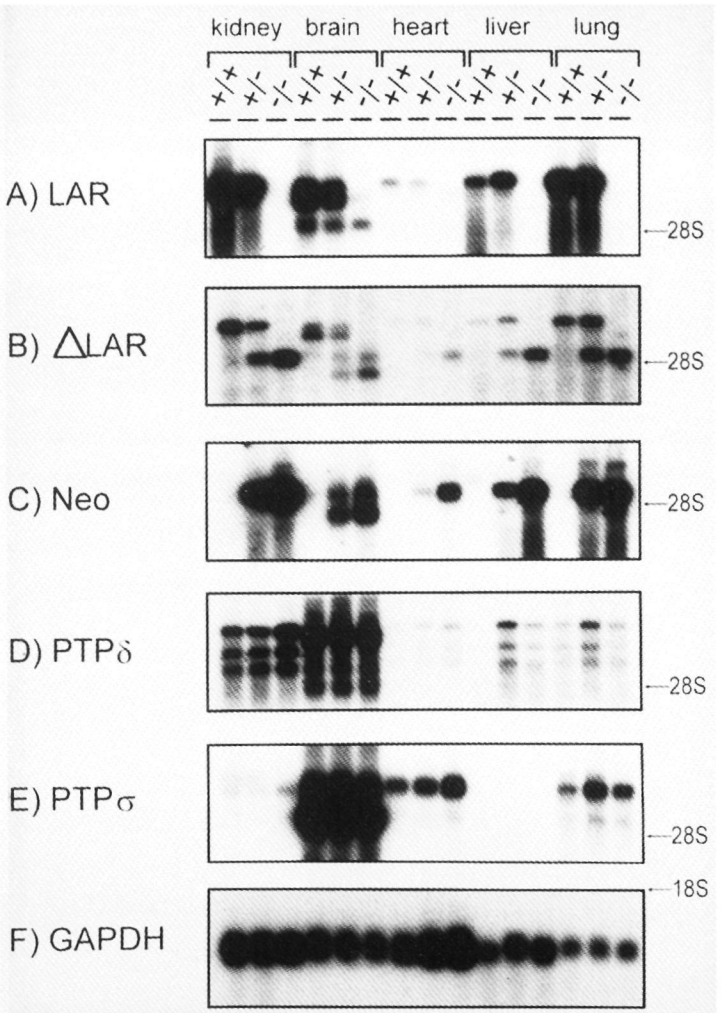


**Figure 2.** Northern blot analysis of poly(A)$^+$ RNA isolated from various tissues from LAR$^{+/+}$, LAR$^{+/-}$, and LAR$^{-/-}$ mice using different probes: (**A**) A mouse LAR cDNA encompassing both PTPase domains of LAR; (**B**) A mouse LAR cDNA *Eco*RI fragment upstream of the deleted region (ΔLAR); (**C**) A Neo cDNA *Xho*I fragment; (**D**) A mouse PTPδ cDNA; (**E**) A mouse PTPσ cDNA clone; and (**F**) A rat GAPDH cDNA *Pst*I fragment is used to verify the integrity of the RNA loaded. The positions of the 28S and 18S ribosomal bands are indicated on the right.

*Histological analysis of LAR mutant mice*

Mice lacking the LAR PTPase domains had normal weight, behaved normally and appeared healthy upon veterinary inspection We then performed an extensive histological survey to look for possible consequences of the absence of functional LAR at the tissue and cellular level A wide variety of tissues, including brain, kidney, adrenal, liver, lung, heart, stomach, colon, ileum, duodenum, testis, uterus, pancreas, muscle, spleen, thymus and mammary gland were isolated from 6, 14, and 21 week old male and female mice, sectioned and stained with haematoxylin-eosin In addition, serial parasagittal and coronal sections of brain were stained with cresyl violet This analysis revealed no significant differences between $LAR^{+/+}$, $LAR^{+/-}$, and LAR mice (data not shown)

*Expression of LAR-like RPTPases*

The LAR-like RPTPases, PTPδ (Mizuno *et al* , 1993) and PTPσ (Ogata *et al* , 1994 Wagner et al , 1994), are ~90% identical to LAR at the amino acid level for both PTPase domains (Schaapveld *et al* , 1995), exhibit similar *in vitro* PTPase activity and all three interact with the LAR-interacting protein, LIP 1, suggesting similar functions in various tissues (Pulido *et al* , 1995b) To determine whether PTPδ and/or PTPσ could compensate for LAR function in the $LAR^{-/-}$ mice, we performed Northern blot analysis using mouse cDNA probes encompassing the cytoplasmic regions of PTPδ and PTPσ (Schaapveld *et al* , 1995) Signal quantitation by densitometry revealed no significant difference in mRNA levels of PTPδ or PTPσ in $LAR^{-/-}$ mice compared with $LAR^{+/+}$ and $LAR^{+/+}$ mice (Fig 2, panels D and E), and the expression distribution of the tissue specific alternative mRNA isoforms was consistent with data published elsewhere (Mizuno *et al* , 1993, Ogata *et al* , 1994, Wagner *et al* , 1994) This finding cannot rule out any redundancy at the enzymatic level, and ultimately crosses between LAR-, PTPδ-, and PTPσ negative mice will be needed to reveal a potential overlap in function between these RPTPase subfamily members

*Increased neonatal mortality for pups from $LAR^{-/-}$ females*

Unexpectedly upon interbreeding $LAR^{-/-}$ males and females normal-sized litters were obtained, but many of the pups died within 1-3 days after birth To substantiate this finding, an extensive breeding program was performed in which four possible intercrosses were examined (Fig 3) We observed an increased neonatal death rate for pups from $LAR^{-/-}$ mothers (>50%) compared to pups from $LAR^{+/+}$ mothers (<10%

t(27,48)=5 13, $p<0.001$), independent of the male genotype and litter size ($F(2,30)=0\ 08$, $p=0\ 921$) This reduced survival rate was a consistent finding for all three independently derived LAR knock-out lines (originating from ES cell lines CA1, CA7, and BC2) and comparable results were observed for the second and third litters from the same $LAR^{-/-}$ females (data not shown). Ongoing studies using female mice that result from multiple backcrosses onto C57BL/6 background point to full penetrance in C57BL/6 mice with respect to the neonatal death. Thus, most likely the heterogeneity of the genetic background (hybrid 129 x C57BL/6) explains the variable penetrance that was observed using F2 animals.

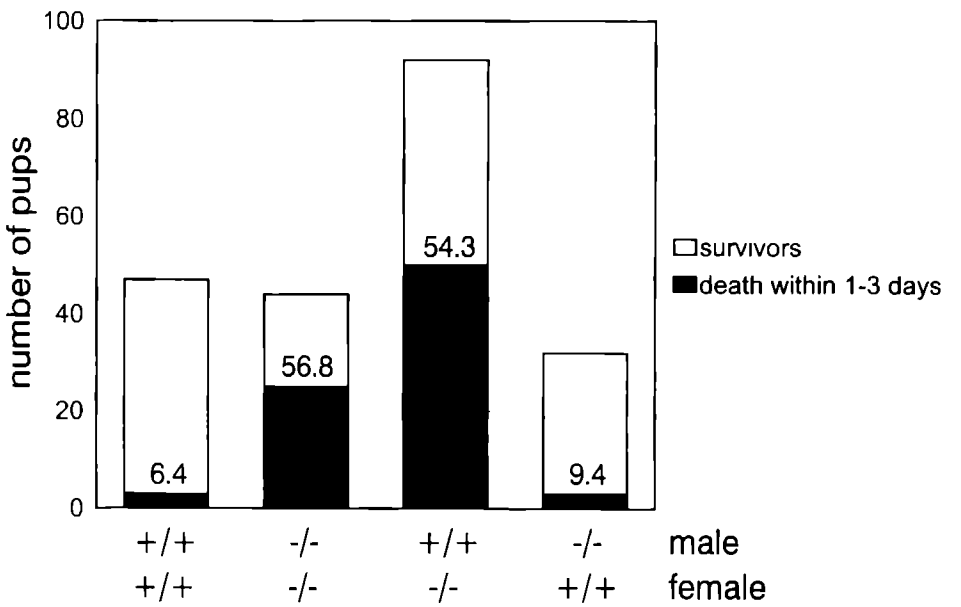


**Figure 3.** Increased neonatal mortality for pups of LAR female mice

Upon breeding, $LAR^{-/-}$ females show normal fertility and give birth to normal-sized litters However, >50% of the pups die within 1-3 days after birth compared to only <10% in the case of $LAR^{+/+}$ females The ratio death/born (%) are indicated reflecting the lack of complete penetrance of the phenotype Comparable results were found for second and third litters (data not shown). Diagram represents the sum of results with mice derived from the CA1 and BC2 ES cell clones.

At autopsy, pups born from LAR females which died at day 1-3 postpartum appeared smaller compared with pups of the same age born from wildtype mice As indicated by the absence of a white coloured belly, no milk was found in their stomachs Because milk ingestion failed despite multiple attempts to suckle the mothers' nipple, we attribute the neonatal death to a defect in the lactating capacity of the LAR$^{-/-}$ mothers rather than to a behavioural abnormality of neonates To validate this observation, one day old LAR$^{-/-}$ pups from LAR$^{-/-}$ parents without white bellies were swapped to lactating wildtype mothers These pups survived demonstrating that their suckling capacity was normal In contrast, one day old healthy looking LAR$^{+/+}$ pups from wildtype parents were accepted by LAR$^{-/-}$ mothers and did suckle their nipples, but dehydrated and died within 5 days (data not shown)

To investigate a possible milk production defect in LAR$^{-/-}$ mothers in more detail, the quantity of the milk produced by these mice was analyzed In contrast to lactating LAR$^{+/+}$, LAR$^{+/-}$, or LAR$^{-/-}$ mothers from which normal amounts of milk (20-50 $\mu$l) could be readily obtained already at postpartum, we never obtained more than 2-3 $\mu$l milk from LAR$^{-/-}$ mothers whose pups died within 1-3 days Neither the injection of higher doses of oxytocin (2-5 I U ) nor the intensive massaging of the mammary glands helped to increase milk production (data not shown), suggesting that the lactational defect in LAR$^{-/-}$ mice may be due to a general lack of milk protein synthesis or impaired milk secretion

***Regulation of LAR expression during pregnancy***

To determine whether LAR has a role in mammary gland development and function, we examined LAR mRNA expression in mammary glands isolated from female mice at various stages during postnatal development LAR expression is already present in mammary glands of virgin females as determined by RT PCR (data not shown) Northern blot analysis of total RNA isolated from glands of wildtype females at various stages during pregnancy (P9-18) and at parturition (L1) revealed that LAR expression during pregnancy is developmentally regulated (Fig 4) LAR expression is evident at day 9 of gestation, increases five-fold during mid-pregnancy and reaches maximum levels at day 16 Since GAPDH expression might vary during pregnancy, as was found during lactation (Buhler et al , 1993), the variations in the amount of RNA loaded was also assessed by hybridizing the blot with a 28S rRNA cDNA probe The obtained LAR expression profile is consistent with a role for LAR during mammary gland development at late pregnancy The expression pattern of the LAR-like RPTPases was also investigated PTPδ transcripts

are undetectable, but PTPσ is expressed, although at very low levels, during mammary gland development (Fig. 4). Compared to LAR, a twenty-fold longer exposure time was needed for PTPσ signals to become visible.

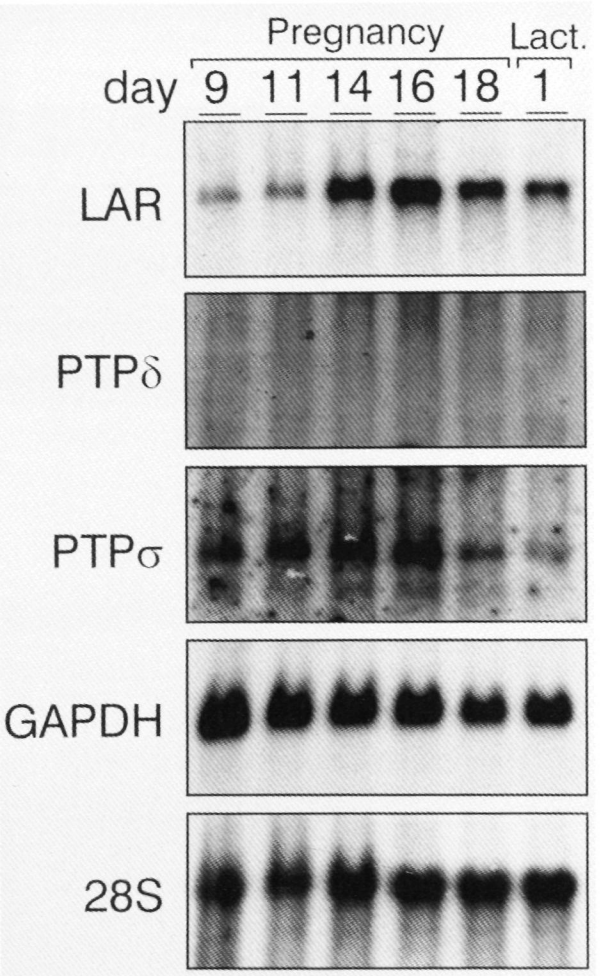


**Figure 4.** Regulation of LAR expression during postnatal development of wildtype mammary gland.
Total RNA isolated from mouse mammary gland at various stages during pregnancy (P) and at parturition (L, lactating) was analyzed by Northern blotting. The blot was subsequently hybridized with cDNA probes for mouse LAR, PTPδ, PTPσ, rat GAPDH, and 28S RNA (28S). The numbers indicate the stage of gestation (in days) at which the mammary glands were dissected.

***Impaired terminal differentiation of mammary alveoli in non-lactating LAR$^{-/-}$ females***

To examine whether the lactational defect found in LAR$^{-/-}$ female mice could result from an impaired mammary gland development, we histologically analyzed mammary glands from virgin, late pregnant, postpartum, lactating, and involuting female mice. We termed LAR$^{-/-}$ females 'non-lactating' if they consistently showed for two or three successive litters that their pups would die within 1-3 days after birth. LAR$^{-/-}$ females which were indistinguishable from wildtype mice regarding the percentage of surviving pups per litter were considered as 'lactating' LAR$^{-/-}$ mice. We observed no significant difference in the number of ducts per unit volume of fat pad in glands of virgin LAR$^{+/+}$, LAR$^{+/-}$, and lactating LAR$^{-/-}$ females (data not shown). Extensive alveolar development was observed during lactation in LAR$^{+/+}$, LAR$^{+/-}$, and LAR$^{-/-}$ females which could lactate (Fig. 5, panel B; and data not shown). The lumina were extended and contained secretion. In contrast, less extended alveoli with filled lumina were found in the exceptional case of a non-lactating LAR$^{-/-}$ female which pups survived until day 7 postpartum (Fig. 5, panel A).

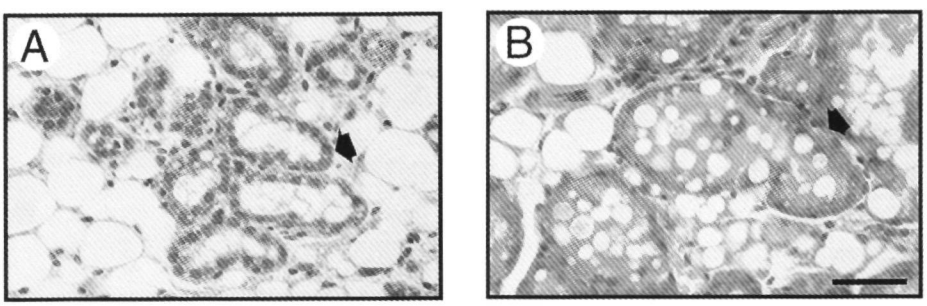


**Figure 5.** Histological analysis of mammary tissue from post-partum non-lactating LAR$^{-/-}$ (**A**) and LAR$^{+/-}$ (**B**). The former litters of the LAR$^{-/-}$ female died within the first three days after birth, but this time the litter was nursed very poorly and the last pups died at day 7. For the ~50% of females that show the non-lactating phenotype, this was the only exception for which we could monitor a lactational state. The fat pad of the LAR$^{-/-}$ mice is not completely filled and the alveoli are overall smaller than those in hemizygous and wildtype mice (not shown). The epithelial cells in the LAR$^{-/-}$ mice do not have a secretory phenotype as witnessed by their cuboidal appearance and flattened apical side (arrow in **A**). In contrast, milk is visible in the hemizygous mice, both as proteinacious luminal content and as intracellular secretory products (arrow in **B**). Bar, 200 $\mu$m.

The alveolar development in LAR$^{-/-}$ females without the ability to lactate appears incomplete, and the epithelial cells in the alveoli do not have the secretory phenotype and apparently milk production and/or secretion coupled with the formation of an extended lumen was abrogated in non-lactating mice

The development of mammary glands from non-lactating LAR$^{-/-}$ females up to late pregnancy (day 18-20) parallels that of wildtype glands, as determined by whole mount analysis (data not shown) Thus, ductal outgrowth and branching is not overtly impaired The formation of the lobuloalveolar structures was examined in more detail by histological analysis using haematoxylin-eosin (Fig 6, panels A and B) or oil-red O staining (data not shown) of mammary glands at late pregnancy Only minor differences in lumen size and alveoli cluster formation were observed between wildtype and non-lactating LAR$^{-/-}$ glands However, clear differences in the morphology of secretory epithelial cells and luminal contents were evident While intraluminal secretions were visible in glands from wildtype mice (Fig 6, panel A), the LAR$^{-/-}$ epithelial cells contained large secretory vesicles and lipid droplets indicating an impaired secretion (Fig 6, panel B)

At parturition, enlarged alveoli with extended lumina were clearly visible in wildtype glands (Fig 6, panel C) In contrast, the number and size of alveoli in non-lactating LAR were reduced, and local interstitial fibrosis and congestion of secretion could be observed (Fig 6, panel D) Whole mount analysis of mammary glands isolated from non-lactating LAR$^{-/-}$ mothers at parturition showed an advanced state of involution (Fig 7, panels C-F) Although there is unavoidable variation in the moment of tissue collection, the involution state is at least comparable with that of glands isolated from wildtype mice two days after the removal of their pups (Fig 7, panel A) and in some cases already resembles complete involution as observed for wildtype mice at two weeks following weaning of the pups which had been nursed for three weeks (Fig 7, panel B)

To test whether altered expression of milk protein genes could explain the failure of non lactating LAR$^{-/-}$ females to switch to the lactational state, RNA was isolated and probed for WDNM1, WAP, $\beta$-casein, and $\alpha$-lactalbumin transcript levels by Northern blot analysis No differences were apparent between non-lactating LAR$^{-/-}$ and wildtype mice (data not shown) Also, immmunohistochemical staining for WAP expression did not point to altered milk protein synthesis (data not shown) Moreover, the protein and triglycerides content of the few microliters that incidentally could be obtained from non-lactating mice is comparable to that of wildtype and lactating LAR$^{-/-}$ mice (data not

shown).

Pups did recognize, attach, and bite the nipple of their mothers, but in about half of the $LAR^{-/-}$ females alveolar development which is characteristic for late pregnancy was not attained and milk secretion was not initiated. Moreover, the transition to involution and apoptosis of alveolar epithelium occured more readily in these mice, even before milk secretion started. These findings suggest an impaired development of mammary alveoli in $LAR^{-/-}$ females at late pregnancy: the failure of the alveoli to acquire secretory activity is paralleled by a rapid involution at parturition.

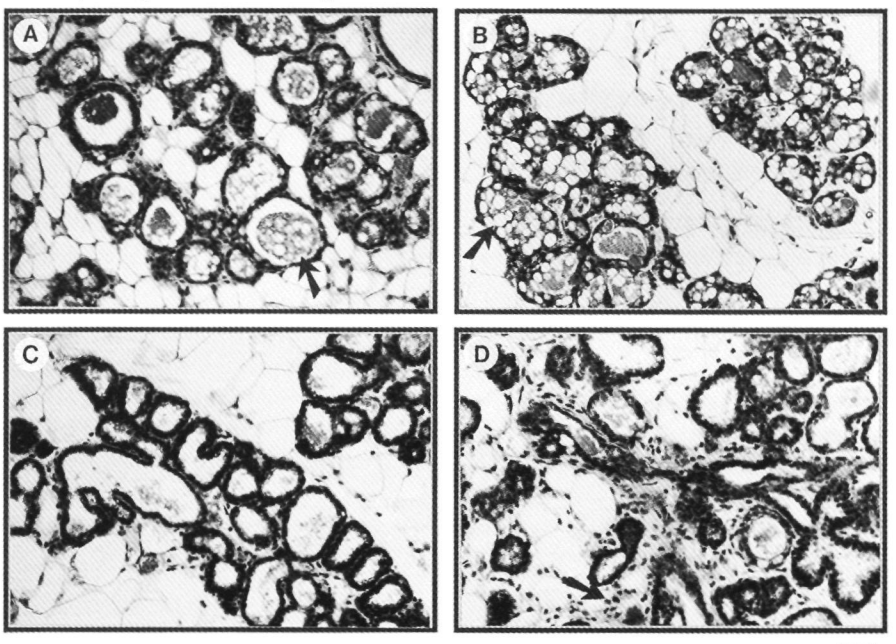


**Figure 6.** Aberrant development at late pregnancy resulting in impaired functional differentiation at parturition in $LAR^{-/-}$ mammary glands. Haematoxylin-eosin staining of mammary glands at late pregnancy (day 18.5; **A** and **B**) and at parturition (**C** and **D**) from a wildtype female (**A** and **C**) and a non-lactating $LAR^{-/-}$ female (**B** and **D**). Only minor differences in lumen size and alveoli cluster formation can be observed at late pregnancy. Tissues from wildtype mice contain more intraluminal secretion (arrow in **A**). In contrast, secretion appears more intracellular in non-lactating $LAR^{-/-}$ glands as indicated by the high content of lipid droplets in epithelial cells (arrow in **B**). Impaired functional differentiation is clearly visible in glands from non-lactating $LAR^{-/-}$ females at parturition. The number and size of alveoli are reduced. Furthermore, signs of local interstitial fibrosis (arrow in **D**) and congestion of secretion are visible. Bar, 30 $\mu$m.

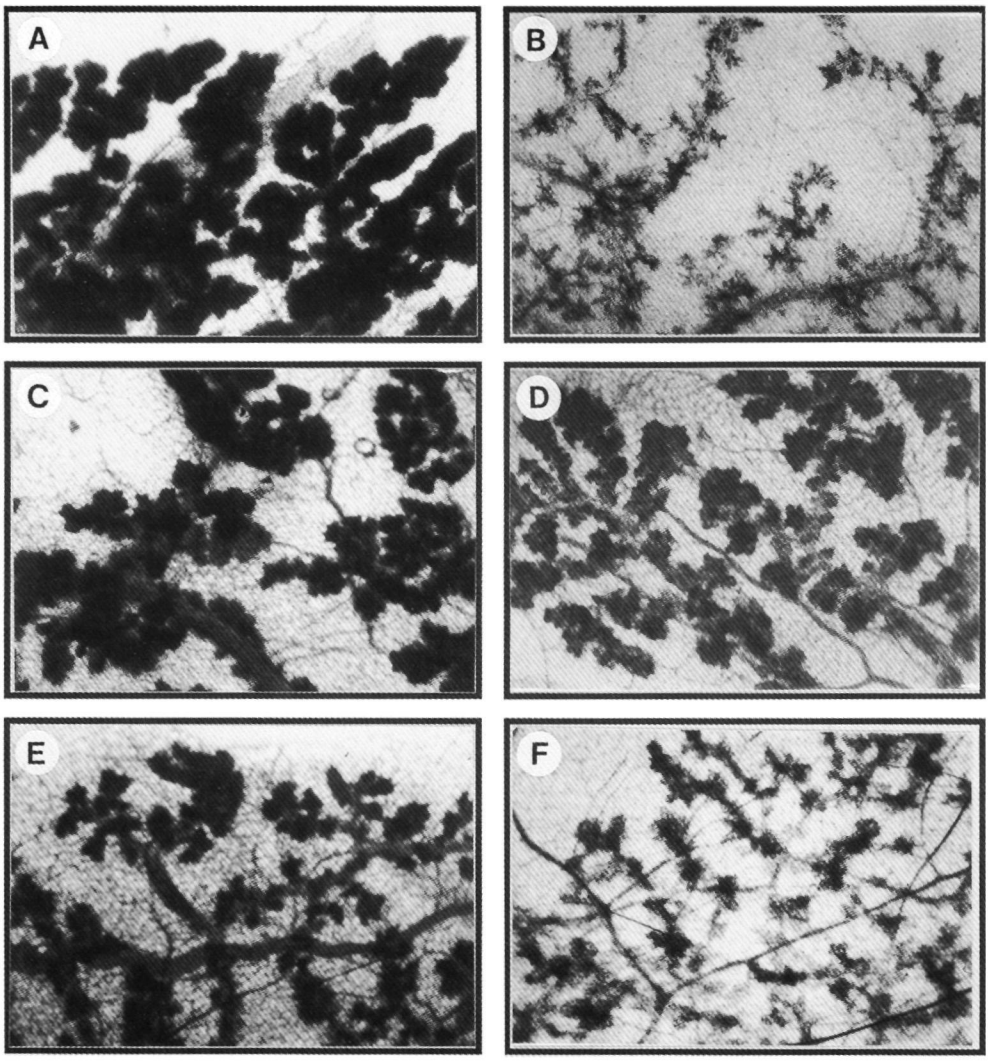


**Figure 7.** Rapid involution postpartum in $LAR^{-/-}$ females.
Whole mount mammary glands were dissected, fixed, stained with carmine, cleared with methyl salicylate, and photographed. An advanced state of involution is observed in mammary glands from non-lactating $LAR^{-/-}$ mothers at parturition (**C-F**). For comparison, a mammary gland from a wildtype mother dissected 2 days after removal of pups at parturition (**A**) and an involuted mammary gland 2 weeks after weaning of pups which were nursed for 3 weeks (**B**) are shown. Bar, 350 $\mu$m.

**Discussion**

To address the biological role of LAR PTPase activity in growth and development we generated mice lacking expression of the LAR PTPase cytoplasmic region The LAR mice are viable, grow, and behave normally Furthermore, histological analysis of a wide variety of tissues at different ages did not reveal structural abnormalities, consistent with a previous report on the generation of LAR-deficient mice using a gene trap-based method capturing the N-terminal signal sequence and generating a $\beta$-galactosidase fusion protein (Skarnes *et al* , 1995) However, our current detailed analysis reveals that LAR has a function during mammary gland differentiation We observed an increased neonatal mortality for pups from LAR$^{-/-}$ mothers, independent of the litter size and the genotype of the male or the offspring The pups suckled the nipples of their mother, but failed to ingest milk, became dehydrated and died Swapping experiments demonstrated that the increased neonatal death was due to a lactational defect of the LAR$^{-/-}$ mothers rather than to an abnormal behaviour of neonatal pups Due to genetic background heterogeneity this phenotype displayed a 50% penetrance in the F2 hybrid 129 x C57BL/6 mice that were used in this study, which might explain why it was not detected by Skarnes *et al* (1995) Interestingly, also in *Drosophila* the inactivation of CAM-like RPTPases resulted in phenotypes with a limited penetrance (Desai *et al* , 1996, Krueger *et al* , 1996) To map potential modifier loci, it will be necessary to cross the LAR allele into different genetic backgrounds Regarding ductal outgrowth and branching in wildtype mammary glands, we did not observe significant background differences between mouse strain 129, C57BL/6, the F2 hybrid 129 x C57Bl/6, and F4 mice on C57BL/6 background, although the ductal system at the virgin state in 129 mice is less developed

Development of the mammary gland progresses gradually during puberty and pregnancy, and acquires full function during lactation (for review see Daniel and Silberstein, 1987) In several natural mouse mutants, as well as transgenic and knock-out mice this process is affected (Gallahan *et al* , 1996, Jhappan *et al* , 1992, 1993, Kordon *et al* , 1995, Li *et al* , 1994, 1996a,b, Robinson *et al* , 1996, Sicinski *et al* , 1995) For example, in transgenic mice expressing TGF$\alpha$ under the control of the mouse WAP gene promoter mammary development and differentiation proceeds during pregnancy, but the secretory cells do not establish and/or maintain a functional state characterized by milk secretion (Robinson *et al* , 1996, Sandgren *et al* , 1995) A similar phenotype was observed in mice which contain a non-functional mCSF1 (Pollard and Hennighausen, 1994) or inhibin ßB gene (Vassalli *et al.*, 1994) The high amounts of intracellular fat

droplets at late pregnancy is also seen in 'milchlos' transgenic mice (Robinson *et al* , 1995) In non-lactating LAR$^{-/-}$ mice a normal epithelial cell proliferation up to late pregnancy is observed, but histological sections of glands at late pregnancy showed a more intracellular storage of secretion products in comparison with the intraluminal secretion in wildtype glands At parturition, mammary glands showed signs of fibrosis and congestion of secretion, and a regression of alveolar structures as is seen normally at a progressed stage of involution following weaning of the pups Taken together, these observations suggest that LAR function is evoked relatively late in mammary gland development and that most likely the LAR$^{-/-}$ phenotype is a combination of a loss of alveolar cells around parturition and the inability of the remaining alveoli to undergo secretory differentiation

Complex molecular interactions are involved in the regulation of lactation (Rillema, 1994) The initiation of lactation following parturition involves many endocrine changes, of which the reduced influence of sex steroid hormones appears to be the most important (for review see Daniel and Silberstein, 1987, Rillema, 1994) Hormonal influences at the level of some milk protein gene promoters are mediated by the mammary gland-specific transcription factor MGF/Stat5 (Wakao *et al* , 1994) Regulation of milk protein expression via regulation of Stat5 activity also requires the basement membrane (Sympson *et al* , 1994, Talhouk *et al* , 1992) which provides signals via interaction with epithelial cells through cell surface receptors (Streuli *et al* , 1995) Mice lacking Stat5a show reduced mammary lobuloalveolar outgrowth and fail to lactate due to an impaired terminal differentiation (Liu *et al* , 1997) The phenotype becomes apparent during mid pregnancy and is reflected by a marked reduction in WAP transcript levels LAR deficient mice show signs of abnormal mammary gland development at a later stage, shortly before parturition, and no reduction in WAP levels could be observed It remains to be investigated whether or not LAR and STAT5a signalling pathways are intertwined at some stage of mammary gland development

LAR is expressed in brain and could perhaps play a role in the regulation of the appropriate hormone balance needed for proper mammary gland development and function However, at the moment it is not known if LAR is specifically localized to regions in the brain that are involved in the hormonal regulation of lactation and further studies will be required to address this issue LAR is predominantly expressed in epithelial cells and localized at regions of association of these cells and the basement membrane (Streuli *et al* , 1992) Consistent with this is the recent finding of co-

localization of LAR and its interacting protein LIP 1 at focal adhesions and its proposed role in disassembly of focal adhesions (Serra-Pagès *et al* , 1995) In view of the developmental regulation of LAR expression in the mammary gland during pregnancy and the phenotypic consequences of the ablation of LAR PTPase activity, these data point to an important role for LAR in cell-matrix interactions that are crucial for mammary gland development and function

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# Chapter V

## Insulin Responsiveness in Mice Lacking LAR Tyrosine Phosphatase Activity

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**Summary**

Insulin binding to the $\alpha$-subunit of the insulin receptor results in phosphorylation and thus activation of its intracellular $\beta$-subunits Subsequent inactivation by dephosphorylation is achieved by protein tyrosine phosphatases (PTPases, protein-tyrosine-phosphate phosphohydrolase, EC 3 1 3 48) The cell adhesion molecule-like PTPase LAR has been implicated in the inactivation of the insulin receptor on the basis of *in vitro* studies Our aim was to test this involvement by studying the insulin response in mice that are deficient in LAR signalling (LAR$^-$) Post-prandial LAR$^-$ and age-matched control male mice were subjected to an insulin tolerance test Blood samples were obtained 120 and 5 min pre-dose and at 30 min intervals for 2 h post-dose enabling calculation of the $\Delta$glucose-AUC LAR$^-$ mice showed no difference in pre-dose plasma glucose levels or insulin concentrations compared to wildtype controls, nor in their response to insulin Adipocytes isolated from the epididymal fat pad were used to determine the $ED_{50}$ for insulin in a lipogenesis assay for both control and LAR$^-$ mice Again, no significant difference for both groups was observed In addition, basal insulin receptor tyrosine kinase activity in soleus muscles did not differ significantly between LAR$^-$ and control mice As our data provide no evidence for increased insulin signalling in absence of I AR tyrosine phosphatase activity, we conclude that PTPase family members other than LAR must be involved

**Introduction**

Insulin plays a pivotal role in metabolic and cellular processes like the control of blood glucose levels, glycogen synthesis, and the regulation of a variety of enzymes and transport systems (White and Kahn, 1994) Binding of insulin to the $\alpha$-subunit of the insulin receptor (IR) results in phosphorylation and thus activation of the intracellular $\beta$-subunits (White *et al* , 1988) Subsequently, several intracellular substrates become tyrosine phosphorylated such as the Insulin Receptor Substrates 1 and 2 (IRS 1, IRS-2), and Shc (Pronk *et al* , 1993, Sun *et al* , 1991, 1995) IRS-1 functions as a multisite docking protein for other signalling molecules like growth factor receptor binding protein 2 (GRB-2), the adaptor protein Nck, and the p85$\alpha$ regulatory subunit of phosphatidylinositol 3 kinase (PI 3'-kinase) via interaction with their Src homology 2 (SH2) domains, leading to the aforementioned various cellular responses mediated by insulin (for review see Myers *et al* , 1994, White and Kahn, 1994)

Dephosphorylation of the IR and its phosphorylated substrates can be achieved by protein tyrosine phosphatases (PTPases), which may act as either positive or negative regulators in the insulin signalling cascade However, little is known about the identity of the PTPases involved, and their regulation and specificity Various PTPases, such as the cytosolic PTP1B, SHP-1 (also known as SHPTP1, SHP, HCP, and PTP1C) and SHP-2 (also known as SHPTP 2, SHPTP-3, PTP1D, Syp, and PTP2C) and the receptor-like PTPases (RPTPases) CD45, LAR, RPTP$\alpha$, and RPTP$\varepsilon$ are all capable of dephosphorylating the insulin receptor *in vitro* (Adachi *et al* , 1996, Goldstein, 1993, Hashimoto *et al* , 1992a, 1992b, Kenner *et al* , 1996, Kulas *et al* , 1995, 1996a, Møller *et al* , 1995, Uchida *et al* , 1994), but data on *in vivo* reactions are scarce

It has been suggested that SHP-2 is a positive regulator of insulin signalling Binding of SHP-2 to the activated IR results in reduced SHP-2 PTPase activity towards the IR (Karitonenkov *et al* , 1995, Maegawa *et al* , 1993, 1994, Staubs *et al* , 1994, Ugi *et al* , 1994), whereas binding to tyrosine phosphorylated IRS-1 leads to activation of SHP 2 (Kuhne *et al* , 1993, Pluskey *et al* , 1995, Sugimoto *et al* , 1994, Ugi *et al* , 1994) It may therefore be responsible for the dephosphorylation of IRS-1 (Kuhné *et al* , 1994, Noguchi *et al* , 1994, Ugi *et al* , 1996) In addition, it acts as a positive regulator in insulin-induced activation of the $p21^{ras}$-MAPK pathway (Hausdorff *et al* , 1995, Milarski and Saltiel, 1994, Noguchi *et al* , 1994, Xiao *et al* , 1994, Yamauchi *et al* , 1995) and in the insulin-mediated dephosphorylation of Focal Adhesion Kinase ($p125^{FAK}$) and paxillin which is accompanied by a reduction of actin stress fibers (Knight *et al* , 1995, Ouwens

*et al* , 1996, Yamauchi *et al* , 1995) However, recently, Arrandale *et al* (1996) showed that mice expressing reduced levels of SHP-2 retain their acute metabolic responses to insulin, suggesting a minor role for SHP-2 in insulin signalling

Several findings support the candidancy of the cell adhesion molecule-like RPTPase LAR as a negative regulator of insulin signalling For example, LAR expression is increased in adipose tissue of obese human subjects (Ahmad *et al* , 1995, Ahmad and Goldstein, 1995) Also, LAR has been found to be active against the autophosphorylated IR *in vitro*, preferentially dephosphorylating the phosphotyrosine residues that have been implicated in the regulation of the receptor kinase (Hashimoto *et al* , 1992a) Antisense inhibition of LAR leads not only to increased ligand-dependent IR activation, but also to increased insulin-dependent PI 3'-kinase activity (Kulas *et al* , 1995, 1996b) Conversely, overexpression of membrane-bound LAR decreased the tyrosine phosphorylation of the IR upon insulin stimulation (Zhang *et al* , 1996) Finally, upon chemical cross-linking on the surface of rat hepatoma cells the IR and LAR could be co-immunoprecipitated (Zhang *et al* , 1996) In these cells LAR may influence the phosphorylation status of the insulin receptor and its substrates IRS-1 and IRS-2 (Li and Goldstein, 1996) To further substantiate the indirect evidence from these *in vitro* and cell culture-based studies, we have generated mice lacking LAR tyrosine phosphatase activity (Schaapveld *et al* , 1997) and used these mice to test the involvement of LAR in insulin signalling *in vivo*

## Materials & Methods

### *Generation of LAR knock-out mice*

The generation and phenotypic analysis of mice lacking LAR tyrosine phosphatase activity is described elsewhere (Schaapveld *et al* , 1997) For all experiments only male mice were used The strain, genotype, and age of the mice are indicated with each experiment All animals were screened for viruses, including MHV, using enzyme-linked immunosorbent assays (ELISA) and trained for handling over three days prior to each experiment to avoid stress

### *Plasma glucose and insulin levels*

Plasma glucose and insulin levels were measured in fasted (18 h on water only) and fed LAR (F3(129 x C57BL/6), n=12, 38-44 wks old) and age matched control mice (C57BL/6, n=10, 43 wks old) Post-prandial mice (fed *ad libitum*) were subjected to an insulin tolerance test (ITT) by s c injection of 1 U human insulin (Actrapid, Novo Nordisk) per kg body weight Blood samples

were collected for determination of post-prandial levels of insulin and glucose at 120 min and 5 min pre-dose and at 30 min intervals for 2 h post-dose, enabling calculation of the Δglucose $AUC_{ITT}$ (AUC, area under curve) (Brand *et al* , 1996, Tai, 1994)

Whole blood and plasma glucose levels were measured on a glucose autoanalyzer (immobilized glucose oxidase method, EBIO, Eppendorf, Germany) using 10 μl blood samples diluted into 500 μl EBIO analysis buffer For the determination of fasting and post-prandial plasma insulin levels, blood was collected into chilled heparinized tubes containing Aprotinin, centrifuged at 4°C and plasma was stored at -20°C Insulin was assayed using an ELISA consisting of GP114 and PO conjugated GP116 anti-insulin antibodies (C Binder, B Dinesen, and M Deckert, Novo Nordisk, personal communication) and a 2 1 mixture of rat insulin I and II (Novo Nordisk) as standard Rat and mouse insulin I and II are identical

***Lipogenesis/Glucose uptake assay***

Adipocytes isolated from epididymal fat pad (Livingston *et al* , 1984) were used to determine the $ED_{50}$ for insulin in 2 months old LAR$^-$ (F3(129 x C57BL/6), n=4) and control (F6(129 x C57BL/6) x C57BL/6), n=4) mice, and 4 months old LAR$^-$ (F3(129 x C57BL/6), n=4) and control (C57BL/6, n=4) mice For $^3$H-glucose incorporation measurements, aliquots of pooled fat cells (2 x $10^5$ cells/ml) were incubated for 2 h at 37°C in the presence of varying concentrations of insulin (K41100, Novo Nordisk), with or without adenosine (a well-known insulin sensitizer, $10^{-5}$ M final concentration), and with or without bisperoxovanadium 1,10-phenanthroline (bpV(phen), a non-specific PTPase inhibitor, $10^{-5}$ M final concentration) (Novo Nordisk)

A toluene-based scintillation fluid was added directly to the cells and after vortexing the $^3$H-labeled lipid was allowed to separate into the fluid phase Samples were then counted in a scintillation counter (Packard)

***Insulin receptor tyrosine kinase (IRTK) activity***

After sacrificing the mouse by cervical dislocation, *M Soleus* were quickly excised from both hindlimbs, freeze clamped, and stored at -80°C until analyzed Muscles were homogenized on ice in solobilization buffer (20 mM Hepes, 8 mM EDTA, 0 2 mM $Na_3VO_4$, 10 mM $Na_4P_2O_7$, 2 5 mM PMSF, 1 mg/ml aprotinin, 2 5 mg/ml benzamidine, 2 5 μg/ml pepstatin, 2 5 μg/ml leupeptin, 160 mM NaF, 2 mM dichloro-acetic acid, 1% Triton X-100, pH 7 4) using a Potter-Elvehjelm homogenizer After 20 min at 4°C, the samples were processed and basal IRTK activity was measured as described previously (Hansen *et al* , 1996)

***Statistical analysis***

Statistic analysis was performed using the Students' t-test (on pooled data from LAR$^-$ vs wildtype controls) or ANOVA All values are mean±SEM For the lipogenesis assay a four

parameter estimation of $ED_{50}$, basal, maximal response, and the slope of the linear part of the curve is performed.

**Results**

As LAR has been implicated in insulin signal transduction, we examined the loss of function of LAR with respect to the insulin response. Insulin plays a pivotal role in the metabolic processes that regulate plasma glucose levels. Therefore, we determined blood glucose levels in response to fasting, feeding, and insulin administration (ITT). Fasting and post-prandial LAR$^{-/-}$ mice showed no difference in plasma glucose levels compared to sex- and age-matched control animals (Table I.). There was also no significant difference in plasma glucose levels in response to insulin between LAR-deficient mice and wildtype controls (Fig. 1).

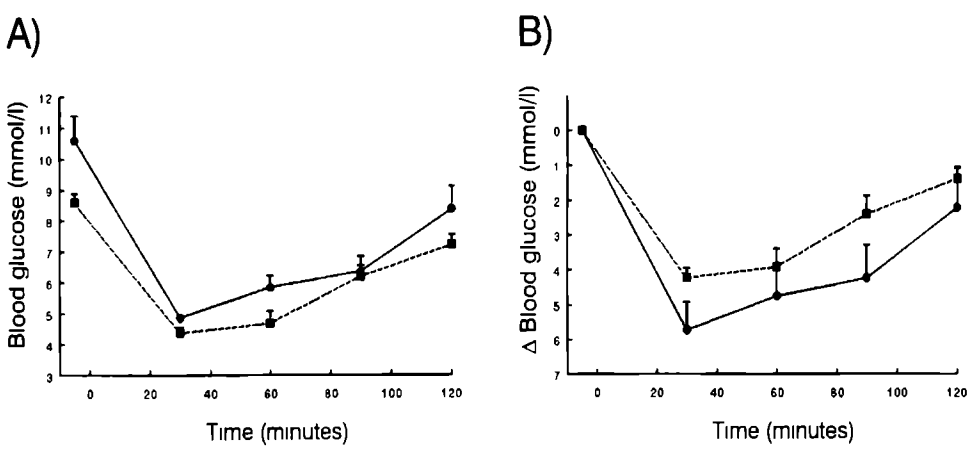


**Figure 1.** Blood glucose levels in LAR$^{-/-}$ and wildtype mice upon insulin administration
Post-prandial LAR$^{-/-}$ (■, n=12) and wildtype (•, n=10) mice were subjected to an insulin tolerance test (ITT) by s.c. injection of 1 U human insulin per kg bodyweight. Absolute levels (panel **A**) and Δglucose values corrected for pre-dose levels are shown (panel **B**). The Δglucose-$AUC_{ITT}$ values are given in Table I.

Although the insulin levels in the fasting LAR$^{-/-}$ animals appear higher than in the fasting control mice, the absence of LAR tyrosine phosphatase activity has clearly no influence on the *in vivo* insulin responsiveness and does not lead to a higher degree of hyperinsulinemia in post-prandial mice However, this does not rule out the possibility of an insulin resistance via desensitization of insulin signalling at the post-receptor level in the fasted mice (Marshall and Olefsky, 1980)

**Table I.** Plasma glucose and insulin concentrations and insulin tolerance in LAR$^{-/-}$ mice

| Mice | [glucose] | | [insulin] | | $\Delta AUC_{ITT}$ |
|---|---|---|---|---|---|
| | fasting | fed | fasting | fed | |
| wildtype (n=9) | 9 3±0 5 | 9 5±0 3 | 36±10 | 393±55 | -452±93 |
| LAR$^{-/-}$ (n=12) | 8 9±0 4 | 8 8±0 3 | 82±14 (*) | 316±54 | -344±38 |

[glucose] in mmol/l, [insulin] in pmol/l, $\Delta$glucose-$AUC_{ITT}$ in min x mmol/l
(*) P=0 05 vs wildtype (ANOVA)
Average weight of mice wildtype, 32 8±1 1 g, LAR$^{-/-}$, 28 5±0 7 g

An important aspect of the regulation of plasma glucose levels is the stimulation by insulin of glucose uptake via glucose transporters from blood into insulin-responsive tissues We investigated a possible role for LAR in the insulin-stimulated translocation of glucose transporters and subsequent increase in hexose uptake Rates of lipogenesis in response to insulin were measured to evaluate the glucose metabolism in primary adipocytes isolated from epididymal fat pads (Fig 2, and Table II) We designed the assay in such a way that the different effects of either adenosine, a well known insulin sensitizer, or bpV(phen), a non-specific PTPases inhibitor, could be observed If LAR activity is necessary for turning off the insulin signal, we would expect to maintain the adenosine effect, but to see no effect of bpV(phen) in adipocytes isolated from LAR$^{-/-}$ mice Figure 2 (panels A-D) shows that there is no significant difference in lipogenesis in fat cells isolated from LAR$^{-/-}$ and control mice over the complete range of insulin concentrations, as measured for 2 (Fig 2, panels A and B) and 4 months old mice (Fig 2, panels C and D) It is also clear that there is no influence of loss of LAR function on either the adenosine, nor the bpV(phen) effect (Fig 2, and Table II) Accordingly, the basal lipogenesis is not higher in the LAR$^{-/-}$ mice (Fig 2) and the $ED_{50}$ for insulin is not

significantly different for both groups of mice (Table II).

In various reports LAR has been put forward as a key regulator of IR tyrosine phosphorylation and activation upon insulin stimulation (Hashimoto *et al.*, 1992a, Kulas *et al.*, 1995, 1996b; Zhang *et al.*, 1996). However, when the basal IR tyrosine kinase (IRTK) activity was determined in soleus muscle no significant difference in basal IRTK activity could be observed between LAR$^{-/-}$ and control mice (2.0±0.5 vs. 3.2±1.1). Further studies will be necessary to address possible differences in IRTK activity in response to insulin.

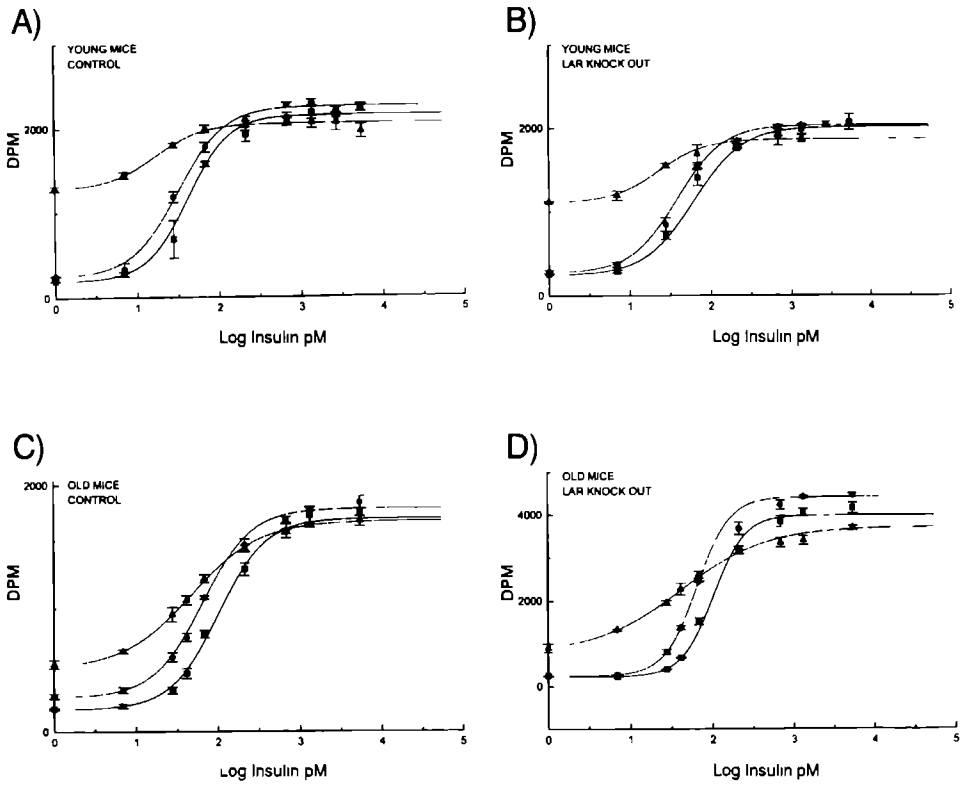


**Figure 2.** Insulin-stimulated $^{3}$H-glucose incorporation into lipid in epididymal adipocytes.
Adipocytes were isolated from young (2 months of age, panels **A** and **B**) and old (4 months of age, panels **C** and **D**) wildtype (n=4) and LAR$^{-/-}$ (n=4) mice. Triplicate samples were incubated with varying concentrations insulin in the absence (■) or presence of adenosine (•) or bpV(phen) (▲).

**Table II.** $ED_{50}$ for insulin stimulated lipogenesis in adipocytes

| | | young mice (n=4) | | old mice (n=4) | |
|---|---|---|---|---|---|
| | | wildtype | LAR$^{-/-}$ | wildtype | LAR$^{-/-}$ |
| Body weight (g) | | 23 5±0 1 | 23 0±0 4 | 32 5±1 1 | 29 2±1 0 |
| Insulin | $ED_{50}$ | 42 | 62 | 101 | 103 |
| + Adenosine | | 32 | 38 | 65 | 65 |
| + bpV(phen) | | 17 | 23 | 47 | 42 |

$ED_{50}$ in pM

**Discussion**

Over the past few years numerous reports have suggested PTPases as important regulators of insulin action Although the cytoplasmic PTPase SHP-2 and the transmembrane RPTPase LAR have been suggested as being candidate positive and negative regulators, respectively, their physiological relevance in the IR signal transduction has not been established yet

Of the family of insulin-responsive glucose transporters (GLUT), GLUT1 and GLUT4 are considered as the most relevant in basal hexose uptake and insulin-stimulated glucose transport, respectively (for review see Mueckler, 1994, Stephens and Pilch, 1995) In response to insulin, GLUT4 translocates from a cytoplasmic pool to the plasma membrane where it catalyses the rate-limiting step for glucose uptake and metabolism in skeletal muscle and adipose tissue (Stephens and Pilch, 1995) Insulin activates hexose transport via a $p21^{ras}$-dependent pathway, leading to an increase in the amount of cell surface GLUT1 Also a metabolic, $p21^{ras}$-independent pathway, leading to the translocation of GLUT4 to the cell surface is implicated in this activity (Haruta *et al* , 1995, Hausdorff *et al* , 1994) This insulin-stimulated translocation of GLUT4 involves activation of PI 3'-kinase, but may be independent of phosphorylated IRS-1 (Morris *et al.*, 1996, and references therein) SHP-2 was shown to be necessary for insulin-stimulated expression of the glucose transporter GLUT1, but is not required for activation of the pathway leading to GLUT4 translocation (Hausdorff *et al* , 1995) In addition, no major role for SHP-2 as a positive regulator in the acute *in vivo* metabolic actions of insulin could be demonstrated (Arrandale *et al* , 1996) However, since homozygous knock-out mice were embryonic lethal, these metabolic studies were performed with hemizygous mice expressing 50% of

the SHP-2 protein level compared to wildtype mice (Arrandale *et al* , 1996) Probably, as the divergence of the mitogenic $p21^{ras}$-MAPK and metabolic signalling pathways occurs at or near the IR (Pang *et al* , 1994), SHP-2 acts as a positive mediator in insulin-induced mitogenesis (Arrandale *et al.*, 1996, Hausdorff *et al* , 1995)

We have used mice lacking LAR tyrosine phosphatase activity (Schaapveld *et al* , 1997) to address the possible negative regulatory role of LAR in insulin signalling We observed no difference for LAR$^{-/-}$ mice in body weight, plasma glucose and insulin levels in fasting and post-prandial mice, and glucose levels after insulin injection when compared to wildtype control mice (Fig 1, and Table I) In addition, our results suggest no dominant role for LAR in the insulin induced expression and translocation of glucose transporters LAR$^{-/-}$ mice show no increased insulin signalling resulting in enhanced $^{3}$H-glucose incorporation into lipids (Fig 2, and Table II)

While the work described in this manuscript was ongoing, a physical and functional association of LAR and the IR was found in intact cells, suggesting a negative regulatory role for LAR in insulin action at the IR level (Ahmad and Goldstein, 1997) In muscle of LAR$^{-/-}$ mice, however, basal IRTK activity is not increased Hauguel-de Mouzon *et al* (1993) have shown for diabetic (insulinepenia) as well as for pregnant (hyperinsulinemia) rats the association of defective liver membrane-associated PTPase activity with decreased IRTK autophosphorylation In addition, impairment of insulin-stimulated IRTK activity has been proposed as an explanation for defective insulin sensitivity and thus insulin resistance *in vivo* (Chang *et al.*, 1994) Therefore, analysis of the IRTK activity upon insulin stimulation in LAR$^{-/-}$ mice will be subject of further study and may give more insight in a direct regulatory role for LAR at the IR Sofar, despite all previously published *in vitro* data, our data clearly show that LAR is not a major determinant in the negative regulation of the IR As also the levels of LAR expression in the insulin sensitive tissues liver and muscle show great variability (Hashimoto *et al* , 1992b, Longo *et al* , 1993, Pulido *et al* , 1995, Schaapveld *et al* , 1996, Streuli *et al* , 1992, Zhang and Goldstein, 1991), the involvement of other (R)PTPases in down-regulating the IR signal *in vivo* is most likely The LAR-like RPTPases RPTPδ and/or RPTPσ could serve this role and may provide a possible functional compensation for LAR in the LAR$^{-/-}$ mice

However, in this study we have not addressed the possible involvement of LAR in the dephosphorylation of a subset of phosphorylated substrates further downstream in insulin initiated signalling cascade(s) For example, recent data justify future studies on a regulatory role for LAR, like SHP-2, in insulin-induced rearrangements of the

cytoskeleton Activation of the IR also leads to tyrosine dephosphorylation of $p125^{FAK}$ and paxillin, two major components of Focal Adhesions (FAs), and the reduction of actin stress fibers (Ouwens *et al* , 1996, Pillay *et al* , 1995, Yamauchi *et al* , 1995) The small GTP binding protein Rho is a key regulator in the formation of these cytoskeletal structures for which tyrosine phosphorylation is essential (Ridley and Hall, 1994) Inhibiton of PTPases with vanadate mimics the stimulation of FA assembly by Rho, suggesting a regulatory role for PTPases in FA turnover (for review see Craig and Johnson, 1996) LAR has been localized to FA and sites of association of cells and the basement membrane (Serra-Pages *et al* , 1995, Streuli *et al* , 1992) More intriguing, LAR is able to bind TRIO, a protein harboring guanine nucleotide exhange factor (GEF) domains for Rho and Rac (Debant *et al* , 1996) Therefore, it is tempting to speculate that LAR is involved in the signalling and/or activation of Rho and Rho-mediated FA assembly and stress fiber formation LAR and TRIO may integrate diverse signals involved in cell migration, a process that requires cell-matrix and cytoskeletal rearrangements As antisense inhibition of LAR leads to increased activity of PI 3'-kinase (Kulas *et al* , 1995), which is an upstream regulator of Rac, and, hence, regulates indirectly Rho (Nobes *et al* , 1995), an inhibitory role for the LAR-TRIO complex upstream or even downstream of PI 3' kinase is not unlikely Finally, insulin and cytoskeletal rearrangements necessary for cell-cell and cell matrix interactions are required for mammary gland development and maintenance of lactation (Rillema, 1994) Female mice lacking LAR tyrosine phosphatase activity show a defect in terminal differentiation of the mammary gland epithelial cells and as a consequence are unable to nurse their pups (Schaapveld *et al* , 1997) The involvement of LAR in insulin mediated cytoskeletal rearrangements during mouse mammary gland development will therefore be subject of further studies

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# Chapter VI

## General Discussion & Future Prospects

## GENERAL DISCUSSION & FUTURE PROSPECTS

Protein tyrosine phosphorylation is now recognized as an important component of the control of cellular function, including growth and differentiation, cell cycle, cytoskeletal integrity, and cell death The net level of phosphorylation of tyrosyl residues in a target substrate reflects the balance between the competing action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases) Since the cloning of the first PTPase in 1988, considerable progress has been made in identifying new members of an ever growing family of enzymes that rival the PTKs in their structural diversity and complexity (see Chapter I) Unfortunately, although it is assumed that the extensive structural diversity reflects a broad range of functions for the PTPase family members *in vivo* (Neel and Tonks, 1997), relatively little is known about the physiological function(s) of individual PTPases

The membrane-bound, receptor-like PTPases (RPTPases) have a putative extracellular ligand-binding region, a transmembrane domain, and, with a few exceptions, two repeated cytoplasmic phosphatase domains The striking variation in the extracellular segments presumably reflects an equivalent diversity of ligands to which these enzymes may respond (see Chapter I) The study as described in this thesis is the first example of a mouse model to study specifically the catalytic tyrosine phosphatase function of a RPTPase, while leaving the possible ligand function of its extracellular part intact The outcome of the experiments performed justifies further studies on the role of LAR in signal transduction

Through our knowledge of the mouse LAR gene and mRNA structure, the coding information, and the feasibility of 'double replacement' gene targeting approaches at the mouse LAR gene (*Ptprf*) locus (see Chapters II and III), tools to study the biological significance of each individual PTPase domain are now available As already discussed in Chapter III, the LAR negative ES cell lines are suitable to perform structure-function studies with respect to the involvement of LAR PTPase domains in early embryonic development and differentiation, by creating catalytic inactive and so-called 'substrate-trapping' LAR mutants (Flint *et al* , 1997) Differences between wildtype and LAR$^{-/-}$ cells in responses to diverse extracellular matrices (ECM), growth factors and hormones, and

in tyrosine phosphorylation patterns may give future directions towards the identification of possible ligand(s) and substrate(s) The elucidation of ligand(s) and/or substrate(s) is a prerequisite to set up a cell biological read out system for LAR tyrosine phosphatase activity Once established, the study of the significance of the association of, for example, LIP 1 and TRIO to the second, C-terminal PTPase domain of LAR becomes possible Furthermore, to study the role of each individual catalytic domain, the 'double replacement' strategy can now be used to generate mice expressing full-length LAR with either a catalytic inactive first, membrane proximal, or an inactive second PTPase domain

Quite unexpectedly, disruption of LAR signalling resulted in impaired mammary gland development during pregnancy as the sole detectable deviation from wildtype control mice (see Chapter IV) The discovery of this specific phenotype has now paved the way to study the role of LAR signal transduction in mammary epithelial cells and the possible involvement of LAR in breast tumorigenesis Monitoring the proliferative and apoptotic index, and the expression of differentiation and apoptosis markers in mammary glands at various stages of pregnancy for both wildtype and LAR$^{-/-}$ mice may provide clues to the precise role of LAR signalling for survival of mammary epithelial cells *in vivo* In conjunction, study of the susceptibility of LAR$^{-/-}$ mice towards DMBA-induced mammary tumors is warranted, as cell survival is of high importance for tumor growth

Mammary epithelial cell (MEC) lines can be derived from LAR$^{-/-}$ and control mice to investigate more precisely the involvement of LAR in diverse signal tranduction pathways When grown on an artificial basement membrane, called Matrigel®, and in the presence of the proper hormones like hydrocortisone and insulin, these MECs are able to form three-dimensional structures representing alveolar outgrowth Using this *in vitro* system one can study the possible roles of LAR in the insulin-induced rearrangments of the cytoskeleton, and in the extracellular matrix-integrin pathway which, like the prolactin signal transduction pathway, leads to the expression of milk proteins It may even well be that, in analogy to what is now known for RPTK signalling (Weiss *et al* , 1997), LAR may not only be a transmembrane signal transducer of specific extracellular ligands but may also, directly or indirectly, participate in the signal modulation of a variety of stimuli More specific, LAR might have its function at the crossroad(s) of the diverse signalling pathways important for functional differentiation of MECs

As said, the specific mammary gland phenotype of the LAR$^{-/-}$ mice was a surprising outcome, especially when taking into account the broad expression pattern of the molecule. The question now arises what the phenotype of a total LAR knock-out mouse will be With the data available to date this is hard to predict Observed defects could result from loss of the ability of LAR, as all other RPTPases, to function as a ligand, independent of its capacity to dephosphorylate target substrates (Neel and Tonks, 1997).

The LAR-deficient mice made by Skarnes *et al.* (1995) using a gene trap-based method were reported as being completely devoid of LAR expression, due to an insertion of the $\beta$-galactosidase reporter gene in the 5' region of the gene encoding the N-terminal Ig-like domains. For these mice, no defects in mammary gland development and function have been described, although it is known that the breeding of these mice is also hampered by a considerable loss of newborns (M. Streuli and D. Rotin, personal communications) It now appears that at least in brain this gene trap is leaky, i.e. full-length LAR transcripts are still detectable (Yeo *et al.*, 1997). Leakiness of the gene trap in the mammary gland was not reported, but may explain that no phenotype was observed in this tissue (Skarnes *et al.*, 1995). On the other hand, leakiness of the gene trap gives rise to transcripts in which the presence of reporter gene sequences may affect proper alternative splicing, resulting in a-specific phenotypic effects.

It is of note therefore, that Yeo *et al.* (1997) describe an abberant morphology of the basal forebrain cholinergic neurons and a reduced cholinergic innervation of the dentate gyrus in the hippocampal region of the brains of these mice. As only a minor decrease in full-length LAR expression and an apparantly normal expression of the smaller brain-specific LAR transcript was reported, the data as presented in this paper are puzzling. In our LAR$^{-/-}$ mice moreover, we could not detect morphological abnormalities in parasagittal and coronal sections of the hippocampal formation (see Chapter IV; Schaapveld *et al.*, manuscript submitted). It is of course possible that, like in our case, the heterogeneity in genetic background obscures the phenotype displayed by the 'Skarnes' mice.

Why are no abnormalities resulting from the loss of LAR function observed in other tissues than the mammary gland? One explanation for the lack of a more general phenotype might be redundancy in LAR-like PTPase function. Recent data showed that LAR and the highly homologous RPTPases RPTP$\delta$ and RPTP$\sigma$ undergo similar pro-protein processing and posttranslational modifications, exhibit similar *in vitro* PTPase

activity, and that all three are capable to interact via the second PTPase domain with the coiled-coil protein LIP 1 (see Chapter I) Hence, it is not unlikely that these three look-a-likes exert similar functions in various tissues At this moment it is an open question whether one might expect adaptations in tissue- and cell type-specific distribution or expression levels of RPTPδ and RPTPσ in LAR$^{-/-}$ mice and what mechanisms would serve to initiate this rewiring in the PTPase network The fact remains that we observed no appreciable differences in mRNA expression levels of the LAR-like RPTPases RPTPδ and RPTPσ in the LAR$^{-/-}$ mice (see Chapter IV) In addition, our RNA *in situ* hybridization data (Schaapveld *et al* , manuscript submitted) indicate also no differences between wildtype and LAR$^{-/-}$ mice in temporal and spatial expression of these two RPTPases during embryonic development

As these three RPTPases show distinct and overlapping expression patterns, it is important to investigate changes in PTPase activity of RPTPδ and RPTPσ in the diverse tissues of the LAR$^{-/-}$ mice Therefore, in analogy with experiments performed on *Drosophila* mutants, intercrossing LAR$^{-/-}$ mice with RPTPδ and RPTPσ knock-out mice may reveal redundant functions of these three closely related molecules and explain the specific mammary gland phenotype of the LAR$^{-/-}$ mice In addition, these studies may lead to the identification of modifier loci in the 129 mouse genome as discussed in Chapter IV, or may point to the existence of yet another member of this subclass of RPTPases

This issue might also play a role in explaining the results of our studies on the involvement of LAR in insulin signal transduction (Chapter V) Over the past few years *in vitro* data on LAR as a negative regulator in signalling via the insulin receptor (IR) are piling up (see Chapter V) Our data, however, indicate no functional correlation between absence of LAR PTPase activity and increased insulin responsiveness At this moment, it is thus not clear whether other PTPases or specific isoforms thereof, including RPTPδ and RPTPσ, are more important in the downregulation of the IR signal

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## Summary

## SUMMARY

Protein tyrosine phosphorylation is now recognized as an important component of the control of cellular function, growth and differentiation, cell cycle, and cytoskeletal integrity The net level of phosphorylation of tyrosyl residues in a target substrate reflects the balance between the competing action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases) On the basis of their overall structures, two classes of PTPases can be distinguished, namely (i) those found in the cytoplasm and nucleus that have only one tyrosine phosphatase domain and (ii) membrane bound, receptor-like PTPases (RPTPases) that have a putative extracellular ligand-binding region, a trans membrane domain, and, with a few exceptions, two related cytoplasmic phosphatase domains (see Chapter I)

One of the best studied RPTPases is the Leukocyte common Antigen-Related molecule LAR LAR is composed of two cytoplasmic phosphatase domains, a transmembrane segment, and an extracellular region which shares homology to immunoglobulin (Ig) like and fibronectin type III (Fn-III) domains This arrangement of extracellular motifs renders LAR a prototype of the cell adhesion molecule (CAM)-like RPTPases Using bacterially expressed proteins and artificial substrates it was shown that the first, membrane proximal phosphatase domain is catalytically active, whereas the second, C terminal phosphatase domain may regulate the phosphatase activity of the first domain

Since LAR-specific ligands and substrates still remain to be identified, we set out to study the consequences of the loss of LAR PTPase function *in vivo* by means of gene targeting using homologous recombination in mouse embryonic stem (ES) cells

As a first step towards site-directed mutagenesis studies, we used the human LAR cDNA as a probe to isolate mouse LAR cDNA and genomic clones (Chapter II) The cytoplasmic region of the mouse LAR protein is encoded by eleven exons which span only 4 5 kb of genomic DNA Compared to the, at that time, known exon-intron struc tures of other mammalian RPTPase genes, such as LRP and Ly 5 (the mouse homologs of RPTPα and CD45, respectively), the gene segment encoding the cytoplasmic region of LAR contains not only smaller, but also fewer introns The mouse LAR cDNA sequence was found to be nearly identical to the cDNAs for rat and human LAR Strikingly, among

species the second, 'catalytically inactive' domain shows a slower mutation rate than the first, 'catalytically active' domain In addition, the homology to the LAR-like RPTPases RPTPδ and RPTPσ is also higher in the second phosphatase domain This is not seen for the other more distant members of the RPTPase family The LAR gene (*Ptprf*) maps to a distinct chromosome region in mouse, C6 D1, demonstrating that LAR, RPTPδ, and RPTPσ, although closely related family members, are indeed separate entities

In Chapter III we describe the use of 'double replacement' gene targeting in mouse ES cells to generate cell lines lacking the gene sequences essential for LAR phosphatase activity We show that the use of replacement-type targeting vectors in two consecutive rounds of targeting does not interfere with germline competence By exploiting the process of gene conversion, we were also able to generate ES cells containing two mutated LAR alleles These double knock-out cell lines were analyzed by RT-PCR and a polyclonal antiserum directed against both phosphatase domains to confirm the absence of the LAR phosphatase domains at the RNA and protein level Loss of LAR expression is apparently not harmful for early cell growth Cells that had undergone two rounds of homologous recombination also contribute normally to development

In Chapter IV we describe the generation of mice lacking both phosphatase domains of LAR Chimaeras showed germ line competence and heterozygote and homozygote mutants deficient in LAR tyrosine phosphatse activity develop and grow normally, do not show an overt phenotype, and display no appreciable tissue abnormalities at the histological level Northern blot analysis of various tissues revealed the presence of a truncated LAR mRNA lacking the part encoding the cytoplasmic signalling moiety and indicated that this mutation of LAR is not accompanied by changes in expression levels of one of the LAR-like RPTPases RPTPδ or RPTPσ However, upon breeding we observed an increased neonatal death rate for pups (more than 50%) from $LAR^{-/-}$ females compared to offspring from $LAR^{+/+}$ females Later, it appeared that full penetrance of the phenotype was hampered by the heterogeneity of the genetic background of the mice Mammary glands of $LAR^{-/-}$ females were incapable of delivering milk due to an impaired terminal differentiation of alveoli at late pregnancy Histological examination showed that the alveolar development which is characteristic for late pregnancy is not attained, the glands fail to switch to a lactational state, and showed a rapid involution postpartum Milk secretion is not initiated because the transition to involution and apoptosis of the

alveolar epithelium occurs more rapidly in these mice, even before milk secretion starts. In wild-type mice, LAR expression is regulated during pregnancy reaching maximum levels around day 16 of gestation. Expression of the LAR-like RPTPases RPTPδ and RPTPσ are not or hardly detectable during mammary gland development. Taken together, these findings point to a highly specific role for LAR-mediated signalling in mammary gland development and function.

In Chapter V we describe how we have used the $LAR^{-/-}$ mice to study insulin signalling in the absence of LAR tyrosine phosphatase activity. *In vitro* studies had implicated LAR as a negative regulator in the signal transduction via the insulin receptor (IR). In contast to expectation, our *in vivo* data provide no evidence for differences in metabolic processes in response to insulin, nor for an increased tyrosine kinase activity of the IR (IRTK) in the absence of LAR activity. Although a more detailed study of the IRTK activity in response to insulin is warranted, we may conclude that PTPases other than LAR, possibly RPTPδ and RPTPσ, are involved in the downregulation of the IR signal.

The study described in this thesis is one of the first to address the specific role of the catalytic segments of an RPTPase *in vivo*, in the proper cellular context and within the entire animal. Further studies, considering the ligand and extracellular receptor functions of LAR should complete our picture on the functioning of this PTPase family member in cell signalling and development.

# Samenvatting

## SAMENVATTING

Het '"**L**eukocyte common **A**ntigen-**R**elated molecule" **LAR** is het prototype van een cel adhesie molecuul achtig receptor-type proteine tyrosine fosfatase (RPTPase, zie Hoofdstuk I) LAR is opgebouwd uit twee cytoplasmatische homologe tyrosine fosfatase domeinen, een transmembraan domein en een extracellulair deel dat 3 immunoglobuline-achtige en 8 fibronectine type III domeinen bevat In de intracellulaire fosfatase domeinen bevindt zich een onder alle PTPases geconserveerde sequentie van ongeveer 11 aminozuren (-(I/V)HCXAGXXR(S/T)G ) waarin het cysteine residue van essentieel belang is voor de enzymatische activiteit Hoewel het tweede, C terminale domein deze sequentie ook bevat, werd met behulp van *in vitro* assays (gebruikmakend van bacterieel geproduceerde eiwitten en artificiele substraten) aangetoond dat dit tweede domein geen enzymatische activiteit bezit Mogelijk speelt het een rol bij de substraat-specificiteit van het eerste, membraan proximale domein De extracellulaire liganden en intracellulaire substraten zijn echter nog onbekend Dit maakt *in vitro* studie ter bestudering van de biologische functie van LAR ontoereikend

Wij besloten *in vivo* het verlies van functie van beide fosfatase domeinen te ondezoeken, door middel van gen "targeting" middels homologe recombinatie in muizen embryonale stam (ES) cellen

In Hoofdstuk II wordt beschreven hoe met behulp van het humane LAR cDNA genomische en cDNA klonen van muis LAR geisoleerd werden De beide fosfatase domeinen van muis LAR worden gecodeerd door 11 exonen die slechts 4 5 kb van genomisch DNA beslaan Vergeleken met de, op dat moment bekende, exon-intron structuren van andere RPTPases, LRP (muizen homoloog van RPTP$\alpha$) en Ly-5 (muizen homoloog van CD45), bevat dit deel van het LAR gen niet alleen kleinere, maar ook minder intronen Op aminozuur-niveau is dit gedeelte van LAR nagenoeg identiek met de rat en humaan LAR cDNAs, en zeer homoloog aan twee andere cel adhesie molecuul-achtige RPTPases, RPTP$\sigma$ en RPTP$\delta$ Opvallend is dat voor LAR tijdens de evolutie het tweede, "enzymatisch inactieve" fosfatase domein het meest geconserveerd blijft Onderlinge vergelijking van LAR, RPTP$\delta$ en RPTP$\sigma$ laat zien dat, uniek voor deze drie RPTPases, de homologie ook het hoogst is in het tweede domein De bepaling van de

chromosomale localisatie op muis chromosoom 4, regio C6-D1, bevestigd dat we inderdaad de muizen homoloog van LAR gekloneerd hebben en dat drie verschillende genen coderen voor LAR, RPTPδ en RPTPσ

In Hoofdstuk III wordt het gebruik van "double replacement gene targeting" in muizen embryonale stam (ES) cellen beschreven We tonen aan dat het mogelijk is met behulp van replacement type" targeting vectoren en twee opeenvolgende rondes homologe recombinatie ES cellijnen te genereren die de genomische sequenties coderend voor de beide fosfatase domeinen van LAR missen Tevens wordt bevestigd dat na de eerste ronde van homologe recombinatie gen-conversie gebruikt kan worden om het tweede allel te muteren Met behulp van RT-PCR en een polyklonaal antiserum gericht tegen beide fosfatase domeinen van LAR wordt aangetoond dat deze "double knock-out" ES cellen het cytoplasmatische deel van LAR missen Deze mutatie heeft blijkbaar geen negatieve gevolgen voor de celgroei Cellen die beide rondes van homologe recombinatie hebben ondergaan behouden hun kiembaan-competentie en kunnen op normale wijze bijdragen aan de embryonale ontwikkeling

De generatie van de LAR knock-out muis staat beschreven in Hoofdstuk IV Bij deze muizen zijn de sequenties die coderen voor beide tyrosine fosfatase domeinen van LAR vervangen door het neomycine fosfotransferase (Neo) gen RNA analyse maakt duidelijk dat deze muizen een getrunceerd LAR transcript tot expressie brengen, dat gebruik maakt van het polyadenylerings signaal in de Neo cassette en geen functioneel LAR eiwit meer kan coderen Deze mutatie van LAR gaat niet gepaard met significante veranderingen in de expressieniveau's van RPTPδ en RPTPσ De verkregen muizen zijn levensvatbaar, zien er gezond uit en vertonen geen noemenswaardige histologische afwijkingen

Echter, na verder fokken blijkt dat ongeveer 50% van de nakomelingen die afkomstig zijn van een homozygoot mutant vrouwtje binnen drie dagen na geboorte doodgaan Het blijkt dat dit percentage toeneemt tot 100% indien de bijdrage van muis 129 DNA in de genetische achtergrond van de muizen afneemt Door uitwisseling van nesten pasgeborenen kwamen we erachter dat LAR-deficiente vrouwtjes niet in staat zijn jongen te voeden Na uitgebreide analyse van de melkklieren blijkt dat de ontwikkeling van deze klieren op het einde van de zwangerschap verstoord raakt De alveolaire epitheliale cellen maken wel secreet, maar deze hoopt zich op intracellulair en wordt niet afgegeven aan het lumen Er vindt geen terminale differentiatie plaats die op de dag van geboorte moet

leiden tot afgifte van melk Het gevolg is dat de hele alveolaire structuur niet in stand wordt gehouden en te gronde gaat door het vervroegd in gang zetten van geprogrammeerde celdood Het uiteindelijke resultaat is dat het vrouwtje haar jongen niet kan voeden Gezien ook de regulatie van LAR expressie in de melklier tijdens de zwangerschap, en het feit dat expressie van RPTPδ en RPTPσ niet tot nauwelijks aantoonbaar is gedurende de melklier ontwikkeling, concluderen we dat LAR in een laat stadium van de zwangerschap een rol speelt in de signaaltransductie vanuit de extracellulaire matrix Deze is immers van belang voor de melkeiwit synthese en functionele differentiatie van de alveolaire epitheliale cellen

De experimenten zoals beschreven in Hoofdstuk V zijn een voorbeeld hoe de LAR knock out muizen te gebruiken voor verder onderzoek Als onderdeel van een samenwerkingsverband gericht op de identificatie van PTPases in insuline signaaltransductie werden de muizen gebruikt om de invloed van verlies van LAR PTPase activiteit op insuline-geinduceerde metabole processen te meten Op dit moment zijn onze resultaten geen bevestiging van de hypothese dat LAR als een negatieve regulator functioneert in de signaaltransductie via de insuline receptor Er zijn geen verschillen meetbaar in bloed glucose en insuline gehaltes, de insuline tolerantie is vergelijkbaar, en de basale tyrosine activiteit van de insuline receptor is niet veranderd Alhoewel de activiteit van de insuline receptor na toediening van insuline nog gemeten moet worden, kunnen we op dit moment slechts concluderen dat er waarschijnlijk (ook) andere PTPases, waaronder mogelijk RPTPδ en RPTPσ, betrokken zijn bij de regulatie van het signaal dat door de insuline receptor wordt doorgegeven na stimulatie met insuline

Het in dit proefschrift beschreven onderzoek is het eerste voorbeeld van een *in vivo* model om zowel in de cel als in een heel organisme de biologische implicaties van de tyrosine fosfatase activiteit van een RPTPase te bestuderen Verder onderzoek naar de mogelijke ligand(en) en de functie van het extracellulaire deel van LAR zal bijdragen tot een beter begrip van rol van dit RPTPase in signaal transductie en ontwikkeling

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Dan is daar Jan (met de gouden handjes) De manier waarop jij mij hebt ingewerkt getuigt van grote klasse en jouw betrokkenheid bij elk hoofdstuk is onontbeerlijk geweest Zonder jouw hulp zouden de coupes van de RNA *in situ* hybridisaties bij wijze van spreken nu nog in de fotoemulsie liggen

Voor de social talk (het bieren en de borrels) en de brainstorm sessies was er Edwin (den Cup) Alhoewel jouw fosfatase in het geheel niet leek op die van mij, heb ik het "meedenken" altijd zeer gewaardeerd Succes in je laatste jaar en tot ziens op het NKI

Belangrijk was ook de goede sfeer in de fosfatase-groep Wiljan's groepje werd alsmaar groter en het beruchte lab 65 mocht al gauw lab 72 gaan bevuilen De "mannenpraat", en al het geouwehoer wat daaronder valt, van Peter, Henri, Patrick, Johan, Arn en Jan op 65 kon mooi ongestoord worden voortgezet op 72 met Edwin, Rick en Barry ondanks de aanwezigheid van Wilma, die ons altijd voorzag van kilo's restrictie enzymen En natuurlijk waren er de studenten, Robin (Gobin), Ilonka, Ton en Joline die ieder hun steentje bijdroegen Zonder de anderen tekort te willen doen wil ik met name jou, Joline, bedanken voor je niet aflatende hulp bij het analyseren van het borstklier fenotype en de enorme hoeveelheid coupes die je hebt gesneden

Mijn kamergenoten, Toine (die ontzettend rijke vent met snor) en Joke maakten het verblijf op "kantoor" tijdens de schrijfperiodes aangenaam Toine, hartstikke bedankt voor je support bij de totstandkoming van dit boekje

Onmisbaar voor het muizenwerk waren Frank en Walther, en natuurlijk de collega's van het CDL Voor het leren snijden van coupes konden Joline en ik altijd terugvallen op

Hein en Huib In de laatste fase heeft Coby ook nog enkele essentiele blokjes weggesneden Vervolgens kon ik dan naar Jack Fransen en Paul (je bent afgevallen!) Jap voor een cursusje Histologie en de beoordeling van diezelfde coupes Hans Smits zorgde voor de berg fotorolletjes en Foto Verweij voor het perfect ontwikkelen en afdrukken ervan Jack en Hein, ook bedankt voor alle hulp als er weer eens een PC vastliep of een printer weigerde

During my PhD I had a very nice time in Boston Dear Michel, thank you very much for giving me the opportunity to spent some months in your lab and to experience some protein chemistry And thank you Carles, Quint, Andreas, Stephen, Katja, and May, for making my stay in Boston as a very pleasant one In addition, a quick visit to the NIH in Washington broadened my knowledge on mammary glands Lothar and Gertraud, thank you for your help with the analysis of the mamamry gland phenotype Finally, I would like to thank the people at Novo Nordisk for their contibutions to the LAR-Insulin story

Afsluitend realiseer ik me dat ik nooit volledig ben Echter, allen die op enige andere wijze (dan hier vermeld staat) hebben bijgedragen, weten dit immers al Ik hoop dan ook iedereen te ontmoeten op het feest

# Curriculum Vitae

## CURRICULUM VITAE

Roeland Quirinus Jozef Schaapveld werd geboren op 15 november 1965 te Stein als derde kind in een gezin van vier Na de lagere school doorliep hij de middelbare school en slaagde hij in 1984 voor het eindexamen Atheneum-B aan de Scholengemeenschap "Groenewald", te Stein In datzelfde jaar begon hij met de studie Biologie aan de Katholieke Universiteit Nijmegen (KUN) Na het propedeutisch examen (1986) liep hij tijdens de doctoraal fase bijvakstages op het gebied van de Radiobiologie (Instituut voor Radiotherapie en Afdeling Bloedziekten, Sint Radboud ziekenhuis, Nijmegen, o.l v. Prof. dr A J van der Kogel, Dr. J. Hoogenhout, Dr J Wessels, Dr J.H M. Schwachöfer) en Moleculaire Celbiologie (Department of Molecular Genetics, Roche Research Center, Hoffmann-La Roche Inc., Nutley, New Jersey, USA, o l v Dr R Narayanan, Dr P T Lomedico, Prof. dr. E J.J. van Zoelen), en een hoofdvakstage op het gebied van de Dierfysiologie (*eerste deel* Neuroimmunoendocrinologie, Afdeling Experimentele Dierkunde, Fac Natuurwetenschappen, KUN, o.l v Prof dr S E Wendelaar Bonga, Dr P.H M Balm; *tweede deel* Immunologie, Immunological Research & Development Labs, Organon Int B V , Oss, o l v Drs H van den Berg, Dr F T.M Rotteveel) Het doctoraal examen in de richting fysiologische en biochemische Biologie legde hij eind 1991 met goed gevolg af.

In april 1992 trad hij in dienst als Onderzoeker In Opleiding bij de afdeling Celbiologie & Histologie, Faculteit der Medische Wetenschapen, KUN (hoofd: Prof. dr. Bé. Wieringa) alwaar het in dit proefschift beschreven onderzoek werd verricht onder begeleiding van Dr Wiljan Hendriks Gedurende deze periode bracht hij werkbezoeken aan de laboratoria van Dr Michel Streuli (Division of Tumor Immunology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, USA) en Dr Lothar Hennighausen (Laboratory of Biochemistry and Metabolism, NIDDK, National Institutes of Health, Bethesda, USA) Tevens werden de cursussen preparatieve en analytische flowcytometrie, Proefdierkunde (Artikel 9 functionaris), en Stralingshygiene (deskundigheidsniveau 3) met goed gevolg afgesloten Hij nam met veel plezier deel aan de congressen FEBS Advanced Course, "Protein Phosphatases in Cellular Regulation" (Bressanone/Brixen, Italy), the NATO/FEBS Advanced Study Institute, "Molecular Mechanisms of Transcellular Signalling From the Membrane to the Gene" (Island of Spetsai, Greece), en the Cold Spring Harbor Laboratory Meeting, "Tyrosine Phosphorylation and Cell Signalling" (Cold Spring Harbor, New York, USA)

Per 1 november 1996 is hij in dienst getreden als post-doctoraal medewerker op de afdeling Celbiologie van Het Nederlands Kanker Instituut/Antoni van Leeuwenhoek ziekenhuis te Amsterdam. In de groep van Dr Arnoud Sonnenberg verricht hij onderzoek aan het integrine $\alpha 6\beta 4$, met name de rol van dit celadhesie molecuul in cel-matrix interacties en signaaltransductie in relatie tot de aggressiviteit van bepaalde typen tumoren.

# Publications

## PUBLICATIONS

Schwachofer, J H M , Schaapveld, R Q.J , Crooijmans, R.P.M A , Hoogenhout, J., and van der Kogel, A J. (1989). In vitro detection of radiation-induced DNA-damage in human tumor cells *Cell Differentiation and Development*, **27**suppl s47.

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Schaapveld, R Q J , Brand, C L , Hansen, B F , Møller, N P H , Hansen, B , Rasmussen S , Jensen, P , Hendriks, W J A.J , and Sørensen, A R (1997) Insulin responsiveness in mice lacking LAR tyrosine phosphatase activity. *Manuscript to be submitted*

Schaapveld, R Q J , Schepens, J.T G , Bachner, D., Attema, J , Jap, P H K , Wieringa, B., and Hendriks, W.J A J (1997) Distinct and overlapping expression patterns of the CAM-like tyrosine phosphatases LAR, RPTPδ and RPTPσ in the embryonic and adult mouse *Manuscript submitted*

# STELLINGEN

behorende bij het proefschrift

**The Biological Role of the Receptor-like Protein Tyrosine Phosphatase LAR**

- a mouse model to study loss-of-function of LAR tyrosine phosphatase activity -

Roel Schaapveld
Utrecht, 7 oktober 1997

The inherent problem of background genes linked to the mutation of interest, might severely hamper the scientific interpretation of the results of transgenic and knockout studies
*Jaqueline N Crawley* Trends in Neuroscience (1996), 19 181 182

Establishing C57BL/6-type ES cell lines represents the optimal solution
*Robert Gerlai* Trends in Neuroscience (1996), 19 177-181

The effect of the RPTP$\beta/\zeta$ ectodomain on contactin (Peles *et al* , 1995) argues for caution in interpreting the phenotypes of RPTP mutants, some or even all of the observed defects could result from loss of ability of RPTPs to function as ligands, independent of their capacity to dephosphorylate target substrates
*Benjamin G Neel, and Nicholas N Tonks* Current Opinion in Cell Biology (1997) 9 193 204

The identification of *PTEN* as a tumor suppressor gene (Li *et al* , 1997) is the first evidence for a long-held speculation that tyrosine phosphatases would be important in cancer
*Stephen Friend* Science (1997), 275 1876-1878

Geen enkel kwaad dat het communisme pretendeerde te bestrijden, is verdwenen nadat het aan de macht kwam, en geen enkel kwaad dat het heeft geschapen, is verdwenen nadat het ten onder ging Daarom is er niets tegen terugkeer van het communisme "
*Henry Kissinger*

"In een lab dat op zijn gat ligt en dat slaapt, daar gebeurt ook qua feesten en cabaret niks "
*Prof dr Dirk Bootsma*, in Vrij Nederland, 15 april 1995

"Kwaliteit is toeval uitsluiten "
*Louis van Gaal*

"Wie een fout compenseert met een fout, maakt twee fouten "
*Frank Snoeks*, voetbalcommentator tijdens Frankrijk-Italie, 11 juni 1997

Het initiatief van de Universiteit Utrecht een deel van het salaris uit te betalen in natura, bijvoorbeeld in de vorm van een extra vliegtuigticket bij een buitenlandse dienstreis, zal het aantal vakanties van universiteitsmedewerkers in plaatsen waar congressen worden gehouden sterk doen toenemen

Ondanks het feit dat als je gaat studeren je vrijwel zeker een baan krijgt, gaat werken, geld verdienen, een huis en een auto kan kopen, een vrouw en kinderen hebt, zijn de voordelen niet geheel duidelijk
Naar *Sigmund*, in De Volkskrant

Wie niet beschikt over intuitie, kan geen verstandige beslissingen nemen

Het verhogen van de leeftijdsgrens tot 55 jaar om in aanmerking te komen voor reageerbuisbevruchting vraagt om een nadere bepaling van de minimumleeftijd

De toename in betuttelende snelheidsbeperkingen op de Nederlandse autosnelwegen leidt tot agressiever weggedrag van de Nederlandse automobilist
*Eigen ervaring*

Bij het lopen van de Nijmeegse Vierdaagse is het verkrijgen van blaren een grotere zekerheid dan het behalen van het felbegeerde kruisje

De nieuwe generatie promovendi (de zgn beursstudenten) is een dief van de eigen portemonnee